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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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.[1–4] 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.[2,5–7] In fact, inhibiting Na^+/H^+ exchange with specific amiloride-derivative antagonists decreases neointimal proliferation in the rat carotid injury model.[7] 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.[8] Of interest, Na^+/H^+ exchange activity[9] and phosphorylation of NHE-1[10] 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.[10,11] We have reported previously that a 90-kd kinase identified as p90RSK is stimulated by angiotensin II and can phosphorylate NHE-1 in vitro.[12] 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)[3] and

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c-Jun *N*-terminal kinase (JNK) and has minimal effects on a third member (big MAP kinase 1 [BMK1]).[13] However, the effects of angiotensin II on p38 remain poorly characterized. p38 is a homolog of the yeast HOG 1 kinase and is involved in the response to extracellular stress.[14,15] Because p38 is involved in the adaptation to osmotic stress, it may be important in cell volume regulation, which is a function that NHE-1 mediates in VSMC after angiotensin II stimulation.[5] Other investigators have suggested that ERK1/2 may be upstream regulators of NHE-1 by either directly phosphorylating 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 incubation 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 Na_3VO_4, 0.5 mmol/L PMSF, 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 $MgCl_2$, 1 mmol/L DTT, 0.1% Triton X-100).

Protein Phosphorylation

Kinase 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 resuspended in 25 mmol/L HEPES, pH 7.4, 10 mmol/L $MgCl_2$, and 10 mmol/L $MnCl_2$. The kinase reaction then was initiated by addition of 5 μg substrate protein, 15 μmol/L ATP, and 0.5 mCi/mL of ^{32}P-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 (pH_i) recovery after acid loading as previously described using BCECF fluorescence.[5,21] Cells were grown on coverslips, loaded with 3 μmol/L BCECF, and alkalinized with 20 mmol/L NH_4Cl as described in a HEPES-Tris–balanced salt solution containing 130 mmol/L NaCl, 5 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 1.0 mmol/L $MgCl_2$, 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 NH_4Cl to acid load the cells, and the rate of recovery was measured in pH units per minute. The rate of pH_i recovery was converted to mmol H^+/min per liter cells (J_H) by multiplying by the buffering power. Buffering power was determined by stepwise reduction of NH_4Cl in the presence of agonists or inhibitors under conditions in which ion fluxes were completely inhibited (5 mmol/L $BaCl_2$, 30 μmol/L EIPA). Data were then plotted as J_H versus pH_i, enabling pH_{50} (pH for half-maximal recovery) and V_{max} 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 mmol/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 *t* test, with a difference of $P<0.05$ considered significant.

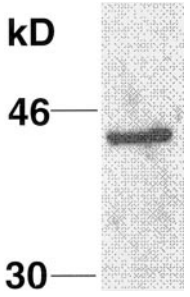

Figure 1. p38 is present in VSMC. Growth-arrested VSMCs were harvested, and cell lysate proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with antibody to p38, and then detected by ECL. A single band of ≈43 kd is present.

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.[14,22] 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.[23] 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,[14] 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

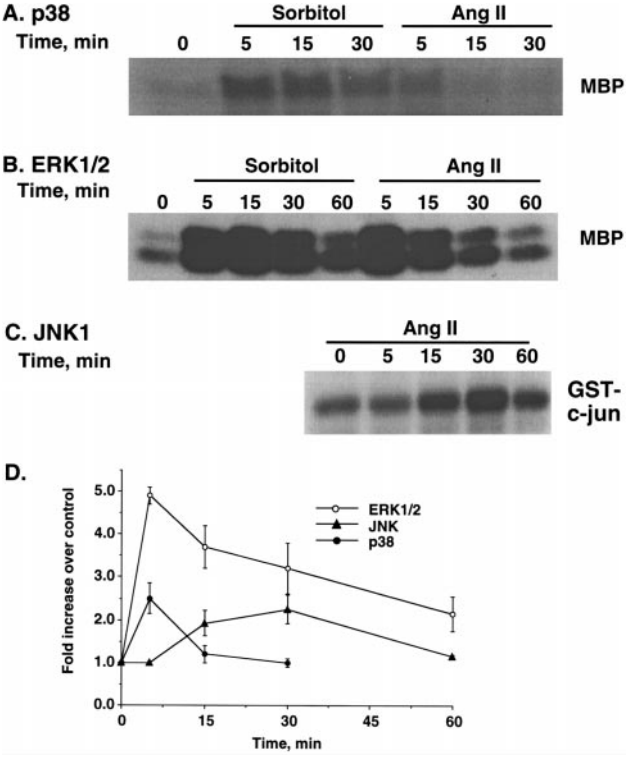

Figure 2. Angiotensin II (Ang II) and sorbitol stimulate ERK1/2, p38, and JNK in VSMC: time course. Growth-arrested VSMCs were stimulated with 0.4 mol/L sorbitol or 100 nmol/L angiotensin II for the indicated times, cells were harvested, and ERK1/2, p38, and JNK activities were determined as described in Materials and Methods. A, p38 activity was measured by an immune complex kinase assay using MBP as substrate. B, ERK1/2 was measured by an in-gel kinase assay using MBP as substrate. MBP phosphorylation was detected after SDS-PAGE by autoradiography. C, JNK activity was measured by an affinity complex kinase assay using GST-Jun as substrate. D, Comparison of magnitude of angiotensin II stimulation of MAP kinases in VSMC. Results of 3 to 6 experiments were normalized to control levels of activity, which were arbitrarily set to 1.0.

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_i recovery in VSMC is completely dependent on Na^+/H^+ exchange in the absence of bicarbonate.[5,7] Because the only isoform of the Na^+/H^+ exchanger present in VSMC is NHE-1,[11] these cells may be used as a model to study regulation of NHE-1 activity. Both angiotensin II[5] 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).[5] We first determined the effect of the p38 inhibitor SKF-86002 on angiotensin II–stimulated Na^+/H^+ exchange by fluorescence pH measurement.[5,9,24] In brief, growth-arrested VSMCs were loaded with BCECF and acid-loaded by the NH_4Cl prepulse

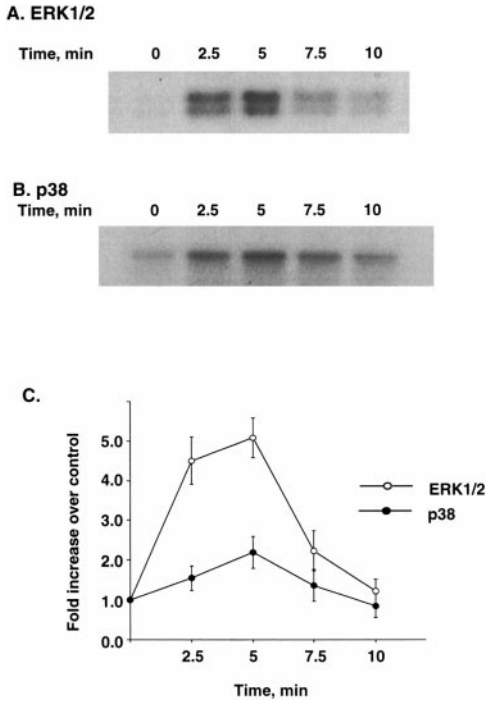

Figure 3. Angiotensin II stimulates ERK1/2 and p38 in VSMC: time course. Growth-arrested VSMCs were stimulated with 100 nmol/L angiotensin II for the indicated times, cells were harvested, and ERK1/2 and p38 activities were determined as described in Materials and Methods. A, ERK1/2 was measured by an in-gel kinase assay using MBP as substrate. MBP phosphorylation was detected after SDS-PAGE by autoradiography. B, p38 activity was measured by an immune complex kinase assay using MBP as substrate. C, Comparison of magnitude of angiotensin II stimulation of MAP kinases in VSMC. Results of 3 experiments were normalized to control levels of activity, which were arbitrarily set to 1.0.

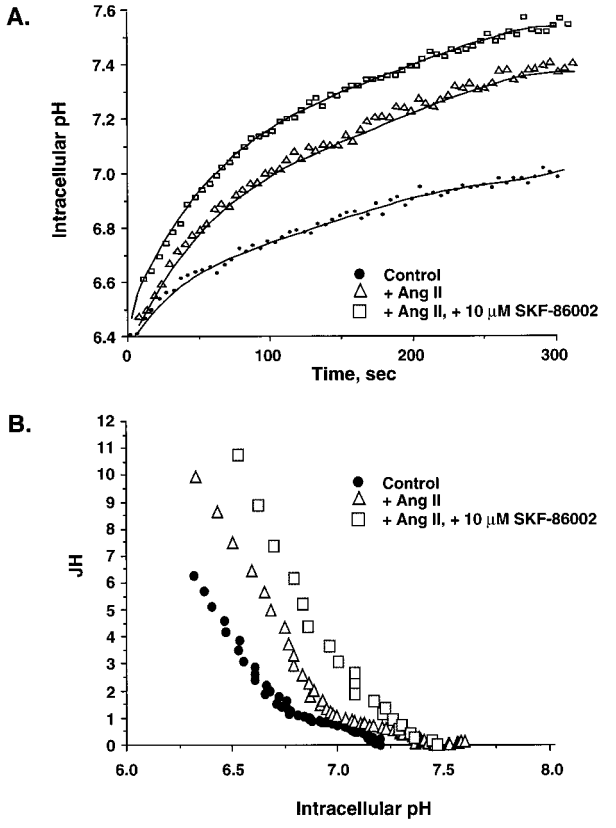

Figure 4. Effects of angiotensin II and SKF-86002 on Na^+/H^+ exchange in VSMC. A, Growth-arrested VSMCs were acid-loaded with NH_4Cl for 5 minutes, and the rate of pH_i recovery was measured using BCECF. As indicated, cells were pretreated during the NH_4Cl pulse with vehicle (●), 100 nmol/L angiotensin II alone (△), or 100 nmol/L angiotensin+10 μmol/L SKF-86002 (□). pH_i recovery was inhibited by >90% when 10 μmol/L EIPA was added, as previously reported.[7] B, Data from 6 separate experiments comparing pH_i recovery vs time were analyzed. The rate of pH_i recovery was expressed as J_H by multiplying the dpH_i/dt by the buffering power at each pH. Data were then plotted as pH_i vs J_H. The standard errors were <10% and were deleted for clarity. SKF-86002 had no significant effect on buffering power, and the pH_i vs J_H plot for this compound alone did not differ significantly from control (not shown).

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 J_H versus pH_i plot, consistent with a decrease in K_m for H^+ as previously reported.[25] SKF-86002 increased angiotensin II–stimulated Na^+/H^+ 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 J_H 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^+/H^+ exchange, we used the MEK-1 inhibitor, PD98059. As previously reported by other investigators,[18,19,26,27] PD98059 inhibited Na^+/H^+ 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^+/H^+ 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)

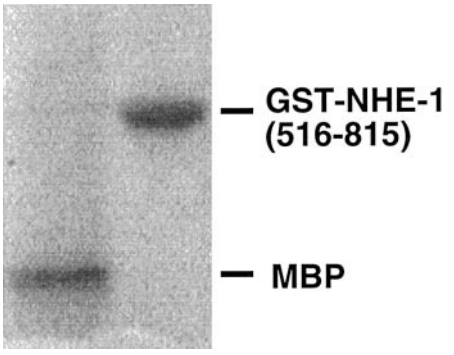

Figure 5. p38 phosphorylates NHE-1(516 to 815). Recombinant His-p38 (0.25 mg/assay) was incubated with 35 pmol/L NHE-1(516 to 815) fusion protein or 35 pmol/L MBP in the presence of ^{32}P-γ-ATP for 20 minutes at 30°C. The reaction was terminated by addition of Laemmli sample buffer. Phosphorylated proteins were identified by autoradiography after SDS-PAGE.

(Figure 5, right) to an extent similar to phosphorylation of MBP (Figure 5, left), under these conditions.

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 determined the effect of the p38 inhibitor SKF-86002.[22] VSMCs were stimulated with 0.4 mol/L sorbitol for 15 minutes, p38 immunoprecipitated, and an immune complex kinase assay then was performed with NHE-1(625 to 747) in the presence or absence of 10 μmol/L SKF-86002.

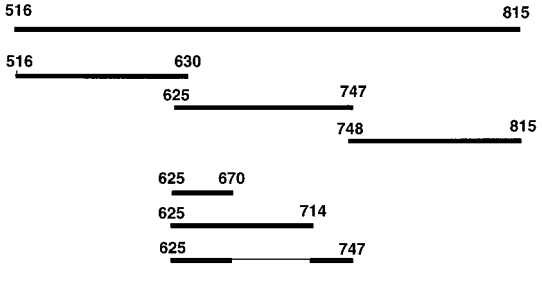

Figure 6. Schema for constructs of human NHE-1. A total of 6 GST-fusion protein constructs were prepared spanning amino acids 516 to 815 of human NHE-1.

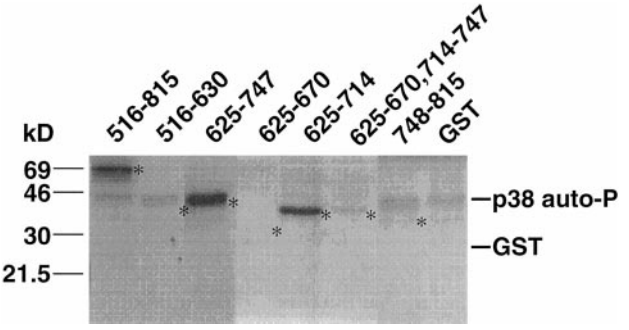

Figure 7. NHE-1(625 to 747) contains serines and threonines phosphorylated by p38. Recombinant His-p38 (0.25 mg/assay) was incubated with 35 pmol/L of the indicated GST-NHE-1 fusion proteins or GST alone in the presence of ^{32}P-γ-ATP for 20 minutes at 30°C. The reaction was terminated by addition of Laemmli sample buffer. Phosphorylated proteins were identified by autoradiography after SDS-PAGE. *Phosphorylated GST-NHE-1 fusion protein. Autophosphorylation of His-p38 (molecular weight=46 kd) occurred to a small extent (p38 auto-P). Note that the molecular weight of NHE-1(625 to 747) is similar to His-p38.

As shown in Figure 8, SKF-86002 completely inhibited p38 phosphorylation of NHE-1(625 to 747). In contrast, neither the drug vehicle (1% dimethyl sulfoxide [DMSO]) nor SKF-105809 (an inactive prodrug of SKF-86002) had any effect on NHE-1(625 to 747) phosphorylation by p38. Because SKF-86002 is specific for p38 relative to other MAP kinase members,[22] inhibition of NHE-1 phosphorylation proves specificity of the antibody for immunoprecipitation and validates that p38 is the immunoprecipitated kinase responsible for phosphorylation of NHE-1. It was not possible to perform this experiment with SKF-86002 in intact cells, because during immunoprecipitation of p38 and subsequent washing, the inhibitor was removed and p38 autophosphorylation (and hence activation) occurred (not shown).

Comparison of NHE-1 Phosphorylation by Endogenous MAP Kinases

Angiotensin II and sorbitol activated p38 with different time courses and magnitude when assayed by MBP phosphorylation (Figure 2). We repeated this experiment using NHE-1(625 to 747) as the substrate (Figure 9). Angiotensin II rapidly and transiently stimulated p38 activity with peak activity (≈2.5-fold increase) at 5 minutes. In contrast, sorbi-

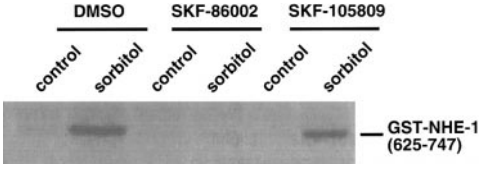

Figure 8. The p38 inhibitor, SKF-86002, blocks phosphorylation of NHE-1(625 to 747) by endogenous p38. Growth-arrested VSMCs were stimulated with 0.4 mol/L sorbitol for 15 minutes, and p38 was immunoprecipitated from cell lysates. Immunoprecipitated p38 then was incubated for 15 minutes at room temperature with 1% DMSO, 100 μmol/L SKF-86002, or 100 μmol/L SKF-105809 (structurally related to 86002 but inactive). p38 activity was measured using NHE-1(625 to 747) as a substrate. Similar results were obtained after angiotensin II stimulation (not shown).

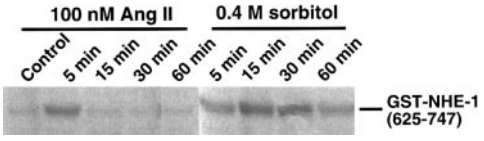

Figure 9. Angiotensin II and 0.4 mol/L sorbitol activate p38 in VSMC: time course. Growth-arrested VSMCs were stimulated with 100 nmol/L angiotensin II or 0.4 mol/L sorbitol for the indicated times, and cells were harvested. p38 was immunoprecipitated from cell lysates, and p38 activity was then measured using NHE-1(625 to 747) as a substrate. Results are representative of 3 experiments.

tol stimulated a larger, slower, and more sustained activation with peak activity (≈11-fold) at 15 minutes. These results confirm those previously obtained with MBP as substrate.

To gain insight into the relative potency of endogenous VSMC kinases as NHE-1 kinases, we compared NHE-1 kinase activity of immunoprecipitated p38, JNK, and ERK1/2 after stimulation of VSMC by 100 nmol/L angiotensin II (Figure 10). The rank order of potency of these kinases as NHE-1 kinases (after angiotensin II stimulation), measured by phosphorylation of GST-NHE-1(625 to 747) was ERK1/2>p38≫JNK (Figure 10; normalized densitometric values relative to p38 of 2.0, 1.0, and 0.05, respectively). Specifically, JNK showed no significant basal or angiotensin II–stimulated activity as an NHE-1 kinase. Both p38 and ERK1/2 showed minimal activity toward NHE-1 at baseline and a 2- to 3-fold increase in activity after angiotensin II treatment. To prove the specificity of the immunoprecipitation, Western blot analysis was performed with antibodies to each of the 3 MAP kinases. This analysis showed that only the appropriate kinase was present in the immunoprecipitates (not shown).

Inhibition of p38 Augments Angiotensin II Stimulation of ERK1/2

A possible explanation for the increase in NHE-1 activity after treatment of VSMC with SKF-86002 (Figure 4) is that p38 inhibits another NHE-1 kinase. Several reports suggest that ERK1/2 and downstream kinases regulated by ERK1/2, such as p90RSK, are involved in activation of NHE-1.[12,18,19,26,27] To determine whether activation of p38 by angiotensin II inhibited activation of ERK1/2, VSMCs were stimulated by angiotensin II±1 μmol/L SKF-86002, ERK1/2 were immunoprecipitated, and an immune complex kinase assay then was performed with MBP as substrate (Figure 11A). In the presence of SKF-86002, angiotensin II stimulated a 2.5±0.4-fold greater increase in ERK1/2 activity than

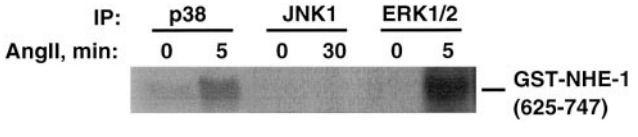

Figure 10. NHE-1(625 to 747) is a better substrate than JNK for endogenous p38 and ERK1/2. Growth-arrested VSMCs were stimulated with 100 nmol/L angiotensin II for 5 or 30 minutes as indicated, and cells were harvested. p38, JNK1, and ERK1/2 were immunoprecipitated from cell lysates, and kinase activity was then measured using NHE-1(625 to 747) as a substrate. Results are representative of 3 experiments.

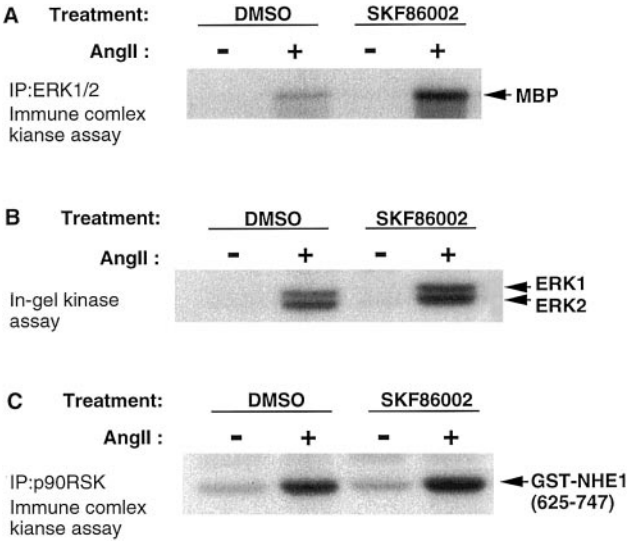

Figure 11. SKF-86002 increases angiotensin II stimulation of ERK1/2. Growth-arrested VSMCs were preincubated for 30 minutes with 0.1% DMSO or 10 μmol/L SKF-86002, stimulated with 100 nmol/L angiotensin II for 5 minutes, and then harvested. A, ERK1/2 were immunoprecipitated from cell lysates, and ERK1/2 activity was measured using MBP as a substrate. B, Total cell lysates were size-fractionated by SDS-PAGE and an in-gel kinase assay performed with MBP as substrate. C, p90RSK was immunoprecipitated from cell lysates, and p90RSK activity was measured in an in-gel kinase assay using GST-NHE-1(625 to 747) as a substrate. Results are representative of 3 experiments.

in the presence of vehicle (DMSO) demonstrating that angiotensin II activation of p38 is associated with inhibition of ERK1/2 activation. 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[12] 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 GST-NHE-1(625 to 747) as substrate (Figure 11C). As previously reported,[12] 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; (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-α, 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-γ 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.[30] 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[12,33] and has a stoichiometry of phosphorylation ≈ 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 carboxyl-terminal domain of NHE-1 was phosphorylated by p38 (Shrode et al[17]). 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-α,[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,[36,37] and cross-talk among upstream regulatory kinases,[16,18,19] as demonstrated in the present study.

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