Activation of MAPKs in Proximal Tubule Cells From Spontaneously Hypertensive and Control Wistar-Kyoto Rats

Astrid Parenti, Xiao-Lan Cui, Ulrich Hopfer, Marina Ziche, Janice G. Douglas

Abstract—The aim of this study was to test the hypothesis that differences exist in the activity and/or expression of mitogen-activated protein kinases (MAPKs) between spontaneously hypertensive rats (SHR) and control Wistar-Kyoto rats (WKY) and that these differences may account for the enhanced activity of the Na+/H+ exchanger (NHE) previously observed in the renal proximal tubule of SHR. Therefore, the activities of c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 were investigated. A reduced amount of ERK1 and JNK1 protein was found in renal cortex specimens of SHR as compared with WKY; however, their activities were the same. To study the cellular basis of this difference, immortalized proximal tubule cell lines were grown on Millicell-CM filter inserts where the cell lines organize as polarized monolayers with separate access to apical and basolateral compartments. Although basal JNK1 and ERK1/2 activities were not significantly different between WKY and SHR cells, anisomycin stimulated JNK1 activity in WKY cells more than in SHR cells (eg, at 15 minutes 300% versus 30%, respectively). Similarly, angiotensin II increased JNK1 and ERK1/2 activity in a time- and concentration-dependent manner in WKY cells but not in SHR cells. Western blot analyses showed a deficit in JNK1 and ERK1 protein in SHR (0.25 and 0.5, respectively, of the levels in WKY cells), although ERK2 and p38 protein levels were the same. These observations suggest that, although angiotensin II activates MAPKs and MAPKs have been shown to regulate NHE, this regulatory pathway is unlikely to account for the increased activity of NHE in the proximal tubular epithelium of SHR. (Hypertension. 2000;35:1160-1166.)

Key Words: epithelial cells • protein kinases • hypertension, essential • angiotensin II

The genetic basis for essential hypertension in humans and animal models, such as the spontaneously hypertensive rat (SHR), has not been elucidated. In the SHR model, the pathogenesis of hypertension appears to be related to changes in the renal set point for Na+ reabsorption or cellular Na+ homeostasis, in general. The long-term control of Na+ homeostasis appears to depend on factors that influence the renal set point for Na+ reabsorption or cellular Na+ homeostasis in the kidney (ie, the renal set point for Na+ reabsorption is set such that hypertension is necessary to achieve a normal extracellular fluid volume). Evidence for the crucial role of the kidneys comes from transplantation experiments of SHR kidneys into normotensive recipient strains: SHR kidneys exhibit an altered pressure-natriuresis relationship that is maintained after transplantation,2 and the transplanted SHR kidneys confer hypertension to animals from normotensive strains.3 The proximal tubule is the major site for Na+ reabsorption4 and thus is potentially a site for changes in the set point for Na+ reabsorption. Considerable evidence points toward intrinsic differences in tubule function between SHR and the normotensive Wistar-Kyoto (WKY) control strain. Of particular importance to the pathogenesis of hypertension appears to be abnormalities of the 2 most important regulatory agents of Na+ transport: dopamine and angiotensin II (Ang II). D1 dopaminergic receptor signaling is impaired resulting in enhanced Na+ reabsorption.5 Alterations in responses to Ang II are more complex and age dependent, but they generally also result in enhanced proximal tubule Na+ reabsorption.6,7 In young, prehypertensive SHR, type 1 Ang II receptor numbers are increased, although their sensitivity is decreased. In contrast, in 12-week old rats, the sensitivity to Ang II is increased. With cell lines from SHR and WKY proximal tubular epithelial cells, it has been possible to confirm that there is enhanced Ang II-dependent activation of the Na+/H+ exchanger (NHE).8 Moreover, isolated brush border membranes9 and cultured proximal tubule cells10 from SHR exhibit higher NHE activity even during the prehypertensive state, which is consistent with enhanced proximal tubular Na+ reabsorption as a cause of hypertension in SHR. These observations suggest that there may be an underlying defect in NHE activity (basal and Ang II–stimulated) that may be responsible for enhanced Na+ reabsorption in SHR. Interestingly, mitogen-activated protein kinases (MAPKs) have been linked to Na+ homeostasis, although the nature of this connection has not been completely unraveled. For

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example, extracellular signal-regulated kinase (ERK) activation is a major mediator of growth factor–induced activation of NHE-1. Moreover, cells that overexpress a dominant negative mutant of MAPK have a 50% reduction in NHE activity.

Because MAPKs have been linked to regulation of NHE activity and epithelial cell Ang II signaling, the present studies were designed to test the hypothesis that enhanced basal and/or Ang II–stimulated activity of MAPKs in proximal tubular epithelium may be associated with SHR cells. Basal levels as well as stress- and Ang II–dependent activation of endogenous ERK1/2, c-jun N-terminal kinase (JNK1), and p38 were assessed in renal cortex specimens, and well-differentiated, proximal tubule cell lines were derived from SHR and WKY.

### Methods

#### Renal Cortex Specimens

Male 4- to 8-week-old WKY and SHR (Charles River Laboratories, Wilmington, Mass) were anesthetized with pentothal (40 mg/kg body weight), and their kidneys were perfused through the renal artery with DMEM/F12 (1:1 vol/vol) medium supplemented with 5% fetal bovine serum, 5 μg/mL transferrin, 5 μg/mL insulin, 10 ng/mL epithelial growth factor, 4 μg/mL dexamethasone, and 2 mM/L L-glutamine (rat–renal tubular epithelial [RTE] medium). After complete washout of blood, small pieces of outer renal cortex were excised and promptly frozen in liquid N2 until use. To measure protein levels and activities of MAPK, the frozen specimens were homogenized on ice in lysis buffer followed by centrifugation at 14 000×g for 10 minutes at 4°C. Aliquots of 100 μg of protein from the harvested supernatants were used for immunoprecipitation of ERK, and JNK; and for Western blot analyses of JNK, p38, and phosphorylated p38. Fifty micrograms of lysate protein was used for Western blot analysis of ERK.

#### Protein Determination

Protein concentrations were measured by the BCA method (Pierce Chemical Co).

#### Cell Culture

Immortalized epithelial cell lines derived from renal proximal tubules of normotensive WKY 1292 (clone 8) and SHR 0193 (clone 2) rats were grown to confluence on Ethicon collagen-coated 30-mm Millicell-CM culture plate inserts. The culture medium was rat-RTE.

#### Monolayer Resistance

Confluence of monolayers was assessed quantitatively by measuring the electrical resistance with a Millicell ERS probe as previously described. The electrical resistance of the monolayers for the reported experiments was 510±18 Ωcm and 390±17 Ωcm for the SHR and WKY cell lines, respectively, which is in accordance with the values reported previously.

#### [35S]-Labeling

Cells were uniformly labeled by growing them for 8 to 12 hours in methionine-free rat-RTE medium in the presence of 100 μCi/mL of [35S]-l-methionine (1000 Ci/mmol, New England Nuclear [NEN]).

#### MAPK Activity

MAPK protein levels and activities were measured in confluent, polarized monolayers. Before each experiment, serum and EGF were omitted from the medium for 1 night. Stimuli were added to either the inside or the outside of the insert to differentially stimulate the apical or basolateral side of the polarized monolayers, respectively. JNK, and ERK, activities were measured by immunoprecipitation, with polyclonal antibodies against JNK, and ERK, and immune-complex kinase assays as previously described. The anti-JNK antibody recognized all JNK isoforms, and the anti-ERK antibody recognized both ERK and ERK, but preferentially bound to ERK. The activated form of p38 was measured by immunoblotting with anti-phospho p38 antibody.

#### Western Blot Analysis

Cell lysates containing 50 to 100 μg proteins were subjected to 8% SDS-polyacrylamide gel electrophoresis (PAGE) and proteins were then transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting. The blots were treated with rabbit polyclonal antibodies against JNK, ERK, or p38 overnight at 4°C. Immunoreactive proteins were detected by enhanced chemiluminescence. The intensities of the bands corresponding to MAPKs were quantified by densitometric analysis (scanned on UMAX MagicScan with Adobe Photoshop and analyzed with the software package IMAGE, United States Biochemical).

#### Statistical Analysis

Results are expressed as mean±SEM for (n) experiments with duplicate measurements. Differences between groups were tested for significance by Student’s t test for unpaired data, and a P value <0.05 was considered significant.

#### Materials

Purified rabbit IgG, anti-rabbit IgG coupled to agarose beads, myelin basic protein, PMSF, dithiothreitol, R24571, okadaic acid, and aminosycon were purchased from Sigma Chemical Co. [γ32P]ATP and [35S]-l-methionine were from NEN. Rabbit polyclonal antibodies against JNK, ERK, and p38 as well as the recombinant activating factor 2 (ATF-2) protein were purchased from Santa Cruz Biotechnology, Inc. Anti-phospho p38 polyclonal antibody was from New England Biolabs. Aprotinin, leupeptin, fetal bovine serum, and trypsin-EDTA were purchased from Boehringer Mannheim. Collagen dispersion was from Ethicon, Inc. Millicell-CM culture plate inserts (diameter 30 mm) were from Millipore. Acrylamide, TEMED, ammonium persulfate, and Coomasie brilliant blue were from Bio-Rad Laboratories. [Sar]-Ang II was from Upstate Biotechnology.

#### Results

### MAPK Expression and Activity in Renal Cortex of WKY and SHR

A comparison of the relative concentrations and activities of different MAPKs was performed in freshly isolated renal cortex from 5-week-old WKY and SHR. ERK1/2 and JNK, activities were measured by immunocomplex kinase assays, whereas p38 activity was quantified as a phosphorylated isoform of the enzyme on Western blots. The activities were normalized to lysate protein. The results are summarized in Table 1. No differences were found in the activities of ERK1/2, JNK, and p38 between SHR and WKY. However, when MAPK protein levels were measured by Western blot analysis and normalized to actin levels, smaller amounts of JNK and ERK were found in SHR than in WKY, whereas ERK and p38 protein expression levels appeared to be identical in both strains (Table 1).

Taken at face value, the observation of similar levels of MAPK activities in SHR and WKY renal cortex seems inconsistent with the hypothesis that these enzymes are responsible for elevated Na transport in the proximal tubules in SHR. However, the finding that the fraction of activated to total ERK and JNK was higher in SHR than in WKY suggests intrinsic strain differences that complicate the interpretation of the results. To get insight into the cellular basis of
the differences between SHR and WKY strains and to study MAPKs under more controlled conditions than is possible in vivo, further experiments were performed with proximal tubule cell lines derived from these 2 rat strains.8

MAPK Activities in WKY and SHR Cell Lines

Basal Activities
Similar to renal cortex specimens, baseline activity of ERK1/2 and JNK, were the same in both cell lines (ERK1, 1026 ± 200 cpm and 830 ± 95 cpm with n = 5; JNK1, 128 ± 9 cpm and 122 ± 15 cpm with n = 9, for WKY and SHR, respectively).

Stimulated JNK1 Activity
JNK1 is activated by a number of different stresses, including application of certain drugs, such as anisomycin.18 First, we evaluated whether differences existed between apical and basolateral stimulation in response to anisomycin because epithelial monolayers often exhibit different properties when stimulated from different sides.19,20 Activation of JNK1 by apical application was significantly greater than by basolateral stimulation in response to anisomycin because epithelial monolayers often exhibit different properties when stimulated from different sides.19,20 Activation of JNK1 by apical application was significantly greater than by basolateral application, as shown for WKY monolayers in Figure 1A. Furthermore, 100 nmol/L anisomycin stimulated JNK1 more in WKY cells than in SHR cells, although basal levels of JNK1 activity were the same. Figure 1B illustrates the time-dependence of activation in WKY cells whereby the maximal activity was observed at 15 minutes with stimulation of 300% over basal levels. In contrast, anisomycin did not significantly stimulate JNK1 in the SHR cells (Figure 1B) as the increase was ~30%. At a higher concentration of 500 nmol/L, anisomycin was able to increase JNK1 activity in SHR cells, although this activation was still considerably less than in WKY cells (179 ± 45% and 444 ± 80% increase over basal activity at 15 minutes for SHR and WKY, respectively, n = 3).

We next compared the response to Ang II, which represents a physiological stimulus involved in the regulation of salt reabsorption. Experiments throughout this study were performed with [Sar]-Ang II, although the plain term Ang II is used. At 0.1 μmol/L, Ang II significantly stimulated JNK1 activity in the WKY cell line (Figure 2a). Interestingly, the time course of activation was different with apical and basolateral stimulation. With basolateral stimulation, JNK1 was maximally activated within 5 minutes, whereas with apical stimulation, activation increased more slowly and became stable at 30 minutes. Moreover, the Ang II stimulation of JNK1 activity in WKY cells was concentration dependent with maximal activation at 1 μmol/L [Sar]-Ang II (50% over basal levels, Figure 2b). Interestingly, Ang II did not change JNK1 activity in SHR cells (Figure 2c), even at concentrations as high as 10 μmol/L (data not shown). To test whether the differences can be explained on the basis of regulation of MAPKs by phosphatases,21 JNK1 activity was measured in the presence of phosphatase inhibitors. The addition of R24571 (10 μmol/L) and okadaic acid (100 nmol/L), which inhibit phosphatase 2B and phosphatases 1 and 2A, respectively, did not augment JNK1 activation by Ang II, suggesting that phosphatases are not responsible for the weaker JNK1 activation in SHR cells (Table 2).

ERK. Activity in WKY and SHR Proximal Tubule Cells
Because Ang II has been reported to stimulate ERK in rabbit proximal tubular epithelial14 and opossum kidney epithelial
cells and has been suggested to be an important signaling modulator of ion transport, the ability of Ang II to stimulate ERK 1 in proximal tubule cell lines was assessed. Ang II stimulated ERK 1 activity in a time- and concentration-dependent manner in confluent WKY epithelial monolayers. Significant stimulation of ERK 1 activity was already seen by 5 minutes when Ang II (0.1 μmol/L) was added to the basolateral side, an effect that persisted for at least 15 minutes (data not shown). Dose-response relationships differed be-

### MAPKs Levels in SHR and WKY Proximal Tubule Cell Lines

One reason JNK 1 and ERKs were not activated in SHR cells by either stress or Ang II might be that the protein levels of these particular MAPKs were reduced in SHR; thus, the fractional activation under basal conditions was already close to the maximum. This explanation is supported by the findings in cortex specimens (ie, the greater fractional activation in SHR reported above). Therefore, the protein levels of MAPKs were also determined in the cell lines by immunoblot analysis.

Interestingly, SHR cells contained lower levels of both JNK 1 isoforms (p46 and p54), than WKY cells. Figure 4a illustrates representative data for the p46 JNK 1 isoform. Densitometric analysis of all immunoblots performed showed that, despite using the same amount of protein from cell lysates, JNK 1 expression in SHR cells was approximately one fourth of that observed in WKY cells (SHR/WKY = 0.25 ± 0.03 densitometric units, P < 0.01, n = 3). A second approach to measure JNK 1 expression was to label cells for 8 to 12 hours with [35S]-methionine, immunoprecipitate JNK 1, and then visualize it by means of autoradiography. Again, [35S]-methionine–labeled JNK 1 was less in SHR than in WKY cells (Figure 4b).

The same experimental approach was used to measure ERK 1 expression. As shown in Figure 4c, Western blot analysis revealed a lower amount of ERK 1 in SHR compared with WKY cells. Densitometric analysis produced a ratio of 0.58 ± 0.15 for ERK 1 expression in SHR relative to WKY cells (P < 0.05, n = 4). The anti-ERK 1 antibody also recognized ERK 2; however, the expression of ERK 2 was the same in both cell lines. Similar results were obtained with [35S]-methionine labeling (Figure 4d).

### p38 MAPK Activity and Expression

The levels of activated p38 were determined by immunoblotting with anti-phospho p38 antibody. In contrast to what was
found for JNK$_1$ and ERK$_1$, p38 activity appeared the same in WKY and SHR cells stimulated for 20 minutes with 100 nmol/L anisomycin (Figure 5a). Moreover, immunoblot analysis did not reveal any difference in the amount of this member of the MAPK superfamily (Figure 5b). The ratio of protein levels of ERK$_1$ in SHR/WKY was 1.00$\pm$0.05 $(n=3)$.

**Discussion**

The present studies were aimed at testing the hypothesis that proximal tubule cells from SHR possess enhanced basal or stimulated activity of some members of the MAPK superfamily as compared with WKY. Such enhanced activity could be an explanation for enhanced Na$^+$ reabsorption. Previous studies from this laboratory had documented enhanced Ang II–stimulated activity of NHE and Na$^+$ reabsorption in SHR as compared with WKY epithelial cell lines. Others have documented that NHE is regulated by members of the MAPK superfamily. This report represents an assessment of activity and expression levels of many members of the MAPK superfamily comparing renal cortex specimens and proximal tubule cells from SHR and WKY. With the same cell lines and culture conditions (growth on filters, same medium) as used for ion transport earlier, we observed that the basal activities of ERK$_{1,2}$, p38, and JNK$_1$ were similar in these cells. However, stimulation by Ang II or anisomycin was reduced in SHR. This lower capacity for activating both ERK$_1$ and JNK$_1$ may be a result of the lower amount of enzymes expressed. Greater fractional activation of ERK$_1$ and JNK$_1$ in SHR was seen in both experimental preparations used (cell lines, cortex) under baseline conditions (no stimuli), suggest-
ing that the cell lines reflect in vivo behavior and are useful model systems.

Activation of MAPKs had been associated with higher NHE activity.\textsuperscript{9–14} Therefore, the results also suggest that neither ERK\textsubscript{1} or JNK\textsubscript{1} can be responsible for enhanced activation of NHE by Ang II in SHR epithelial cells. This conclusion differs strikingly from vascular smooth muscle cells, wherein increased activity of ERKs or a difference in the time-dependency of activation was observed in SHR as compared with WKY. Moreover, the differences in VSMC were due to altered regulation of MAPK phosphorylation and dephosphorylation rather than a difference in the relative abundance of the enzyme.\textsuperscript{22,23} Of interest is the observation that p38 and ERK\textsubscript{2} levels were the same in both the cell lines and the tissue samples, which demonstrated that deficit protein expression does not involve all members of the MAPK superfamily.

The pathogenesis and pathophysiology of essential hypertension are complex and are influenced by many interrelated factors.\textsuperscript{24} The kidney is central to the pathogenesis of high blood pressure in salt-sensitive individuals, because its dominant role in the regulation of Na\textsuperscript{+} homeostasis. Studies on SHR suggest proximal tubular abnormalities in several signal transduction pathways, apart from MAPKs, that can affect NHE-3 activity and Na\textsuperscript{+} absorption.\textsuperscript{5–7} Our data document comparable basal levels of JNK\textsubscript{1} and ERK\textsubscript{1} activities and deficient activation in SHR cells and therefore suggest that the MAPK superfamily is unlikely to be a crucial regulatory factor for enhanced activity of proximal tubular NHE-3. This conclusion leaves other altered signaling pathways as possible explanations, such as defective receptor/G-protein coupling. Evidence for an abnormal G-protein coupling in SHR is emerging in the case of D\textsubscript{1} agonist inhibition of NHE-3.\textsuperscript{3,25}

The present study demonstrates different time-course and dose-response relationships when Ang II was added to the apical versus basolateral side of the polarized monolayer. This is not surprising given that the Ang II receptor substrates differ in the 2 compartments.\textsuperscript{18,26,27} The apical angiotensin type 2 (AT\textsubscript{2}) receptor is linked to phospholipase A\textsubscript{2} and arachidonic acid and inhibits Na\textsuperscript{+} reabsorption. Arachidonic acid is also critical for MAPK activation.\textsuperscript{14,15} By contrast, the basal angiotensin type 1 (AT\textsubscript{1}) receptor has been linked to enhanced Na\textsuperscript{+} reabsorption, decrements in cAMP, and activation of MAPK as well.\textsuperscript{28–30} The mechanism of AT\textsubscript{1}-mediated MAPK activation has not been determined. Several reports have documented that the AT\textsubscript{1} receptor increases activity of NHE,\textsuperscript{31,32} whereas the AT\textsubscript{2} receptor decreases activity of the NHE-3.\textsuperscript{33} However, the importance of MAPKs as modulators of ion transport under physiological circumstances has not been resolved. This question remains of significant potential interest despite the exclusion of MAPKs as crucial factors for increasing proximal tubule Na\textsuperscript{+} transport in SHR.

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