Abstract—Activation of the Na+/H+ exchanger isoform-1 (NHE-1) by angiotensin II is an early signal transduction event that may regulate vascular smooth muscle cell (VSMC) growth and migration. Many signal transduction events stimulated by angiotensin II are mediated by the mitogen-activated protein (MAP) kinases. To define their roles in angiotensin II–mediated NHE-1 activity, VSMCs were treated with angiotensin II and the activities of p38, c-Jun N-terminal kinase (JNK), and extracellular signal–regulated kinases 1 and 2 (ERK1/2) were measured. Angiotensin II rapidly (peak, 5 minutes) activated p38 and ERK1/2, whereas JNK was activated more slowly (peak, 30 minutes). Because angiotensin II stimulated Na+/H+ exchange within 5 minutes, the effects of p38 and ERK1/2 antagonists on Na+/H+ exchange were studied. The MEK-1 inhibitor PD98059 decreased ERK1/2 activity and Na+/H+ exchange stimulated by angiotensin II. In contrast, the specific p38 antagonist SKF-86002 increased Na+/H+ exchange. Two mechanisms were identified that may mediate the effects of p38 and SKF-86002 on angiotensin II–stimulated Na+/H+ exchange. First, angiotensin II activation of ERK1/2 was increased 1.5- to 2.5-fold (depending on assay technique) in the presence of SKF-86002, demonstrating that p38 negatively regulates ERK1/2. Second, the ability of angiotensin II–stimulated MAP kinases to phosphorylate a glutathione S-transferase fusion protein containing amino acids 625 to 747 of NHE-1 in vitro was analyzed. The relative activities of endogenous immunoprecipitated p38, ERK1/2, and JNK were 1.0, 2.0, and 0.05 versus control, respectively suggesting that p38 and ERK1/2, but not JNK, may phosphorylate NHE-1 in VSMC. These data indicate important roles for p38 and ERK1/2 in angiotensin II–mediated regulation of the Na+/H+ exchanger in VSMC. (Circ Res. 1998;83:824-831.)

Key Words: mitogen-activated protein kinase • Na+/H+ exchange • angiotensin II • vascular smooth muscle

Angiotensin II is a multifunctional agonist for vascular smooth muscle cells (VSMC), stimulating ion fluxes, protein phosphorylation, contractility, gene expression, and cell growth. Our laboratory has characterized the signal transduction events stimulated by angiotensin II in VSMC to gain insight into its mechanisms of action. Many of the signal transduction events stimulated by angiotensin II are mediated by members of mitogen-activated protein (MAP) kinase family of protein kinases. Characterization of substrates for the MAP kinases should provide important insights into the mechanisms by which angiotensin II regulates VSMC function.

The Na+/H+ exchanger isoform-1 (NHE-1) is an important membrane protein whose activity may be regulated by protein kinases activated by angiotensin II. Stimulation of Na+/H+ exchange by angiotensin II is an early event that is required for VSMC growth, migration, and contraction. In fact, inhibiting Na+/H+ exchange with specific amiloride-derivative antagonists decreases neointimal proliferation in the rat carotid injury model. The Na+/H+ exchanger is a phosphoprotein, and growth factors (including angiotensin II; E.T. and B.C.B., unpublished observations, 1998) have been shown to increase phosphorylation of specific NHE-1 tryptic peptides. Of interest, Na+/H+ exchange activity and phosphorylation of NHE-1 are increased in VSMC isolated from the spontaneously hypertensive rat compared with the normotensive Wistar-Kyoto rat. Based on these findings, it has been suggested that an abnormality in an NHE-1 kinase may be pathogenic in this model of genetic hypertension.

We have reported previously that a 90-kd kinase identified as p90RSK is stimulated by angiotensin II and can phosphorylate NHE-1 in vitro. Because p90RSK activity is regulated by extracellular signal–regulated kinases 1 and 2 (ERK1/2), it appears likely that the MAP kinases are important in regulating angiotensin II–mediated activation of NHE-1.

Our laboratory has shown that angiotensin II stimulates at least 2 members of the MAP kinase family (ERK1/2) and
creased 1.5- to 2.5-fold (depending on assay technique) in the finding that angiotensin II activation of ERK1/2 was in-
cross-talk between ERK1/2 and p38 was suggested by the
angiotensin II, only p38 and ERK1/2 exhibited significant
recombinant NHE-1, and examined the effects of inhibiting
angiotensin II, determined their activity as kinases toward
kinases activities of p38, ERK1/2, and JNK in response to
stimulation of NHE-1 in VSMC, we measured the relative
the roles of the MAP kinases in angiotensin II–mediated
effects of angiotensin II on p38 remain poorly characterized.14,15 Because p38 is
involved in the adaptation to osmotic stress, it may be
involved in the upstream regulators of NHE-1 by either directly phosphory-
lating NHE-1,16,17 or phosphorylating other kinases or regulatory proteins,16,18,19 that modulate NHE-1 activity. To define the roles of the MAP kinases in angiotensin II–mediated stimulation of NHE-1 in VSMC, we measured the relative kinases activities of p38, ERK1/2, and JNK in response to angiotensin II, determined their activity as kinases toward recombinant NHE-1, and examined the effects of inhibiting p38 and ERK1/2 on regulation of intracellular pH. We found that whereas p38, ERK1/2, and JNK were activated by angiotensin II, only p38 and ERK1/2 exhibited significant activity as NHE-1 kinases in vitro. Physiologically relevant cross-talk between ERK1/2 and p38 was suggested by the finding that angiotensin II activation of ERK1/2 was increased 1.5- to 2.5-fold (depending on assay technique) in the presence of the p38 inhibitor SKF-86002.

Materials and Methods

Cell Culture

VSMCs were isolated from the aortae of 200- to 250-g male Sprague-Dawley rats (Harlan, Indianapolis, Ind) and maintained in 10% calf serum (Hyclone, Gaithersburg, Md)/DMEM as described previously.20 Passages 5 to 15 VSMC at 70% to 80% confluence were growth arrested by induction in DMEM supplemented with 0.4% calf serum for 48 hours before use.

Immunoprecipitation and Western Blot Analysis

Cells were harvested in a lysis buffer containing 10 mmol/L HEPES, pH 7.4, 50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 μmol/L NaVO₃, 0.5 mmol/L PMSE, 10 μg/mL leupeptin, and 0.1% Triton X-100. Western blot analysis, SDS-PAGE, transfer to nitrocellulose, and detection by chemiluminescence were performed as described.20 p38, ERK1/2, p90RSK, and JNK were immunoprecipitated from 200 μg protein by incubation for 1 hour at 4°C with polyclonal antibodies (Santa Cruz; p38 antibody was from J.H.) and an additional 2 hours’ incubation with protein A or protein G-Sepharose (Gibco-BRL). The immunoprecipitates were washed 2 times with 1 mL lysis buffer, 2 times with 1 mL LiCl wash buffer (500 mmol/L LiCl, 100 mmol/L Tris-HCl, pH 7.6, 0.1% Triton X-100, 1 mmol/L DTT), and 2 times in 1 ml. Buffer A (HEPES 20 mmol/L, pH 7.2, 2 mmol/L EGTA, 10 mmol/L MgCl₂, 1 mmol/L DTT, 0.1% Triton X-100).

Protein Phosphorylation

Kinet assays were performed using bacterially expressed p38 and ERK2 or immunoprecipitates of p38 and ERK1/2. JNK was assayed by an affinity complex kinase assay using glutathione S-transferase (GST)-Jun. His-p38 was prepared using expression vector pET14b and a polymerase chain reaction (PCR) fragment containing the coding region of p38 and ERK2 cDNA. His-p38 was purified by affinity chromatography using His-‐Bind metal chelation resin (Novagen). Protein concentrations were checked by Coomassie staining of SDS-PAGE–separated proteins. Precipitated kinases or His-p38 were re suspended in 25 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl₂, and 10 mmol/L MnCl₂. The kinase reaction then was initiated by addition of 5 μg substrate protein, 15 μmol/L ATP, and 0.5 μCi/mL of 32P-γ-ATP (final volume, 30 μL), and the reaction proceeded for 10 minutes at 30°C. The phosphorylation reaction was terminated by addition of Laemmli sample buffer, and proteins were analyzed on 15% SDS-PAGE, followed by autoradiography. The radioactivity in the spot corresponding to substrate protein was determined by densitometry (in the linear range of film exposure) using NIH Image 1.60.

Intracellular pH Measurement

Na⁺/H⁺ exchange was determined by ethylisopropyl amiloride (EIPA)–sensitive intracellular pH (pHᵢ) recovery after acid loading as previously described using BCECF fluorescence.12 Cells were grown on coverslips loaded with 3 μmol/L BCECF, and alkalized with 20 mmol/L NH₄Cl as described in a HEPES-Tris–balanced salt solution containing 130 mmol/L NaCl, 5 mmol/L KCl, 1.5 mmol/L CaCl₂, 1.0 mmol/L MgCl₂, and 20 mmol/L HEPES buffered to pH 7.4 at 25°C with Tris. After 5 minutes, the solution was replaced with 130 mmol/L NaCl lacking NH₄Cl to acid load the cells, and the rate of recovery was measured in pH units per minute. The rate of pHᵢ recovery was converted to mmol H⁺/min per liter cells (JᵢH) by multiplying by the buffering power. Buffering power was determined by stepwise reduction of NH₄Cl in the presence of agonists or inhibitors under conditions in which ion fluxes were completely inhibited (5 mmol/L BaCl₂, 30 μmol/L EIPA). Data were then plotted as JᵢH versus pHᵢ, enabling pHᵢo (pH for half-maximal recovery) and Vₑᵦᵢ to be calculated.

GST-NHE-1 Fusion Protein Construction and Purification

Bacterial expression plasmids containing different domains of the human NHE-1 were prepared by subcloning PCR-generated Eco RI fragments of NHE-1 cDNA (cloned in pBluescript) into pGEX-KG. Three overlapping constructs were prepared by PCR: NHE-1(516 to 630), NHE-1(625 to 747), and NHE-1(748 to 815), as previously described.12 The orientation and reading frames of all constructs were confirmed by sequencing. Three additional fusion proteins were prepared from NHE-1(625 to 747). These proteins—NHE-1(625 to 670), NHE-1(625 to 714), and NHE-1(625 to 670,714 to 747)—were prepared by digesting NHE-1(625 to 747) with the following restriction enzymes: Bse AI, Psp 5II, Bse AI, and Psp 5II, respectively. After blunting the ends, the fragments were religated and cloned in pGEX-KG. After transformation of GST-NHE-1 constructs into the BL21 strain of E. coli, cultures were grown to sublog phase and induced for 3 hours at 37°C with 1 mol/L isopropyl-β-D-thiogalactopyranoside. Cells were collected, sonicated, and centrifuged. The supernatants were incubated with glutathione-agarose for 60 minutes at 4°C. Bound fusion proteins were washed extensively and eluted with 20 mmol/L reduced glutathione, 100 mmol/L Tris-HCl, pH 7.4, and 100 mmol/L NaCl. Protein concentrations were checked by Coomassie staining of SDS-PAGE–separated proteins. NHE-1(516 to 815) was not synthesized by E. coli in the soluble fraction, so this fusion protein was purified from inclusion bodies. In brief, after induction with 1 mol/L isopropyl-β-D-thiogalactopyranoside, cells were collected, sonicated, and centrifuged. The pellet was washed once with 1 mol/L sucrose, resuspended with 10 mol/L Tris, pH 7.4, 2% Triton X-100, 5 mmol/L EDTA, and 100 mmol/L NaCl and was incubated overnight at 4°C. After centrifugation, the pellet was resuspended with 3% SDS, and the SDS then was removed by chromatography on Extracti-Gel D Detergent Removing gel (Pierce). Protein concentrations were determined by Coomassie staining of SDS-PAGE–separated proteins.

Statistical Analysis

For experiments performed at least 3 times, results were compared by Student’s t test, with a difference of P<0.05 considered significant.
Results

p38 Is Present in VSMC
To verify that p38 was present in VSMC and that the antibody used was specific, Western blot analysis was performed on whole-cell lysates. A single band of ~43 kd (Figure 1) was detected, which is the same mass as previously reported in other cell types. Immunoprecipitation using this antibody yielded predominantly a 43-kd protein, whereas preimmune serum failed to precipitate this protein (not shown).

Angiotensin II Stimulates p38, ERK1/2, and JNK Activity With Different Time Courses
We have shown previously that angiotensin II rapidly stimulates ERK1/2 in VSMC with peak activation at 5 minutes. To compare activation of MAP kinase family members, we determined the time course for activation of p38, ERK1/2, and JNK by angiotensin II. Angiotensin II (100 nmol/L) stimulated a rapid increase in p38 with peak at 5 minutes (Figure 2A). Angiotensin II also stimulated a rapid increase in ERK1/2 (peak, 5 minutes; Figure 2B), which returned much more slowly to baseline than p38 (60 minutes versus 15 minutes). In contrast, angiotensin II activation of JNK was much slower (peak, 30 minutes; Figure 2C). A more detailed analysis of ERK1/2 and p38 was performed to determine the precise time course for activation and their relationship. Both p38 and ERK1/2 were activated within 2.5 minutes with peak at 5 minutes, suggesting that these 2 MAP kinases are both rapidly activated in response to angiotensin II (Figure 3). The magnitude of p38 activation by angiotensin II at the peak time (2.0-fold at 5 minutes) in VSMC was smaller than activation of ERK1/2 (4.9-fold at 5 minutes), using myelin basic protein (MBP) as substrate (Figure 3C).

Hyperosmolar stress has been shown to stimulate p38, and its mechanism of activation may be different from angiotensin II. Therefore, we compared activation of p38 and ERK1/2 by 0.4 mol/L sorbitol to activation by angiotensin II. As shown in Figure 2, 0.4 mol/L sorbitol stimulated ERK1/2, but the time course was slower (peak, 15 minutes) and more sustained (≥120 minutes) than angiotensin II. Similar to the time course for ERK1/2, sorbitol stimulated a slow activation of p38 (maximum 5.5-fold at 15 minutes) that was sustained for ≥60 minutes. Thus both angiotensin II and sorbitol stimulate ERK1/2 and p38 activity in VSMC, but activation of p38 by angiotensin II was of smaller magnitude and shorter duration than activation by sorbitol.

Effect of p38 and ERK1/2 Inhibition on Regulation of Na+/H+ Exchange
We previously have demonstrated that pH recovery in VSMC is completely dependent on Na+/H+ exchange in the absence of bicarbonate. Because the only isoform of the Na+/H+ exchanger present in VSMC is NHE-1, these cells may be used as a model to study regulation of NHE-1 activity. Both angiotensin II and sorbitol (B.C.B., unpublished data, 1998) stimulate Na+/H+ exchange in VSMC. To characterize the relative roles of the MAP kinases as potential NHE-1 kinases, we used pharmacologic concentrations of inhibitors. We focused on the roles of p38 and ERK1/2 in angiotensin II activation of NHE-1, because the increase in JNK activity (peak, 30 minutes) was much slower than stimulation of NHE-1 activity (peak, 1 to 5 minutes). We first determined the effect of the p38 inhibitor SKF-86002 on angiotensin II–stimulated Na+/H+ exchange by fluorescence pH measurement. In brief, growth-arrested VSMCs were loaded with BCECF and acid-loaded by the NH4Cl prepulse.
technique, and the rate of acid recovery was measured in the presence of 100 nmol/L angiotensin II (5-minute pretreatment) ±0.1 to 30 μmol/L SKF-86002. Angiotensin II stimulated the rate and extent of pH i recovery (Figure 4A and 4B). Angiotensin II also shifted the pH 50 to higher pH i (control = 6.51 ± 0.08; angiotensin II = 6.70 ± 0.12; Figure 4B; N = 6; P < 0.001) as shown by a shift to the right in the JH versus pH i plot, consistent with a decrease in K m for H i as previously reported. 25 SKF-86002 increased angiotensin II–stimulated Na i/H i exchange (10 μmol/L shown in Figure 4A and 4B) and pH 50 (angiotensin II + 10 μmol/L SKF-86002 = 6.82 ± 0.11; N = 6; P = 0.016 versus angiotensin II alone). There was no significant effect of SKF-86002 alone on JH versus pH i (data not shown). The time dependence for the SKF-86002 effect (1 to 120 minutes) showed that the maximal effect of 10 μmol/L SKF-86002 occurred at 5 minutes. The effect of SKF-86002 to increase pH 50 was concentration dependent with an EC 50 of 1 μmol/L (not shown). These results suggest that p38 exerts an inhibitory effect on angiotensin II stimulation of NHE-1. To study the role of ERK1/2 in angiotensin II activation of Na i/H i exchange, we used the MEK-1 inhibitor, PD98059. As previously reported by other investigators, 18,19,26,27 PD98059 inhibited Na i/H i exchange in a concentration-dependent manner with an EC 50 of ~1 μmol/L (not shown). Thus both p38 and ERK1/2 appear to be involved in angiotensin II–mediated regulation of NHE-1 activity.

Recombinant Histidine Tagged p38 (His-p38) Phosphorylates GST-NHE-1 In Vitro

The Na i/H i exchanger has been suggested to be a physiological substrate for ERK1/2 based on studies by Fliegel’s group (Wang et al 16). However, the domains of NHE-1 phosphorylated by ERK1/2 and the potential of other MAP kinases to phosphorylate the exchanger have not been well characterized. We chose to study recombinant His-p38 for these experiments because of its high level of activity as measured by phosphorylation of MBP (see below). In addition, a specific inhibitor for p38 is available (SKF-86002), whereas inhibitors that inactivate ERK1/2 and JNK directly have not been identified. We first tested the ability of His-p38 to phosphorylate the entire NHE-1 COOH tail (NHE-1[516 to 815]), which contains 25 serines and 8 threonines. Recombinant His-p38 readily phosphorylated NHE-1[516 to 815]
To characterize domains of NHE-1 phosphorylated by p38, we prepared 3 overlapping GST-NHE-1 fusion proteins (Figure 6) described as NHE-1(516 to 630), NHE-1(625 to 747), and NHE-1(748 to 815). When utilized as substrates for His-p38, only NHE-1(625 to 747) was phosphorylated in vitro (Figure 7). This region of the NHE-1 COOH tail contains several prolines near serine/threonine residues (T686, S694, T696, S703, S724, and S727) that may serve as phosphorylation motifs for serine/threonine kinases such as MAP kinase family members. Based on the location of these prolines, we constructed 3 additional GST-NHE-1 fusion proteins (Figure 6) overlapping NHE-1(625 to 747): NHE-1(625 to 670), NHE-1(625 to 714), and NHE-1(625 to 670,714 to 747). Recombinant His-p38 phosphorylated NHE-1(625 to 714) but did not phosphorylate NHE-1(625 to 670) or NHE-1(625 to 670,714 to 747) (Figure 7). These results indicate that p38 phosphorylated serine/threonine residues located between amino acids 671 and 714 of NHE-1.

Specificity of NHE-1(625 to 747) as a p38 Substrate
To confirm the specificity of NHE-1(625 to 747) phosphorylation by p38, we prepared 3 overlapping GST-NHE-1 fusion proteins (Figure 6) described as NHE-1(516 to 630), NHE-1(625 to 747), and NHE-1(748 to 815). When utilized as substrates for His-p38, only NHE-1(625 to 747) was phosphorylated in vitro (Figure 7). This region of the NHE-1 COOH tail contains several prolines near serine/threonine residues (T686, S694, T696, S703, S724, and S727) that may serve as phosphorylation motifs for serine/threonine kinases such as MAP kinase family members. Based on the location of these prolines, we constructed 3 additional GST-NHE-1 fusion proteins (Figure 6) overlapping NHE-1(625 to 747): NHE-1(625 to 670), NHE-1(625 to 714), and NHE-1(625 to 670,714 to 747). Recombinant His-p38 phosphorylated NHE-1(625 to 714) but did not phosphorylate NHE-1(625 to 670) or NHE-1(625 to 670,714 to 747) (Figure 7). These results indicate that p38 phosphorylated serine/threonine residues located between amino acids 671 and 714 of NHE-1.

Figure 6. Schema for constructs of human NHE-1. A total of 6 GST-fusion protein constructs were prepared spanning amino acids 516 to 815 of human NHE-1.
In the presence of vehicle (DMSO) demonstrating that angiotensin II stimulation of p38 is associated with inhibition of ERK1/2 activity. To confirm that the increase in ERK1/2 activity after treatment with SKF-86002 (measured by immune complex MBP phosphorylation) was not due to an associated protein in the immunoprecipitate, the same experiment was performed by in-gel kinase assay (Figure 11B). With this technique, there was a 1.5±0.2-fold greater increase in ERK1/2 activity in the presence of SKF-86002. Finally, we studied the effect of SKF-86002 on p90RSK, a putative NHE-1 kinase that is regulated by ERK1/2 (Figure 11C). VSMCs were stimulated by angiotensin II±1 μmol/L SKF-86002, p90RSK was immunoprecipitated, and an immune complex kinase assay then was performed with MBP as substrate (Figure 11C). As previously reported, angiotensin II stimulated p90RSK activity, which was 1.4±0.2-fold greater in the presence of SKF-86002. Addition of SKF-86002 (1, 10, or 100 μmol/L) directly to the kinase reaction caused no change in ERK1/2 or p90RSK activity, indicating that there was no direct action of the drug on ERK1/2 or p90RSK. These data indicate that stimulation of p38 by angiotensin II inhibits ERK1/2 activity and downstream kinases such as p90RSK.

**Discussion**

This study establishes a functional role for p38 in angiotensin II signal transduction and suggests that p38 is a negative regulator of NHE-1 function in VSMC. The 4 major findings that support these conclusions are: (1) angiotensin II stimulates p38 in VSMC; (2) inhibiting p38 activity with the specific p38 antagonist SKF-86002 increases angiotensin II-stimulated NHE-1 activity in the presence of vehicle (DMSO) demonstrating that angiotensin II stimulation of p38 is associated with inhibition of ERK1/2 activity. To confirm that the increase in ERK1/2 activity after treatment with SKF-86002 (measured by immune complex MBP phosphorylation) was not due to an associated protein in the immunoprecipitate, the same experiment was performed by in-gel kinase assay (Figure 11B). With this technique, there was a 1.5±0.2-fold greater increase in ERK1/2 activity in the presence of SKF-86002. Finally, we studied the effect of SKF-86002 on p90RSK, a putative NHE-1 kinase that is regulated by ERK1/2 (Figure 11C). VSMCs were stimulated by angiotensin II±1 μmol/L SKF-86002, p90RSK was immunoprecipitated, and an immune complex kinase assay then was performed with MBP as substrate (Figure 11C). As previously reported, angiotensin II stimulated p90RSK activity, which was 1.4±0.2-fold greater in the presence of SKF-86002. Addition of SKF-86002 (1, 10, or 100 μmol/L) directly to the kinase reaction caused no change in ERK1/2 or p90RSK activity, indicating that there was no direct action of the drug on ERK1/2 or p90RSK. These data indicate that stimulation of p38 by angiotensin II inhibits ERK1/2 activity and downstream kinases such as p90RSK.

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II–stimulated NHE-1 activity; (3) among the MAP kinases stimulated by angiotensin II in VSMC, ERK1/2 and p38 exhibit the greatest activity in vitro as NHE-1 kinases; and (4) inhibiting p38 activity enhances ERK1/2 activation, indicating cross-talk between these MAP kinases. These findings demonstrate important roles for p38 and ERK1/2 in angiotensin II–mediated regulation of the Na\(^+/\)H\(^+\) exchanger in VSMC.

The present report is the first to describe p38 activation by angiotensin II and a functional role for p38 in VSMC. p38 was originally described as the mammalian homolog of HOG 1, which is required for the response to hyperosmolar stress in yeast.\(^{14}\) Although p38 complements HOG 1–deficient yeast, its role in mammalian cells remains to be defined. The concept that p38 is important in mediating the response to extracellular stress, especially inflammation, is supported by data that show p38 activation by UV radiation, interleukin-1, tumor necrosis factor-\(\alpha\), and lipopolysaccharide.\(^{28}\) A role for angiotensin II as an inflammatory mediator has emerged from studies of angiotensin II–mediated signal transduction. For example, angiotensin II stimulation of the Janus kinase/signal transducer(s) and activator(s) of transcription pathway resembles the responses elicited by interferon-\(\gamma\) in VSMC.\(^{29}\) The present findings that angiotensin II stimulates p38 further strengthens the concept that angiotensin II may have cytokine and proinflammatory actions. However, it should be noted that angiotensin II also activates JNK,\(^{1}\) which suggests that cytokine-like effects of angiotensin II are likely due to JNK as well as p38.

Several kinases have been suggested to be important for activation of the Na\(^+/\)H\(^+\) exchanger, including calcium-calmodulin–dependent kinase,\(^{30–32}\) ERK1/2,\(^{16,19,27}\) and p90RSK.\(^{12,33}\) There are several findings that suggest that calcium-calmodulin–dependent kinase is a critical regulator of NHE-1 activity.\(^{30–32}\) Binding experiments with calmodulin-Sepharose, as well as fluorescence measurements with dansylated calmodulin, revealed that the NHE-1 cytoplasmic domain strongly binds calmodulin in a Ca\(^{2+}\)–dependent manner.\(^{31}\) Mutations that prevent calmodulin binding to the high-affinity binding region rendered NHE-1 constitutively active.\(^{31}\) These data suggest that the high-affinity calmodulin binding region functions as an “autoinhibitory domain” and that Ca\(^{2+}\)–calmodulin activates NHE-1 by relieving this autoinhibition. There is also evidence that calcium-calmodulin–dependent kinase phosphorylates the exchanger directly,\(^{34}\) but the functional significance is unclear.

The present study agrees with previous reports that ERK1/2 are able to phosphorylate NHE-1 in vitro.\(^{16,17}\) Several recent studies suggest that the MEK-ERK1/2 pathway is important in activation of NHE-1. These studies include inhibition of serum-stimulated NHE-1 activity in fibroblasts by dominant negative ERK1/2,\(^{16,19}\) inhibition of NHE-1 activation by phorbol ester, and vasopressin in platelets with the MEK-1 inhibitor PD98059,\(^{18}\) and demonstration that NHE-1 may serve as a substrate in vitro for ERK1/2.\(^{16}\) In contrast to our study and previous reports,\(^{16,17}\) Bianchini et al\(^{19}\) failed to show significant kinase activity of ERK1/2 toward recombinant NHE-1. The most likely explanations for this difference are the magnitude of ERK1/2 activity achieved by the various cell stimuli and the purity of the recombinant NHE-1 fusion proteins used for assay. However, it should be noted that the stoichiometry of phosphorylation of NHE-1 by ERK1/2 in the present study and previous reports\(^{16,17}\) is quite low (<0.1). In contrast, p90RSK, a downstream substrate of ERK1/2, is also stimulated by angiotensin II\(^{12,23}\) and has a stoichiometry of phosphorylation \(\approx 1.0\) for recombinant NHE-1 (M.T. and B.C.B., unpublished observations, 1998).

The present study also indicates that angiotensin II stimulation of p38 is important in regulating Na\(^+/\)H\(^+\) exchange in VSMC. We propose 2 nonexclusive mechanisms by which p38 may regulate NHE-1 activity. First, p38 may phosphorylate NHE-1 directly, causing a conformational change that inhibits transport activity (or preventing interactions with other regulatory molecules required for transport activity). Second, p38 decreases ERK1/2 activity in VSMC, inhibiting function of downstream kinases regulated by ERK1/2, such as p90RSK,\(^{12}\) that are NHE-1 kinases. In agreement with the present study, Grinstein’s group reported that the carboxy-terminal domain of NHE-1 was phosphorylated by p38 (Shrode et al\(^{15}\)). However, these investigators concluded that p38 was unlikely to be an NHE-1 kinase, because they found that NHE-1 activation preceded p38 activation in U937 cells. We also found that p38 activation was too slow to account for angiotensin II–mediated activation of NHE-1, but the temporal events are consistent with p38 playing a role in inactivation of NHE-1. Finally, Bianchini et al\(^{19}\) found that inhibiting p38 with SB203580 (which is identical to SKF 86002) had no effect on NHE-1 activation in CCL39 fibroblasts stimulated by either thrombin+insulin or sorbitol. A possible explanation for the difference in the present study and Bianchini et al\(^{19}\) is that cross-talk between p38 and ERK1/2 occurs to a lesser extent in CCL39 cells compared with VSMC.

In summary, we propose that angiotensin II simultaneously activates and inactivates NHE-1 in VSMC by stimulating ERK1/2 and p38, respectively. Our data suggest that p38 negatively regulates NHE-1 activity; indirectly by inhibiting ERK1/2 activity and possibly directly by phosphorylating NHE-1. A similar “antagonism” between ERK1/2 and p38 has been proposed for stimulation of apoptosis\(^{35}\) and for IgE receptor–mediated release of arachidonic acid and production of tumor necrosis factor-\(\alpha\),\(^{26}\) suggesting that cross-talk between p38 and ERK1/2 is important in several biological responses. Future work will be required to determine the relative importance of p38 and ERK1/2 in angiotensin II–mediated regulation of NHE-1, as it is clear that NHE-1 regulation is complex, involving phosphorylation of NHE-1,\(^{16,17}\) modification of NHE-1–associated proteins,\(^{15,16,17}\) and cross-talk among upstream regulatory kinases,\(^{15,16,18,19}\) as demonstrated in the present study.

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p38 Kinase Is a Negative Regulator of Angiotensin II Signal Transduction in Vascular Smooth Muscle Cells: Effects on Na+/H+ Exchange and ERK1/2
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