Mitogen-Activated Protein/Extracellular Signal–Regulated Kinase Inhibition Attenuates Angiotensin II–Mediated Signaling and Contraction in Spontaneously Hypertensive Rat Vascular Smooth Muscle Cells

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Abstract—This study investigates the role of extracellular signal–regulated kinases (ERKs) in angiotensin II (Ang II)–generated intracellular second messengers (cytosolic free Ca\(^{2+}\) concentration, ie, \([\text{Ca}^{2+}]_c\), and pH\(_i\)) and in contraction in isolated vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and control Wistar Kyoto rats (WKY) using the selective mitogen-activated protein (MAP)/ERK inhibitor, PD98059. VSMCs from mesenteric arteries were cultured on Matrigel basement membrane matrix. These cells, which exhibit a contractile phenotype, were used to measure \([\text{Ca}^{2+}]_c\), pH\(_i\), and contractile responses to Ang II (10\(^{-12}\) to 10\(^{-6}\) mol/L) in the absence and presence of PD98059 (10\(^{-5}\) mol/L). \([\text{Ca}^{2+}]_c\) and pH\(_i\) were measured by fura-2 and BCECF methodology, respectively, and contraction was determined by photomicroscopy. Ang II–stimulated ERK activity was measured by Western blot analysis using a phospho-specific ERK-1/ERK-2 antibody and by an MAPK enzyme assay. Ang II increased \([\text{Ca}^{2+}]_c\) and pH\(_i\) and contractile responses to Ang II in SHR (10\(^{-12}\) to 10\(^{-6}\) mol/L). \([\text{Ca}^{2+}]_c\) and pH\(_i\) were higher in SHR compared with WKY. 

Key Words: [\text{Ca}^{2+}]_c, pH\(_i\), resistance vessel, PD98059, hypertension

Increased peripheral resistance plays a critical role in blood pressure elevation. In spontaneously hypertensive rats (SHR), this increase in vascular resistance has been attributed to multiple interacting factors, including structural alterations in small vessels, decreased endothelium-dependent vasodilation, and enhanced vascular reactivity to vasoconstrictor stimuli.\(^1\) Of the many vasoactive agonists that have been implicated in vascular hyperresponsiveness in hypertension, angiotensin II (Ang II) appears to play one of the most important roles. Whereas responses to endothelin-1, vasopressin, and norepinephrine have been reported to be decreased, unchanged, or (rarely) increased, vascular reactivity to Ang II has, for the most part, been found to be increased in vessels from adult SHR.\(^2-4\) Altered Ang II–stimulated vascular responsiveness occurs early in the development of hypertension, and we and others have shown increased intracellular free Ca\(^{2+}\) concentration ([\text{Ca}^{2+}]_c) and contractile effects of Ang II in SHR as young as 6 weeks of age.\(^5,6\) Ang II–mediated vascular hyperresponsiveness is not specific to SHR, as similar Ang II–related actions, both in the prehypertensive and established hypertensive phases, have been demonstrated in other models of hypertension, including stroke-prone SHR, renal hypertensive rats, and desoxycorticosterone acetate/salt–hypertensive rats.\(^7-9\) The signal transduction systems responsible for enhanced Ang II–elicited excitation-contraction coupling in hypertension are not completely understood, and the signaling processes involved in the prehypertensive phase may differ from...
those in the phase of established hypertension. In cultured vascular smooth muscle cells from adult SHR, Ang II–stimulated phospholipase C–mediated signaling is increased, with augmentation of [Ca\(^{2+}\)], and pH responses relative to normotensive controls.\(^{5,10}\) These effects are partially due to increased Ca\(^{2+}\) influx and mobilization and to enhanced activity to the Na\(^{+}\)-H\(^{+}\) exchanger.\(^{11,12}\) [Ca\(^{2+}\)], elevation and alkalization are major determinants of vascular contraction.\(^{5,10–12}\) They mediate actin-myosin interaction, crossbridge cycling, and vascular smooth muscle contraction.\(^{11,12}\)

In addition to activation of the classic phosphoinositide-phospholipase C–mediated signaling pathways commonly associated with Ang II, it has become clear that Ang II stimulates pathways dependent on tyrosine kinase and mitogen-activated protein kinase (MAPK).\(^{13–17}\)

Mammalian cells express multiple MAPKs,\(^{18,19}\) including the well-characterized extracellular signal–regulated kinase (ERK) pathway. ERKs (ERK-1 and ERK-2), which are critical in the mitogenic response, are phosphorylated by mitogen-activated protein/ERK (MEK-1/MEK-2) kinase. Although MAPK activation is typically associated with cell growth, recent data indicate that these pathways may also play a role in vascular contraction.\(^{20–23}\) We demonstrated that tyrosine kinases, putative upstream regulators of MAPKs, modulate intracellular second messengers and contraction in Ang II–stimulated vascular smooth muscle cells.\(^{16,21}\) Epstein et al.\(^{22}\) reported that activation of MAPK is temporally associated with sustained vascular smooth muscle contraction.\(^{11,12}\) Watts\(^{23}\) showed that MEK inhibition decreased serotonin-stimulated contraction in rat vessels.

In the present study, we questioned whether MAPKs, and specifically ERKs, may contribute to Ang II–elicited hyperresponsiveness in vascular smooth muscle in SHR. The aims of this investigation were (1) to determine the role of ERKs in Ang II–generated second messengers and associated vasomotor effects, (2) to determine whether increased Ang II–induced [Ca\(^{2+}\)], and pH responses are associated with augmented cellular contraction in isolated vascular smooth muscle cells from SHR, and (3) to assess whether alterations in ERK-dependent pathways underlie altered Ang II–mediated cellular effects in SHR.

The novel MEK inhibitor PD98059 (2-[2'-amino-3'-methoxyphenyl]-ox-anaphthalen-4-one), which inhibits MEK-1/MEK-2,\(^{24}\) was used. We studied primary cultured unpassaged vascular smooth muscle cells that retain their contractile phenotype and have undergone little phenotypic change relative to the native cells in blood vessels. Cells were isolated from small arteries (mesenteric) that contribute to peripheral resistance and consequently to blood pressure.\(^{5,10–12}\) Those in the phase of established hypertension. SHR were studied at 17 weeks of age, at which stage hypertension is established, and compared with age-matched normotensive Wistar Kyoto rats (WKY).

**Materials and Methods**

Ang II was bought from Peninsula Laboratories Inc. Fura-2-acetoxymethyl ester, BCECF-acetoxymethyl ester, and pluronic F-127 were from Molecular Probes Inc. DMEM was from Gibco Canada, and Ham’s F-12 medium was from Flow Laboratories Inc. All other chemicals were from Fisher Scientific Co, BDH Inc, and Sigma.

**Animal Experiments**

The study was approved by the Animal Ethics Committee of the Clinical Research Institute of Montreal and carried out according to the recommendations of the Canadian Council for Animal Care. Seventeen-week-old male WKY rats (n=20) and SHR (n=20) (Taconic Farms Inc., Germantown, NY) were studied. The rats were housed under standardized conditions (constant temperature [22°C], 12-hour light-dark cycle, and relative humidity [60%]) in the animal unit at the Clinical Research Institute of Montreal.

Systolic blood pressure was recorded in pretrained (external temperature 37°C) conscious rats by the tail-cuff method using a photoelectric pulse sensor (model PCPB) and a polygraph (model 7; Grass Instruments Co) a few days before experimentation. Blood pressure was significantly higher (P<0.001) in SHR (190±2.1 mm Hg) than in WKY (112±1.0 mm Hg).

**Cell Culture**

The rats were killed by decapitation. Vascular smooth muscle cells derived from mesenteric arteries were isolated and characterized as described in detail previously.\(^{5,10–12}\) Briefly, mesenteric arteries were cleaned of adipose and connective tissue; smooth muscle cells were dissociated by digestion of vascular arcades; the tissue was filtered; and the cell suspension was centrifuged and resuspended in DMEM containing heat-inactivated calf serum, L-glutamine, HEPES, penicillin, and streptomycin. Cells were plated onto round glass coverslips that were coated with Matrigel basement membrane matrix (Becton Dickinson Labware), which is a cell culture preparation optimized for contractile phenotypic states. The composition of Matrigel matrix is similar to that of the basement membrane that surrounds individual medial smooth muscle cells in vivo. It is a solubilized basement membrane matrix extracted from the Engelbreth-Holm-Swarm mouse sarcoma, with its major component being laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin, and nidogen. Smooth muscle cells cultured using this system have phenotypic characteristics typical of contractile cells.\(^{26}\)

Coverslips were coated with Matrigel basement membrane matrix according to the manufacturer’s instructions. Aliquots of frozen Matrigel basement membrane matrix were thawed and diluted 1:3 in serum-free medium. Under sterile conditions, the gel was placed on glass coverslips (50 μL/cm\(^2\) growth surface) in 6-well plates at room temperature. The coated coverslips were incubated at 37°C. Gels formed within 30 minutes, after which time cells were added. Gels were covered with 2 mL of DMEM containing 2% FCS and incubated in a humidified incubator maintained at 37°C and equilibrated with 5% CO\(_2\), and 95% air. After 48 hours and every 48 hours thereafter, the medium was replaced with 2 mL of DMEM containing 0.5% FCS.

ERK activity was determined in vascular smooth muscle cells from WKY and SHR by Western blot analysis and by a MAPK enzyme assay system. Cells were cultured in DMEM containing 10% FCS. Early passaged cells\(^{4–7}\) were used for these experiments, as primary cultured unpassaged cells did not yield sufficient protein to perform ERK assays. Cells were rendered quiescent by serum deprivation for 48 hours before assay.

**Western Blotting of ERKs**

Quiescent cells, grown on 15-mL culture plates, were stimulated with HBSS (vehicle) or Ang II at a final concentration of 10^{-7} mol/L for 5 minutes. For experiments with the MEK inhibitor, cells were pretreated with vehicle alone or with 10^{-4} mol/L PD98059 for 30
minutes before Ang II addition. In some experiments, cells were stimulated for 5 minutes with PMA (10⁻⁹ mol/L) in the absence or presence of PD98059. The plates were washed with cold PBS, and 800 μL of lysis buffer was added (buffer [in mmol/L]: sodium pyrophosphate 50, NaF 50, NaCl 50, EDTA 5, EGTA 5, NaVO₄ 2, HEPES [pH 7.4] 10, and phenylmethylsulfonyl fluoride 50, and 0.1% Triton X-100). The plates were placed on dry ice for 5 minutes and then thawed to thaw the ice. Cells were scraped off, transferred to Eppendorf tubes, and sonicated for 5 seconds. The protein supernatant was separated by centrifugation, and protein concentrations were determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories). Equal amounts of proteins (5 μg) were loaded on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Boehringer Mannheim) for 1 hour at 100 V. Membranes were blocked with blocking buffer containing Tris-buffered saline and 0.1% Tween-20 with 5% wt/vol nonfat dry milk and incubated for 24 hours at 4°C. Membranes were incubated with a phospho-specific ERK-1/ERK-2 antibody (pp44/p42 MAPK [T202/Y204] E10 monoclonal antibody) diluted 1:1000 for 24 hours at 4°C. They were then washed, incubated with a goat anti-rabbit horseradish peroxidase–conjugated antibody (Bio-Rad Laboratories) diluted 1:2000 for 1 hour at room temperature, and washed extensively. Membranes were then incubated with biotinylated substrate (Boehringer Mannheim) following the manufacturer’s protocol and exposed to film, which was then developed. The film was scanned by a ScanJet 6100C/T scanner (Hewlett Packard), and the images were saved on computer. Band intensity was measured by computer analysis, using the Image Quant program.

**MAPK Activity Assay**

Cells were stimulated for 5 minutes with Ang II (10⁻⁹ mol/L) in the absence and presence of PD98059 (10⁻⁹ mol/L). The MEK inhibitor was added 30 minutes before Ang II addition. Stimulated cells were then lysed in a buffer containing 50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 μmol/L NaVO₄, 10 mmol/L HEPES [pH 7.4], 0.1% Triton X-100, 500 μmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin, then flash-frozen on a dry ice/ethanol bath. After the cells were allowed to thaw, they were scraped off the dish and centrifuged at 14,000 rpm (4°C for 30 minutes), and protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories). MAPK activity was determined using a MAPK assay kit (Upstate Biotechnology). The assay is based on phosphorylation of the specific substrate myelin basic protein (MBP), using the transfer of the γ-phosphate of adenosine-5′-['³²P]triphosphate (γ-³²P]ATP) by MAPK. The methodology was based on the manufacturer’s instructions. Briefly, 10 μL each of substrate mixture, inhibitor mixture, and cell lysate (10 μg/mL protein) was aliquoted into a microcentrifuge tube. The substrate mixture contained 2 mg/mL MBP in assay dilution buffer (in mmol/L: MOPS [pH 7.2], β-glycerol phosphate 25, EGTA 5, sodium orthovanadate 1, and DTT 1), and the inhibitor mixture contained (in μmol/L) protein kinase A inhibitor peptide 2, protein kinase A inhibitor peptide 2, and calmodulin kinase inhibitor peptide 20 in assay dilution buffer. The reaction was initiated by adding 10 μL of Mg²⁺/ATP mixture (75 mmol/L MgCl₂ and 500 μmol/L ATP in assay dilution buffer) containing (γ-³²P]ATP (3000 μCi/mmol) at 0.5 μL/μmol ATP mixture at 30 μmol/L ATP. The reaction was stopped by removing 25 μL of the reaction mixture, which was pipetted onto the corner of a P81 phosphocellulose paper. The papers were washed 3 times for 15 minutes with 0.75% phosphoric acid and finally with acetone. The papers were then added to scintillation vials containing scintillation fluid. The bound radioactivity on the paper was quantitated in a scintillation counter for 1 minute. Activity was determined as pmol phosphate incorporated into MBP per minute per mg protein.

**Simultaneous Measurement of Vascular Smooth Muscle Cell [Ca²⁺], pH, and Contractile Responses**

After 7 to 9 days, Ang II–induced responses of vascular smooth muscle cells were measured. Contraction was measured on the basis of the methods described by Kahn et al.²⁷ and Bodin et al.²⁸ Cells were loaded with fura-2-acetoxyethyl ester (4 μmol/L) for [Ca²⁺], or BCECF (0.2 μmol/L) for pH, as described in detail previously.¹⁶,²⁹ Single cells in cell clusters were investigated using an Axiovert 135 inverted microscope (×40 oil-immersion objective) and an Attofluor digital fluorescence system (Zeiss) using alternating excitatory wavelengths of 343 and 380 nm for [Ca²⁺] measurements and 440 and 495 nm for pH measurements. Video images of fluorescence at an emission wavelength of 520 nm were obtained using an intensified charge-coupled devise camera system (Zeiss) with the output digitized to a resolution of 512×480 pixels. The gel-coated coverslips with attached fura-2– or BCECF–loaded cells was placed on the stage of the Axiovert microscope. Matrigel, diluted 1:3, did not exhibit any autofluorescence. After a 10-minute stabilization period, a field of cells was photographed to obtain baseline images. Ang II (in the absence or presence of inhibitors) was then added, and serial images were taken of the same field of cells at 30-second intervals after Ang II addition. The images, which were saved on computer, were later scanned using a ScanJet 4c/T scanner (Hewlett Packard). The cell lengths of the longest axes of cells were measured in the first image, and lengths of the same cells were measured in the subsequent photographs using the Adobe Photoshop software (version 4.0). The magnitude of cell contraction was expressed as the percentage reduction in cell length relative to initial baseline measurements.¹⁶,²⁷,²⁸ For each cell, the percentage contraction from the baseline was calculated, and these values were averaged for all cells. The average basal cell measurements were between measurements. Cells were photographed simultaneously with the signal of the [Ca²⁺] (or pH)–sensitive fluorescence ratio. [Ca²⁺], was calculated according to the formula of Grynkiewicz et al.⁰ [Ca²⁺] = Kᵢ × B × Rₑ × (Rₑ – Rₑmin)/Rₑmax, where Kᵢ is the dissociation constant for fura-2-Ca²⁺ and taken to be 224 nmol/L, β is defined as the ratio of fluorescence at 380 nm and no Ca²⁺ (Fₑmax - Fₑmin) to saturating Ca²⁺ (Fₑmax) conditions, and R is the ratio of fluorescence obtained with excitation at 343 and 380 nm; and max min respectively, which were not significantly different from values obtained in SHR cells (Rₑmax = 0.74 ± 0.04, Rₑmin = 2.31 ± 0.04, and β = 1.68 ± 0.26). pH was calculated from a calibration curve obtained by determining the fluorescence ratios at pH values from 6.8 to 7.5. pH was set by the percentage reduction in cell length relative to initial baseline measurements. Experimental Protocols

[Ca²⁺], pH, and contractile responses were measured in unstimulated cells and in cells exposed to increasing concentrations (10⁻¹² to 10⁻⁶ mol/L) of Ang II in the absence and presence of the selective MEK inhibitor, PD98059 (10⁻⁵ mol/L), which was dissolved in 0.05% DMSO (final concentration). To determine whether PD98059 modulates [Ca²⁺], transients and contraction through pathways not directly linked to MEK stimulation by Ang II, we also assessed [Ca²⁺], and contractile effects of the phorbol ester, PMA (an activator of classical and novel isoforms of PKC), in the absence and presence of PD98059. In these experiments, cells were pretreated for 30 minutes with vehicle or PD98059 (10⁻⁵ mol/L) before PMA (10⁻⁴ to 10⁻⁶ mol/L) addition. To determine whether Ang II–induced pH effects are mediated via activation of the Na⁺–H⁺ exchanger, cells from 3 separate preparations were pretreated with the selective Na⁺–H⁺ exchange ionophore, nigericin, as well as the pH-sensitive dye BCECF (0.2 μmol/L) for [Ca²⁺], and then exposed to increasing concentrations of Ang II. At various intervals throughout the experiments, effects of vehicle (HBSS or DMSO) were assessed. Vehicle did not alter responses.
ERK activation was determined by the following 2 techniques: (1) Western blot analysis with a monoclonal antibody that specifically recognizes the phosphorylated forms of ERK-1 and ERK-2 (phospho-specific p44/p42 MAPK Thr202/Tyr204, New England Biolabs) and (2) a MAPK enzyme assay. Phospho-p44/p42 MAPK antibody detects doubly phosphorylated Thr202 and Tyr204 of p44 and p42 MAPKs (ERK-1 and ERK-2) but does not cross-react with nonphosphorylated MAPK. Furthermore, the antibody does not appreciably cross-react with the corresponding phosphorylated threonine or tyrosine of either JNK/SAPK or p38 MAPK. Results from Western blots demonstrated 2 bands of 44 and 42 kDa corresponding to phosphorylated ERK-1 and phosphorylated ERK-2, respectively. Ang II (10^{-7} mol/L) stimulation for 5 minutes increased phosphorylation of ERK-1 and ERK-2, with responses being significantly greater in cells from SHR than from WKY (Figure 1). PD98059 pretreatment significantly inhibited Ang II–induced phosphorylation of ERK-1 and ERK-2 (Figure 1). PMA had a potent stimulatory effect on ERK phosphorylation (Figure 1). Preexposure of cells to PD98059 did not alter PMA effects (Figure 1).
Western blots, indicate that Ang II–induced ERK activity is significantly enhanced in cells from SHR.

Effects of PD98059 on Ang II–Stimulated Contractile Responses

Under the conditions described, the vascular smooth muscle cells displayed a contractile phenotype. They had a low proliferation rate, they had a nodular-type morphology under light microscopy, they expressed the cytoskeletal marker α-actin as determined by immunocytochemical analysis, and they contracted in response to Ang II. These features are characteristic of the contractile phenotype as previously described.26 The contractile response rate to Ang II was 79% for WKY cells and 83% for SHR cells. Figure 3a shows a time course of the contractile effects of 10⁻⁶ mol/L Ang II in cells from WKY rats and SHR. Maximal cell shortening occurred within 5 minutes after Ang II application. Cell contraction was sustained, and 10 minutes after stimulation, cells were still significantly contracted. Ang II dose-dependently contracted cells, with responses being significantly greater in SHR than WKY (Figures 3 and 4; Table).

Pretreatment of cells for 30 minutes with the MEK inhibitor PD98059 had no effect on cell shape or size in either group. However, PD98059 significantly reduced Ang II–stimulated contractile responses in WKY and SHR (Figures 3b and 4; Table). MEK inhibition reduced sensitivity to Ang II in WKY and SHR, and the augmented Ang II–stimulated contractile responses in SHR were normalized (Table). PD98059 failed to completely inhibit Ang II–induced contraction, and 10 minutes after stimulation, cells were still slightly contracted (8 to 12%) (Figure 3b).

Figure 3. Line graphs demonstrate the time course of the effects of (10⁻⁶ mol/L) on contraction, [Ca²⁺], and pH in vascular smooth muscle cells from WKY and SHR in the absence (left) and presence (right) of PD98059 (10⁻⁶ mol/L). A [in panels d through f] indicates the time of Ang II addition. *P<0.05 and **P<0.01 vs WKY.

Figure 4. Dose-response curves demonstrate contractile effects of Ang II in the absence and presence of 10⁻⁶ M PD98059 in vascular smooth muscle cells from WKY and SHR. Contraction is presented as the percentage reduction in cell length relative to cell length in the basal unstimulated state. The maximal contractile response obtained for each cell (~240 seconds after Ang II addition) was used to construct the dose-response curve. Each data point is the mean±SEM of at least 3 experiments, with each experimental field comprising 4 to 9 cells. *P<0.05 and **P<0.01 vs WKY.
PD98059 Effects on Ang II–Stimulated $[Ca^{2+}]_i$ Responses

Basal $[Ca^{2+}]_i$ was significantly higher ($P<0.05$) in SHR cells (121±2.5 nmol/L) than in WKY cells (93±3.3 nmol/L). To account for the elevated basal $[Ca^{2+}]_i$ in SHR, the net Ang II–stimulated $[Ca^{2+}]_i$ change was determined as the $[Ca^{2+}]_i$ difference between the stimulated response and the basal response. Ang II–induced $[Ca^{2+}]_i$ responses were rapid, occurring within a few seconds after stimulation (Figures 3c and 5). The acute $[Ca^{2+}]_i$, peak was followed by a second $[Ca^{2+}]_i$, sustained phase that plateaued to levels above baseline (Figure 5). The $[Ca^{2+}]_i$ recovery phase was prolonged in SHR (Figures 3c and 5). Dose-response curves were constructed from $[Ca^{2+}]_i$, peak values and from the sustained plateau phase (Figure 6). Ang II dose-dependently increased $[Ca^{2+}]_i$, with responses significantly greater in SHR than in WKY (Figure 6, Table). Preincubation of cells with PD98059 significantly attenuated $[Ca^{2+}]_i$, responses to Ang II (Figures 3d, 5, and 6). In the presence of PD98059, $pD_2$ values and $E_{max}$ were not different between WKY and SHR (Table).

To evaluate whether PD98059 modulates $[Ca^{2+}]_i$ transients and contraction through mechanisms not directly linked to MEK activation in response to stimulation by Ang II, $[Ca^{2+}]_i$ and contractile effects of PMA were assessed. In these experiments, cells from WKY and SHR were preexposed to PD98059 for 30 minutes before PMA addition. PMA increased $[Ca^{2+}]_i$ and contraction (Figure 7). Responses were not significantly different between SHR and WKY (Figure 7). PD98059 pretreatment had no effect on PMA-stimulated $[Ca^{2+}]_i$ or contractile responses in either rat group (Figure 7).

PD98059 Effects on Ang II–Stimulated $[Ca^{2+}]_i$, Responses

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To evaluate whether PD98059 modulates $[Ca^{2+}]_i$, transients and contraction through mechanisms not directly linked to MEK activation in response to stimulation by Ang II, $[Ca^{2+}]_i$ and contractile effects of PMA were assessed. In these experiments, cells from WKY and SHR were preexposed to PD98059 for 30 minutes before PMA addition. PMA increased $[Ca^{2+}]_i$ and contraction (Figure 7). Responses were not significantly different between SHR and WKY (Figure 7). PD98059 pretreatment had no effect on PMA-stimulated $[Ca^{2+}]_i$ or contractile responses in either rat group (Figure 7).

### PD98059 Effects on Ang II–Stimulated pH$_i$, Responses

Basal pH$_i$ was similar in WKY (7.06±0.02) and SHR (7.10±0.01). Ang II increased pH$_i$ in a dose-dependent fashion, with Ang II–induced alkalinization being significantly greater ($P<0.05$) in cells from SHR than in cells from WKY (Figures 3e and 9). Preincubation of cells with HMA abrogated the Ang II–stimulated alkalinization in WKY and SHR (Figure 10). Pretreatment of cells with PD98059 did not significantly alter basal pH$_i$ in WKY (7.04±0.02) or SHR (7.09±0.03). However, MEK inhibition significantly reduced Ang II–elicited alkalinization in both rat groups (Figures 3f, 8, and 9). In the presence of PD98059, Ang II–stimulated pH$_i$ effects were not different between WKY and SHR (Figures 3f

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**Figure 5.** Representative recordings of $[Ca^{2+}]_i$-sensitive ratio of fluorescence intensities of 343 to 380 nm (F343/F380) in WKY and SHR cells in response to Ang II (10$^{-6}$ mol/L) in the absence and presence of PD98059 (10$^{-6}$ mol/L). Arrowheads indicate time of Ang II addition.
and 9, Table). The sustained pH wave induced by Ang II was unaltered by PD98059 (Figure 8).

**Temporal Associations Between Contraction, \([Ca^{2+}]_i\), and pH in Ang II–Stimulated Cells**

Ang II–elicited contraction was slow relative to the \([Ca^{2+}]_i\) responses, and the bottom panels demonstrate effects on the plateau phase of the \([Ca^{2+}]_i\) response. Data are presented as the Ang II–induced \([Ca^{2+}]_i\) change, measured as the difference between stimulated and basal \([Ca^{2+}]_i\). Dose-response curves were constructed from maximal responses that were obtained \(\sim30\) seconds after Ang II addition. Each data point is the mean \(\pm\) SEM of at least 3 experiments, with each experimental field comprising 4 to 9 cells. \(*P<0.05; **P<0.01\) vs WKY.

Figure 6. Dose-response curves demonstrate \([Ca^{2+}]_i\) effects of Ang II in the absence and presence of PD98059 (10\(^{-5}\) mol/L) in WKY- and SHR-derived vascular smooth muscle cells. The top panels demonstrate Ang II–induced effects on peak \([Ca^{2+}]_i\) responses, and the bottom panels demonstrate effects on the plateau phase of the \([Ca^{2+}]_i\) response. Data are presented as the Ang II–induced \([Ca^{2+}]_i\) change, measured as the difference between stimulated and basal \([Ca^{2+}]_i\). Dose-response curves were constructed from maximal responses that were obtained \(\sim30\) seconds after Ang II addition. Each data point is the mean \(\pm\) SEM of at least 3 experiments, with each experimental field comprising 4 to 9 cells. \(*P<0.05; **P<0.01\) vs WKY.

In cells preincubated with the MEK inhibitor, Ang II–elicited maximum contraction was expedited. In WKY cells, maximum contraction occurred within 3 minutes, and by 8 minutes cells approached their prestimulated state (Figure 3b). In SHR cells, maximum contractile responses were also achieved within 3 minutes, but the recovery phase to baseline was slightly prolonged compared with WKY cells (Figure 3b). Even though PD98059 accelerated contraction, maximum cell shortening was still temporally disassociated from peak \([Ca^{2+}]_i\), (Figure 3b and 3d).

**Discussion**

The major findings of the present study demonstrate that Ang II–stimulated contraction and associated intracellular second messengers, \([Ca^{2+}]_i\), and pH, are amplified in SHR-derived vascular smooth muscle cells, that ERK activity is increased in cells from SHR, and that augmented Ang II effects in SHR are partially mediated by ERK-dependent signaling pathways. Furthermore, using the MEK inhibitor, PD98059, we show that ERKs, which are classically associated with cell growth signaling pathways, may play a role in Ang II–stimulated contraction. Increased ERK activity may underlie hypercontractile responsiveness to Ang II in SHR cells. These abnormal cellular signaling events in SHR are probably postreceptor phenomena, as Ang II receptor density is not different between adult SHR and WKY.\(^{32}\) To our knowledge, this is the first study to investigate the role of ERKs in hypertension-associated Ang II–mediated hyperresponsiveness of isolated contracting vascular smooth muscle cells.

Vascular smooth muscle cells control the dynamic caliber of blood vessels and are the primary effector cells in the regulation of vascular tone and contractility. In hypertension, vasomotor tone is increased. This has been attributed to many underlying factors, including hypersensitivity to the vasoconstrictor Ang II.\(^{5,6}\) In the present study, we have shown that...
Ang II contracted individual vascular smooth muscle cells in a dose-dependent manner and that contractile effects were enhanced in cells from SHR (Figures 3 and 4). These results are in agreement with those of Bodin et al., who reported that the potency of Ang II–induced contraction was greater in isolated aortic vascular smooth muscle cells from adult SHR with established hypertension than in cells from age-matched WKY. Ang II induced sustained contraction with a slow rate of rise in cells from both WKY and SHR. Cells failed to fully relax to their prestimulated state even after Ang II was removed from the buffer. Ten minutes after Ang II stimulation, cells were still significantly contracted, with the magnitude of contraction greater in cells from SHR than in those from WKY (Figure 3a). This prolonged contraction may underlie exaggerated vasoconstriction in hypertension.

Smooth muscle cell contraction is regulated by \([\text{Ca}^{2+}]_i\), which activates myosin light-chain kinase, and by pH, which influences the \([\text{Ca}^{2+}]_i\) sensitivity of myofilaments. Increased cellular contraction in hypertension probably reflects enhanced second-messenger signaling. Our results demonstrating that Ang II–stimulated responses are augmented in cells from SHR are in agreement with other studies, except that the magnitude of \([\text{Ca}^{2+}]_i\) increase in WKY- and SHR-derived cells was higher than previously reported. Unlike in other investigations, the cells in the present study were grown on Matrigel basement membrane matrix and retained their contractile phenotype. Ang II increased pHi and \([\text{Ca}^{2+}]_i\) in a dose-dependent manner (Figures 6 and 9). The magnitude of Ang II–stimulated pHi change, although small, was temporally associated with contraction. Previous studies have demonstrated in intact vessels that even relatively small changes in pHi can significantly alter vascular tone. Ang II induced a biphasic \([\text{Ca}^{2+}]_i\) response with an initial and transient peak phase followed by a prolonged suprabasal phase (Figure 5). In stimulated cells, the first peak \([\text{Ca}^{2+}]_i\) phase preceded maximal contraction, whereas the second \([\text{Ca}^{2+}]_i\) phase and alkalinization were temporally associated with sustained contraction. The initial \([\text{Ca}^{2+}]_i\) transient generated by Ang II
probably sensitizes actin-myosin cross-bridge formation to initiate contraction.\textsuperscript{12,33,34} In SHR, increased [Ca\textsuperscript{2+}i], responses may potentiate activation of the contractile machinery, thereby enhancing contraction. Ang II mediates its biphasic [Ca\textsuperscript{2+}i], effects by inositol triphosphate–induced mobilization from reticular stores inducing the first [Ca\textsuperscript{2+}], transient and via Ca\textsuperscript{2+}, entry through Ca\textsuperscript{2+} channels, resulting in the second [Ca\textsuperscript{2+}], phase.\textsuperscript{35} We recently demonstrated that Ang II–elicited peak [Ca\textsuperscript{2+}], hypervasoresponsiveness in SHR was due primarily to increased [Ca\textsuperscript{2+}], mobilization.\textsuperscript{25} Although it is generally accepted that acutely elevated [Ca\textsuperscript{2+}], initiates contraction, underlying mechanisms for sustained contraction have not been clearly identified. Recent suggestions have implicated alternative signaling events, specifically MAPK-dependent pathways, in the regulation of the long-lasting phase of contraction (the “latch state”).\textsuperscript{22} Because MAPK activity is increased in vascular smooth muscle from various models of hypertension,\textsuperscript{36} including SHR (this study), and because Ang II–mediated MAPK activation is altered in SHR-derived vascular smooth muscle cells, we questioned whether MAPKs and specifically ERKs may play a putative role in contraction, and if so, whether they are involved in augmented Ang II–mediated contractile responses in SHR.

To determine whether ERKs (ERK-1 and ERK-2) influence vascular smooth muscle cell contraction and associated signaling in hypertension, the novel MEK inhibitor PD98059 was used. PD98059 has low affinity for kinases other than MEK-1/MEK-2 and inhibits both tyrosine and threonine protein phosphorylation.\textsuperscript{24,37} Thus, stimulation of the ERK pathway is blocked just before ERK activation. PD98059 inhibited ERK activity, indicating that the inhibitor did in fact block Ang II–induced ERK activation and that it was effective at a concentration of 10\textsuperscript{−5} mol/L. PD98059 significantly reduced the magnitude of contraction but did not completely abolish Ang II–stimulated contraction (Figures 3b and 4), suggesting that ERK-dependent pathways are not the only pathways involved in Ang II–induced contraction. It is possible that the PD98059 inhibitory effect on Ang II–stimulated actions results from nonselective inhibition of other protein kinases, including PKC, which is important for smooth muscle contraction, and other kinases involved in PKC-mediated responses. To examine this possibility, effects of PD98059 on [Ca\textsuperscript{2+}], and contractile responses induced by the PKC agonist PMA, which increased ERK activity in SHR and WKY, were tested. Whereas MEK inhibition significantly attenuated Ang II–induced actions, it did not alter PMA-stimulated [Ca\textsuperscript{2+}], or contractile responses and it failed to completely inhibit PMA-stimulated ERK activation. These data may suggest that effects of PD98059 influence ERK-dependent actions by Ang II but not by other intracellular events, including PKC-dependent mechanisms involved in [Ca\textsuperscript{2+}], and contractile responses to PMA. Similar findings have been reported for agonist-induced contraction in rat tail and mesenteric arteries.\textsuperscript{23} It may also be possible that PD98059 at a concentration of 10\textsuperscript{−5} mol/L is sufficient to inhibit Ang II–stimulated activation of ERKs but that higher concentrations of the inhibitor are needed for PMA-induced actions. The exact association between PKC-mediated contraction and ERKs in SHR is unclear and awaits elucidation.

The results of the present investigation implicate ERK-dependent pathways in Ang II–stimulated vascular smooth muscle cell contraction and, together with our data demonstrating that ERK activity is increased in SHR, suggest that aberrations in these pathways may underlie increased contractility in SHR. Our findings are supported by a recent study that demonstrated a temporal association between tyrosine phosphorylation of MAPK with the sustained phase of agonist-induced vascular smooth muscle contraction.\textsuperscript{22} Furthermore, Lucchesi et al\textsuperscript{38} reported that regulation of MAPK activation by Ang II differs in WKY and SHR. Stimulated MAPK inactivation in SHR is more rapid than in WKY, and SHR vascular smooth muscle cells display a greater dependence on Ca\textsuperscript{2+} mobilization than WKY-derived cells.\textsuperscript{38} Ang II–elicited hypervasoresponsiveness may be agonist-specific, as various studies have demonstrated that other vasoactive agonists, such as endothelin-1, vasopressin, and norepinephrine do not exhibit increased responses in vessels of SHR.\textsuperscript{3–5} Interestingly, all of these peptides mediate effects via similar signaling pathways, including phospholipases and MAPKs. Underlying mechanisms for differential agonist-elicited responsiveness are unclear. It may be possible that there is upregulation of the cellular Ang II–dependent pathways relative to that of vasopressin, endothelin-1 and norepinephrine in SHR with established hypertension. Ang receptor coupling to second messengers may be altered in hypertension, which could manifest as increased downstream signaling with activation of the MAPK cascade.

Mechanisms whereby ERK may regulate contraction are unclear. MAPK activation may lead to long-lasting contraction through interactions with active regulatory proteins, such as caldesmon.\textsuperscript{39,40} ERKs, which are Ca\textsuperscript{2+}-dependent,\textsuperscript{41,42} could also retroactively modulate [Ca\textsuperscript{2+}],. Cellular processes through which ERKs influence [Ca\textsuperscript{2+}], are unknown, but modulation of inositol 1,4,5-trisphosphate production, the primary mediator of intracellular Ca\textsuperscript{2+} mobilization, and activation of Ca\textsuperscript{2+} channels, the major pathway for Ca\textsuperscript{2+} influx, may be important. A recent study demonstrated that Ang II–induced inositol phosphate generation is mediated in part through tyrosine kinase pathways in cardiomyocytes,\textsuperscript{43} and others have implicated tyrosine kinases and MAPKs in vascular smooth muscle voltage-dependent Ca\textsuperscript{2+} channel activation.\textsuperscript{44} MAPKs may open voltage-dependent Ca\textsuperscript{2+} channels, thereby increasing Ca\textsuperscript{2+} influx and consequently raising [Ca\textsuperscript{2+}],. Ca\textsuperscript{2+} channel activation is important for maintenance of vascular smooth muscle contraction. Increased activation of these channels has been implicated in enhanced vascular contractility and structural alterations in hypertension.\textsuperscript{45,46} MEK could also influence contraction by changing pH. Ang II–induced intracellular alkalinization, a consequence of activation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, increases Ca\textsuperscript{2+} sensitivity of the actin-myosin complex, thereby modulating the contractile properties of vascular smooth muscle cells.\textsuperscript{47} Ang II–associated ERK signaling may regulate pH via activation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, as demonstrated by our results with the Na\textsuperscript{+}/H\textsuperscript{+} exchange blocker HMA, which completely abrogated Ang II–induced alkalinization (Figure 10).
vitz and Granot demonstrated in human platelets that MAPks activate Na\(^+\)-H\(^+\) exchange and tyrosine kinase-dependent pathways. Similar regulatory processes may occur in vascular smooth muscle. The exact interaction between inositol triphosphate, Ca\(^{2+}\) channels, Na\(^+\)-H\(^+\) exchange, and ERKs in hypertension is currently unclear and must await further clarification.

In conclusion, the present study demonstrates that in SHR- derived vascular smooth muscle cells, ERK activity is increased, Ang II-induced contraction and associated [Ca\(^{2+}\)]\(_{i}\), and pH, signaling are amplified, [Ca\(^{2+}\)]\(_{i}\), recovery and relaxation are prolonged, MEK inhibition abolishes sustained contraction, and PD98059 normalizes Ang II–elicted hyperresponsiveness. These data, together with the data of others, support a role for altered ERK-dependent signaling pathways in vascular smooth muscle cell function in SHR. Manipulation of this pathway with selective pharmacological inhibitors may provide novel approaches in the treatment of hypertension.

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References
Mitogen-Activated Protein/Extracellular Signal–Regulated Kinase Inhibition Attenuates Angiotensin II–Mediated Signaling and Contraction in Spontaneously Hypertensive Rat Vascular Smooth Muscle Cells
R. M. Touyz, M. El Mabrouk, G. He, X-H. Wu and E. L. Schiffrin

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