Angiotensin II Stimulation of Renal Epithelial Cell Na/HCO₃ Cotransport Activity: A Central Role for Src Family Kinase/Classic MAPK Pathway Coupling

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Abstract. Angiotensin II (AII) plays an important role in renal proximal tubular acidification via the costimulation of basolateral Na/HCO₃ cotransporter (NBC) and apical Na/H exchanger (NHE) activities. These effects are mediated by specific G protein-coupled AII receptors, but their corresponding downstream effectors are incompletely defined. Src family tyrosine kinases (SFKs) contribute to the regulation of both transport activities by a variety of stimuli and are coupled to classic mitogen-activated protein kinase (MAPK) pathway activation in this cell type. We therefore examined these signaling intermediates for involvement in AII-stimulated NBC activity in cultured proximal tubule cells. Subpressor concentrations of AII (0.1 nM) increased NBC activity within minutes, and this effect was abrogated by selective antagonism of AT₁ angiotensin receptors, SFKs, or the classic MAPK pathway. AT₁ directly activated Src, as well as the proximal (Raf) and distal (ERK) elements of the classic MAPK module, and the activation of Src was prevented by AT₁ receptor antagonism. An associated increase in basolateral membrane NBC1 content is compatible with the involvement of this proximal tubule isofrom in these changes. We conclude that AII stimulation of the AT₁ receptor increases NBC activity via sequential activation of SFKs and the classic MAPK pathway. Similar requirements for SFK/MAPK coupling in both cholinergic and acidic costimulation of NBC and NHE activities suggest a central role for these effectors in the coordinated regulation of epithelial transport by diverse stimuli.

Key words: Angiotensin II | Proximal tubular epithelial cell | Src family kinases | Extracellular signal-regulated kinases | Signal transduction | Na/HCO₃ cotransport | Intracellular pH regulation | AT₁ receptor

Introduction

In addition to its classic role as a renal vasoconstrictor, angiotensin II (AII) is a potent stimulator of proximal tubule acidification and Na reabsorption (Harris & Young, 1977; Schuster, Kokko & Jacobson, 1984; Liu & Cogan, 1987). These tubular effects are observed at suppressor AII concentrations (<1 nM) and reflect the direct stimulation of transepithelial NaHCO₃ flux in this nephron segment. Both basolateral Na/HCO₃ cotransport (NBC) and apical Na/H exchange (NHE) play major roles in the regulation of this flux, and the corresponding transporters represent important target effectors of AII action in the proximal tubule. The ability of AII to concomitantly stimulate both NBC and NHE activities in isolated perfused proximal tubules (Geibel, Giebisch & Boron, 1990) is compatible with such an interpretation. AII increases NBC activity in both proximal tubule (Geibel et al., 1990; Coppola & Frömter, 199a, 1994b) and cortical basolateral membrane vesicle preparations (Eiam-Ong et al., 1993; Ruiz et al., 1995a), effects that uniformly mimic those of AII on NHE3 activity in similar models.
(Arruda & Ruiz, 1991). AII has also been shown to stimulate NHE3 activity in both primary (Saccomani, Mitchell & Navar, 1990; Houillier et al., 1996) and continuous cultures of proximal tubule cells (Cano et al., 1994), but, to our knowledge, such a demonstration has not yet been reported for NBC. Thus, to better understand the molecular mechanisms underlying AII-stimulated NBC activity and to identify signaling intermediates contributing to this effect, we examined the ability of AII to stimulate NBC activity in the OK (American opossum kidney) proximal tubule cell line. We also confirmed the expression and basolateral localization of the proximal tubule NBC1 isoform in this cell culture model.

The parallel regulation of proximal tubule cell NBC and NHE activities by diverse stimuli suggests common regulatory mechanisms. Acute acidosis, for example, has been shown to increase both transport activities via activation of nonreceptor Src family tyrosine kinases (SFKs). In the case of NBC activity, the classic mitogen-activated protein kinase (MAPK) pathway has been shown to couple SFK activation by acidosis to increased transporter activity (Ruiz et al., 1999). We have also recently demonstrated that cholinergic stimulation is similarly coupled to NBC activation in these cells (Robey et al., 2001). These findings suggest a central role for SFKs and the classic MAPK pathway in the regulation of NBC activity by diverse stimuli, so we also examined the role of these signaling intermediates in AII-stimulated NBC activity in OK cells.

**Materials and Methods**

**REAGENTS**

The pH-sensitive fluorophore 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was obtained as the cell-permeable acetoxymethyl ester (BCECF-AM) from Molecular Probes (Eugene, OR), and amiloride was purchased from Research Biochemicals (Natick, MA). Irbesartan, an AT1-selective non-peptide AII receptor antagonist (SR 47436; BMS 186295; 2-n-butyl-3-((2′-(1H-tetrazol-5-yl)-1′,1′-biphenyl-4-yl)methyl)-1,3-diazaspiro [4,4]non-1-en-4-one), was obtained from Bristol-Myers Squibb (Princeton, NJ). Herbigycin A, PD98059 (2′-amino-3′-methoxyflavone), PPI (4-amino-5-(4-methylphenyl)-7-(2-butyloxy)pyrazolo[3,4-d]-pyrimidin), PPI (4-amino-7-phenylpyrazolo[3,4-d]-pyrimidin), and hygromycin B were obtained from Calbiochem (San Diego, CA), and recombinant phosphotyrosine-specific RC20 antibodies were purchased from BD Transduction Laboratories (Lexington, KY). ERK1/2 and Src-specific antibodies were obtained from Upstate Biotechnology (Lake Placid, NY), as were the SFK, ERK1/2, and Raf-1 kinase assay kits employed herein. All immunobots were analyzed using commercially available chemiluminescence detection systems from either New England Biolabs (Phototope®; Beverly, MA) or Amersham Pharmacia Biotech (ECL™, Arlington Heights, IL). Immunoblotting reagents, including nitrocellulose membranes, were routinely obtained from BioRad (Hercules, CA), and cell culture reagents, including serum and media additives, were obtained from Invitrogen (Grand Island, NY). All other reagents, including synthetic human AII, were obtained from Sigma (St. Louis, MO) unless noted otherwise.

**CELL CULTURE**

Mycoplasma-free American opossum kidney (OK) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) at passage 37. Cell monolayers were maintained in a humidified 37°C/5% CO2 incubator in Eagle’s minimum essential medium containing Earle’s salts and supplemented with 10% fetal bovine serum (FBS). All experiments were performed on newly confluent cell monolayers between passages 38 and 48 to minimize the effects of phenotypic variation in continuous culture. Cells were also routinely serum-deprived for 24 hr prior to and during testing.

**FLUOROMETRIC ASSAYS OF pHt AND NBC ACTIVITY**

Cell monolayers cultured on uncoated plastic coverslips were loaded with BCECF-AM and continuously monitored for pH-dependent changes in BCECF fluorescence as described previously (Robey et al., 1998). In brief, cells were perfused with a Cl-free physiologic solution (in mM): 25 NaHCO3, 110 sodium gluconate, 5 potassium gluconate, 2 CaSO4, 0.5 MgSO4, 1 M K2HPO4, 10 glucose, and 9 HEPES, pH 7.40 supplemented with 1 mM amiloride to minimize the contributions of cellular Cl/HCO3 and Na/H exchange activities. All experiments were performed at 37°C, and extracellular pH was maintained constant at 7.40 throughout. The equiluminal substitution of choline for Na uniformly resulted in immediate decreases in both pHt and pH-sensitive BCECF fluorescence. Upon the reintroduction of Na, fluorescence rapidly and fully recovered, and NBC activity was taken as the initial rate of this recovery. In the absence of chloride, recovery is primarily attributable to NBC activity (Alpern, 1985; Ruiz et al., 1995b). pH-sensitive BCECF fluorescence at 510 nm was routinely calibrated at the completion of each experiment in the presence of elevated extracellular potassium and the ionophore nigericin (to equilibrate intracellular and extracellular pH). All measurements were performed by dual-wavelength monitoring and ratiometric analysis at pH-sensitive (500 nm) and pH-insensitive (450 nm) excitation wavelengths (I500/I450). By convention, results are presented as both absolute (ΔpHt/Δt) and relative (% change in ΔpHt/Δt) rates of change in pHt. However, it is pertinent to note that changes in pH are exponentially related to changes in [H+] (pH = −log[H+]), so the latter expressions of per cent change have limited quantitative utility.

**ECTOPIC CARBOXYTERMINAL SRC KINASE (CSK) EXPRESSION**

OK cells stably overexpressing carboxyterminal Src kinase (Csk) have been characterized previously (Ruiz et al., 1999) and were uniformly grown to confluence in normal growth medium supplemented with selection antibiotic (100 μg/ml hygromycin B) before testing. Where appropriate, Csk overexpression was confirmed by immunoblot analysis as described previously (Ruiz et al., 1999).

**SRC PHOSPHORYLATION AND KINASE ACTIVITY ASSAYS**

Src phosphorylation was assessed by quantitative immunoblot analysis of whole-cell lysates as described previously (Ruiz et al., 1999). In these studies, both total Src abundance and Src tyrosine