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Angiotensin II Activates Extracellular Signal-Regulated Kinase Independently of Receptor Tyrosine Kinases in Renal Smooth Muscle Cells: Implications for Blood Pressure Regulation

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ABSTRACT

Angiotensin II can cause hypertension through enhanced vasoconstriction of renal vasculature. One proposed mechanism for reduction of angiotensin II-induced hypertension is through inhibition of the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase cascade. MEK/ERK has been shown to phosphorylate the regulatory subunit of myosin light chain at identical positions as myosin light chain kinase. There are multiple mechanisms proposed regarding angiotensin II-mediated ERK activation. We hypothesized that renal microvascular smooth muscle cells (RμVSMCs) signal through a unique pathway compared with thoracic aorta smooth muscle cells (TASMCs), which is involved in blood pressure regulation. Use of epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptor-specific inhibitors 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) and 6,7-dimethoxy-3-phenylquinoxaline (AG1296), respectively, demonstrates that angiotensin II activates ERK in TASMCs, but not RμVSMCs, through transactivation of EGF and PDGF receptors. In addition, inhibition of Src with its specific inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) abolishes angiotensin II-, but not EGF- or PDGF-, mediated phosphorylation of ERK in RμVSMCs, yet it has no effect in TASMCs. The physiological significance of transactivation was examined in vivo using anesthetized Wistar-Kyoto rats with 15 mg/kg 2′-amino-3′-methoxyflavone (PD98059), an MEK inhibitor, as well as 20 mg/kg AG1478 and 1.5 mg/kg AG1296 in an acute model of angiotensin II-mediated increase in blood pressure. None of the inhibitors had an effect on basal blood pressure, and only PD98059 reduced angiotensin II-mediated increase in blood pressure. Moreover, in RμVSMCs, but not TASMCs, angiotensin II localizes phosphorylated ERK to actin filaments. In conclusion, angiotensin II signals through a unique mechanism in the renal vascular bed that may contribute to hypertension.

Extracellular signal-regulated kinases ERK1 and ERK2 (herein referred to as ERK) are involved in smooth muscle cell contraction (Touyz et al., 1999), attenuation of vascular relaxation (Touyz et al., 2002a), and blood pressure control (Muthalif et al., 2000a,b; Hu et al., 2007); however, this is not a universal mechanism (Watts et al., 1998; Touyz et al., 2002a). In addition, the molecular mechanism underlying the role of ERK in control of vascular contraction is not completely understood; currently there are two proposed mechanisms. One pathway is an ERK-mediated phosphorylation of the 20-kDa myosin light chain regulatory subunit (MLC20) at the same position as myosin light chain kinase (D'Angelo and Adam, 2002; Roberts, 2004). A second pathway involves ERK-mediated phosphorylation of caldesmon (Adam and Hathaway, 1993; D'Angelo et al., 1999). Phosphorylation of

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ABBREVIATIONS: ERK, extracellular signal-regulated kinase; MLC20, 20-kDa myosin light chain regulatory subunit; GPCR, G protein-coupled receptor; PLD, phospholipase D; TASMC, thoracic aorta smooth muscle cell; Ang II, angiotensin II; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; RμVSMC, renal microvascular smooth muscle cell; PE, polyethylene; DMSO, dimethyl sulfoxide; PD98059, 2′-amino-3′-methoxyflavone; AG1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; AG1296, 6,7-dimethoxy-3-phenylquinoxaline; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ANOVA, analysis of variance; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene.

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MLC20 has been proposed to be more relevant than caldesmon (D'Angelo and Adam, 2002), but activation of ERK does not always lead to phosphorylation of MLC20 (Tsai and Jiang, 2005). Importantly, both of these targets would increase contraction through modulating the actin-myosin interaction via classically described interactions (for review, see Webb, 2003).

There is no unified theory to describe the molecular mechanisms underlying G protein-coupled receptor (GPCR) activation of ERK. Multiple mechanisms have been proposed, including transactivation (Daub et al., 1996), β-arrestin (Luttrell et al., 2001), or phospholipase D (PLD)-mediated scaffolding (Rizzo et al., 1999), and nonreceptor kinase-mediated activation of ERK (Ettehadieh et al., 1992; Carroll and May, 1994). These proposed mechanisms are not necessarily mutually exclusive (Luttrell et al., 1999; Shah and Catt, 2002); however, the transactivation theory has become predominant, and it has been shown to occur in many (Wetzker and Bohmer, 2003) but not all (Shah et al., 2004) tissues. in brief, transactivation involves GPCR-activating receptor tyrosine kinases that then signal through their respective signaling cascade, leading to ERK activation. In thoracic aortic smooth muscle cells (TASMCs) angiotensin II (Ang II), a GPCR agonist, activates ERK through transactivation of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors (Eguchi et al., 1998).

Ang II is a vasoactive hormone that can cause multiple cardiovascular and renal disorders in experimental models. One such disorder, hypertension, can be caused by Ang II, and interference with either Ang II production (via angiotensin-converting enzyme inhibitors) or signaling (via Ang II receptor blockers) is common antihypertensive therapy. Renal transplantation studies demonstrate that hypertension can be transferred to a normotensive host if receiving a kidney from a hypertensive donor (Grisk and Rettig, 2001). Recently, similar experiments using genetically modified mice demonstrate that kidney angiotensin type 1 receptors are responsible for Ang II-mediated hypertension and associated cardiovascular disorders (Crowley et al., 2006). This is further supported by the finding that the renal vascular bed is the most responsive to Ang II in vivo (Jackson and Herzer, 2001). Therefore, if Ang II-mediated ERK activation is involved in blood pressure regulation, as has been shown previously (Muthalif et al., 2000b), then inhibition of ERK activation, either by inhibiting MEK or through blocking transactivation of EGF or PDGF receptor kinase activity, has to reduce Ang II-mediated increase in blood pressure, if, and only if, transactivation is a universal mechanism.

In this study, we tested the above-mentioned statement and the hypothesis that Ang II-mediated ERK activation is different in TASMCs compared with renal microvascular smooth muscle cells (RμVSMCs). Previous pharmacological data separated Ang II and EGF signaling to ERK in RμVSMCs, but such studies did not directly examine transactivation (Andresen et al., 2003). Here, we confirm previous studies (Muthalif et al., 2000a,b; Hu et al., 2007) that indicate a role for MEK/ERK in Ang II-mediated blood pressure regulation in vivo, and we demonstrate that this is independent of EGF and PDGF receptor activity. Furthermore, the physiological data are supported via cell culture studies demonstrating that Ang II signals to ERK through a Src family member-dependent, yet nontransactivation-mediated pathway in RμVSMCs, compared with a Src-independent transactivation pathway in TASMCs.

Materials and Methods

In Vivo Experiments. All experiments were conducted per National Institutes of Health and Georgetown University Animal Care and Use Committee guidelines. Sixteen Wistar-Kyoto rats, four per group, were obtained from Taconic Farms (Hudson, NY). The rats were maintained in Georgetown University's animal facility on a 12-h light cycle, and they were fed standard rat chow with water ad libitum. On the day of the experiment, one rat was transferred from the animal facility to the laboratory and anesthetized with pentobarbital (Nembutal; 50 mg/kg b.wt. i.p.). Once anesthetized, the rat was placed on a heated table to maintain body temperature at 37°C, which was monitored via a rectal probe. The left femoral vein was catheterized with PE-50 tubing, and this site was used to infuse 0.1 ml of pentobarbital (50 mg/ml) per 100 g of body weight per hour to maintain the rat in an anesthetized state. Next, the right femoral artery was catheterized with PE-50 tubing to continuously monitor blood pressure through a pressure transducer coupled to a Cardiomax II blood pressure recorder (Columbus Instruments, Columbus, OH). An endotracheal tube was inserted using PE-250 to allow the rat to breathe easily. Following the endotracheal tube, the jugular vein was catheterized with PE-50 tubing. This site was used for fluid maintenance at a rate of 0.5 ml per 100 g per hour. Last, a cystotomy was performed, and a heat-flanged PE-240 tube was inserted into the bladder to relieve the bladder and to maintain normal renal function.

The rat was allowed to equilibrate for 1 h postsurgery. Once equilibrated, basal blood pressure was recorded for 20 min before a bolus injection of 2.5 μg/kg Ang II via the jugular catheter. Following the injection, blood pressure was recorded for the next hour, after which either the drug or vehicle was injected i.p. The injections were one of the following: DMSO as a vehicle (Sigma-Aldrich, St. Louis, MO), 15 mg/kg PD98059, an MEK-specific inhibitor; 20 mg/kg AG1478, an EGF receptor-specific inhibitor; and 1.5 mg/kg AG1296, a PDGF receptor-specific inhibitor (all obtained from Calbiochem, San Diego, CA). Thirty minutes after the i.p. injection, a second 2.5-μg/kg dose of Ang II was administered via the jugular catheter. The rat was euthanized 30 min after the second Ang II injection by overdose with pentobarbital (100 mg/kg i.v.). The raw data were calculated by determining the change (Δ) of Ang II-mediated systolic blood pressure by subtraction of the baseline from the maximal systolic blood pressure. The percentage of change was also calculated as -fold change times 100, which incorporates the baseline into the value. All analysis was conducted with the Δ systolic blood pressure values.

Cell Culture. All cells were obtained from Dr. Edwin K. Jackson (University of Pittsburgh, Pittsburgh, PA) and grown in Dulbecco's modified Eagle's medium/Ham's F-12 with 10% fetal bovine serum plus antibiotics (100 U/ml penicillin G, 100 U/ml streptomycin sulfate, and 250 ng/ml amphotericin B). All cell culture materials were obtained from Invitrogen (Carlsbad, CA).

Activation of ERK. TASMCs and RμVSMCs were grown in 60-mm tissue culture plates, serum-starved overnight (at least 12 h), and then treated with the vehicle or drug. The drugs were all dissolved in DMSO, and the cells were pretreated as follows: 20 μM PD98059 was applied for 30 min, 100 nM PP2 was applied for 30 min, 1 μM AG1478 was applied for 2 h, 10 μM AG1296 was applied for 2 h, or DMSO was applied for 2 h. These concentrations and durations of exposure to drug were chosen because each has been demonstrated to specifically inhibit their respective target. The cells were then stimulated with 1 μM Ang II, 50 ng/ml EGF, or 50 ng/ml PDGF for 5 min, washed with ice-cold PBS, and then lysed in 250 μl of a radioimmunoprecipitation assay lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 100 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA, 10 mM $MgCl_2$, 10 mM NaF, and 40 mM β-glycerol phosphate, supplemented with 1 mM phenylmethylsulfonyl fluoride

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and 2 mM Na_3VO_4). The samples were clarified by centrifuging the lysate at 14,000 rpm and removing the supernatant, and then Laemmli sample buffer was added, and the samples were boiled and stored at −20°C before use. The samples were separated on a 10% SDS-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose obtained from Bio-Rad (Hercules, CA). ERK activation was measured via Western blot with phosphospecific ERK1/2 antibodies (Cell Signaling Technology Inc., Danvers, MA), followed by stripping the membrane with 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS and reprobing for total ERK1/2 (Cell Signaling Technology Inc.). Secondary antibodies and enhanced chemiluminescence were obtained from Pierce Chemical (Rockford, IL), and the images were captured on Fujifilm Super RX X-ray film obtained from Crystalgen (Plainview, NY) and developed with a Kodak RPX-OMAT processor (model M6B; Eastman Kodak, Rochester, NY).

Confocal Imaging. TASMCs and RμVSMCs were grown on 22-mm number 1 coverslips (Fisher Scientific, Pittsburgh, PA) coated with poly-D-lysine (Sigma-Aldrich) in six-well plates. The cells were serum-starved overnight (at least 12 h) and stimulated as described above. After stimulation, the cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 30 min at 4°C, and permeabilized with 0.1% Triton X-100 for 2 min. The cells were then washed with PBS, and blocking was performed with 3% BSA (Sigma-Aldrich) in PBS. After blocking, the E10 antiphosphorylated ERK (mouse monoclonal) primary antibody (Cell Signaling Technology Inc.) was diluted at 1:500 in a 3% BSA in PBS solution, and it was applied to the cells for 1 h. The cells were then washed with PBS and stained with a 3% BSA in PBS solution containing 5 μM Hoechst (Invitrogen), 10 units Alexa 488-labeled phalloidin (Invitrogen), and 2.6 μg/ml Cy5-labeled $F(ab')_2$ fragment donkey anti-mouse (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h. The samples were washed with PBS, and then they were mounted on glass slides (Fisher Scientific) with Prolong antifade mounting medium (Invitrogen). Imaging was conducted with an Olympus BX61 Fluoview FV500 laser scanning confocal microscope (Olympus, Melville, NY) with a Plan Apo 60 × 1.40 numerical aperture oil immersion objective. Hoechst was visualized using a 405-nm diode laser, Alexa 488-labeled phalloidin was visualized using a 488-nm argon laser, and the Cy5-labeled E10 antibody was visualized using a 633-nm He-Ne laser. All images were acquired individually to eliminate the possibility of bleedthrough and set to a resolution of 1024 × 1024. Each image is an average of four repetitive scans.

Statistics and Data Analysis. All graphs were generated with GraphPad Prism 4 for Windows (GraphPad Software Inc., San Diego, CA). Paired t tests were used to determine whether the change in the increase in blood pressure was different between the two injections of Ang II for each treatment group with reverse experiment tester (Boss Statistics) (Lew, 2006) and NCSS 2007 (NCSS, Kaysville, UT). Reverse tester has two functions, the t test and a noninferiority test that tests for similarity between groups instead of a difference. This was used to determine whether the two Ang II-generated peaks in blood pressure were significantly similar in each group, and if the drug treatments were significantly similar to vehicle injection. A repeated measures ANOVA with Tukey-Kramer multiple comparison test was used to compare all the raw data, and a second ANOVA with Tukey-Kramer multiple comparison test was used to compare the differences in Δ systolic blood pressure between the four groups via NCSS 2007. All measurements are represented as mean ± S.E.M., followed by the n. Western blots of p44 ERK1 and p42 ERK2 (referred to as ERK) were grouped together and quantified using ImageJ (http://rsb.info.nih.gov/ij/) 1.36b for Windows. Phosphorylated ERK was normalized to total ERK and that ratio was analyzed via an ANOVA with Tukey-Kramer Multiple-comparison post-hoc test to compare the levels of Ang II-mediated ERK activation. The red channel in the confocal images was enhanced using the curves option in Photoshop 7.0 (Adobe Systems, Mountain View, CA), so that they are more visible on the black background. All TASMCs were altered with the same curve, and a similar curve was used on all the RμVSMCs. MetaMorph software (Molecular Devices, Sunnyvale, CA) was used to filter the Hoechst staining in Fig. 5 via use of the median filter to reduce background signal while maintaining visual localization of the nuclei.

Results

To determine the role of ERK in Ang II-mediated increases in blood pressure, we rapidly injected 2.5 μg/kg Ang II in the jugular vein while measuring blood pressure. This method resulted in a rapid, less than 90 s, increase in systolic blood pressure of 106 ± 5 mm Hg, $n = 16$, that quickly dropped and then returned to baseline in 4.7 ± 0.5 min. One hour after injection of Ang II, the drug or vehicle (DMSO) was administered. The MEK inhibitor PD98059 was used at a concentration similar to what has been previously reported to reduce Ang II-mediated blood pressure (Muthalif et al., 2000b). In addition, PD98059 was used instead of U0126 because U0126 seems to be nonspecific in vivo in regard to muscle contraction (Watts, 2000). The EGF receptor inhibitor AG1478 was used at a dose sufficient to clear tumors from mice when administered i.p. (He et al., 2001), and the PDGF receptor inhibitor AG1296 was used at a dose calculated from previous in vivo studies (Dell et al., 2006). On occasion, injection of the drug or vehicle transiently lowered blood pressure due to a stress response, but it had no appreciable effect on baseline blood pressure over time.

Repeated measures ANOVA demonstrated a significant difference between the groups (drug treatments) ($p = 0.016531$) with a power of 0.804994, with no difference between the first and second stimulation with Ang II (period); however, there is a significant interaction between the period and drug treatments ($p = 0.014309$) with a power of 0.823593. Tukey-Kramer multiple comparison test indicates that treatment with DMSO, AG1478, or AG1296 had no significant effect ($p > 0.05$) on the response to the second dose of Ang II, but that PD98059 did have a significant effect ($p <$

TABLE 1

Effect of MEK, EGF receptor, and PDGF receptor inhibition on 2.5 μ g/kg Ang II increases in systolic blood pressure

ΔAng II 1 represents the mean maximum systolic blood pressure (mm Hg) after the first exposure to 2.5 μg/kg Ang II, and ΔAng II 2 occurs after exposure to the treatment (group). The %Δ represents the total increase, baseline included, of the peak Ang II-mediated change in systolic blood pressure. A one-sided paired t test was used to determine whether the treatment caused a decrease in Ang II-mediated changes in blood pressure as has been previously reported for PD98059. The noninferiority test produces a power statistic that describes the percentage of confidence to detect a change equal to that seen with PD98059; higher power designates that the second ΔAng II effect is similar to the first ΔAng II; $n = 4$ for all groups. Power greater than 0.8 is generally acceptable and therefore similar.

Group	ΔAng II 1		ΔAng II 2 with Drug (Group)		Paired t Test (One-Sided), p Value	Noninferiority Test, Power
	mean ± S.E.	*%Δ ± S.E.*	*mean ± S.E.*	*%Δ ± S.E.*		
DMSO	96 ± 10	172 ± 10	97 ± 12	174 ± 11	0.882	0.898
PD98059	111 ± 8	184 ± 7	88 ± 8	162 ± 4	0.024	0.486
AG1478	91 ± 11	167 ± 8	94 ± 8	175 ± 4	0.641	0.501
AG1296	128 ± 9	202 ± 6	132 ± 7	204 ± 5	0.922	0.9984

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0.05) (Table 1). There are differences among period 1, the first stimulation with Ang II, between the groups; however, this seems to be due to random distribution of the individual rats; overall, the 16 period 1 data points do confer to a normal distribution. In addition, paired *t* test also indicates that there is only a significant difference between the PD98059-treated samples (Table 1). Furthermore, the DMSO and AG1296 treatments were significantly similar to their controls using a paired noninferiority test (Table 1). To graphically display this, the maximum change in systolic blood pressure caused by the first dose of Ang II was subtracted from the maximal change in systolic blood pressure of the second dose of Ang II for comparison across groups (Fig. 1). ANOVA analysis of this normalized data demonstrated a significant difference between the treatment groups (p = 0.014457) with a power of 0.822299, and Tukey-Kramer multiple comparison test indicates that only inhibition of MEK reduced Ang II-mediated increases in systolic blood pressure ($p < 0.05$). Furthermore, the AG1478 and AG1296 groups are similar to DMSO (for $p < 0.05$, the power is 0.705 for AG1478 and 0.9707 for AG1296; $n = 4$). The data indicate that MEK, and presumably ERK, play a role in Ang II-mediated increases in blood pressure. Moreover, transactivation of EGF and PDGF receptors does not contribute to the acute increase in blood pressure in response to Ang II administration.

Since in RμVSMCs Ang II and EGF signaling to ERK can be pharmacologically separated (Andresen et al., 2003) and the kidney plays a large role in Ang II-mediated changes in blood pressure (Jackson and Herzer, 2001; Crowley et al., 2006), we examined the role of transactivation of EGF and PDGF receptors in Ang II-mediated ERK activation in RμVSMCs (Fig. 2, A and B). As shown in Fig. 2, A and B, in TASMCs Ang II-mediated activation of ERK is sensitive to inhibition of MEK and EGF and PDGF receptors, which confirms previous reports (Eguchi et al., 1998). However, in RμVSMCs Ang II-mediated ERK activation is sensitive to inhibition of MEK, but it is insensitive to inhibition of EGF and PDGF receptors. Quantification and ANOVA analysis of the Ang II-treated samples ($n = 3$) indicates that basal ERK levels are slightly, yet not statistically, increased in RμVSMCs compared with TASMCs (Fig. 2B). Ang II significantly increases ERK phosphorylation in TASMCs and RμVSMCs to a similar extent, and PD98059 reduces Ang II-mediated ERK phosphorylation to basal levels in both TASMCs and RμVSMCs. Inhibition of EGF and PDGF receptors only inhibits Ang II-mediated activation of ERK in TASMCs, and this inhibition reduces phosphorylated ERK levels to baseline (Fig. 2B). Treatments with EGF and PDGF demonstrate that PD98059 works similarly in both TASMCs and RμVSMCs (Fig. 2A). In addition, treatments with AG1478 and AG1296 inhibit EGF- and PDGF-mediated activation of ERK, respectively, in both cell types (Fig. 2A). These data demonstrate that EGF and PDGF receptors are present and signal to ERK in RμVSMCs. Therefore, Ang II is not signaling through transactivation of EGF and PDGF receptors in the RμVSMCs.

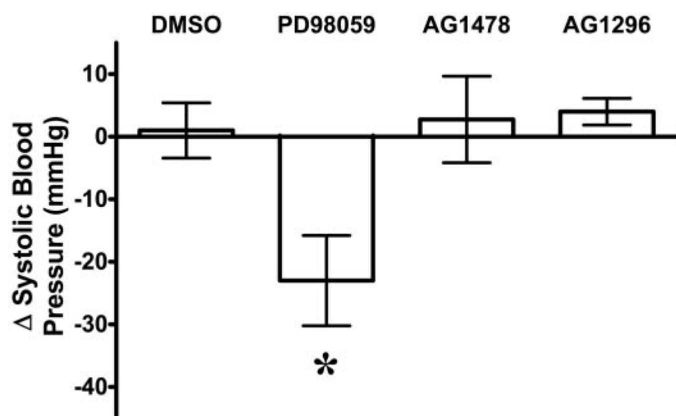

Fig. 1. Inhibition of MEK with 15 mg/kg PD98059, but not EGF (20 mg/kg AG1478) and PDGF (1.5 mg/kg AG1296) receptors, reduces Ang II-mediated increases in systolic blood pressure. The histogram represents the mean difference in systolic blood pressure between the second Ang II-induced peak and the first Ang II-induced peak, shown in Table 1. The asterisk represents that the group is significantly different ($p < 0.05$) from all other groups (n = 4 per group) by ANOVA with Tukey-Kramer multiple comparison post hoc test.

Multiple studies indicate that Src, or a Src family kinase, plays a role in Ang II-mediated ERK activation (Eguchi et al., 1998; Matrougui et al., 2000; Touyz et al., 2001; Shah and Catt, 2002; Shah et al., 2004), and preliminary data indicated that a Src family kinase is involved in Ang II signaling to ERK in RμVSMCs. Therefore, we used the Src family kinase inhibitor PP2 to examine the role of Src family members in Ang II, EGF, and PDGF-mediated activation of ERK in TASMCs and RμVSMCs (Fig. 2, C and D). As shown in the Western blot (Fig. 2C) 100 nM PP2 only inhibited Ang II-mediated activation of ERK in RμVSMCs. Quantification and ANOVA analysis of the western blots ($n = 3$) demonstrates that a Src family member is required for Ang II-mediated activation of ERK in RμVSMCs, but not TASMCs (Fig. 2D). Moreover, EGF and PDGF are not sensitive to 100 nM PP2 in either cell type.

Preliminary data suggested that in RμVSMCs, Ang II caused phosphorylated ERK to localize on actin filaments. Therefore, we examined the cellular location of phosphorylated ERK in TASMCs and RμVSMCs after stimulation with Ang II, EGF, or PDGF for 5 min (Figs. 3 and 4). Hoechst stain was used to identify the nucleus, and phalloidin was used to identify the actin filaments. As shown in Fig. 3, Ang II, EGF, and PDGF all traffic phosphorylated ERK to the nucleus and perinuclear region of TASMCs. Ang II directed phosphorylated ERK to actin filaments in approximately 25% of the TASMCs observed. In contrast, all agonists directed phosphorylated ERK to actin filaments and the nucleus and perinuclear region in RμVSMCs (Fig. 4). Ang II directed phosphorylated ERK to the actin filaments in all of the RμVSMCs cells observed (Figs. 4, E–H, and 5), whereas EGF and PDGF directed phosphorylated ERK to the actin filaments in approximately 50% of the RμVSMCs observed. In addition, as shown in Fig. 5, the alignment of the actin filaments is not in an orientation from the cell surface toward the nucleus, suggesting that active ERK is not using the actin filaments merely as a route to traffic to the nucleus. Therefore, the localization of active ERK is clearly different in RμVSMCs compared with TASMCs, and in RμVSMCs the localization of active ERK suggests that it may mediate phosphorylation of the actin-myosin contractile apparatus.

Discussion

The purpose of this study was to examine the role of transactivation in Ang II-mediated activation of ERK in the renal

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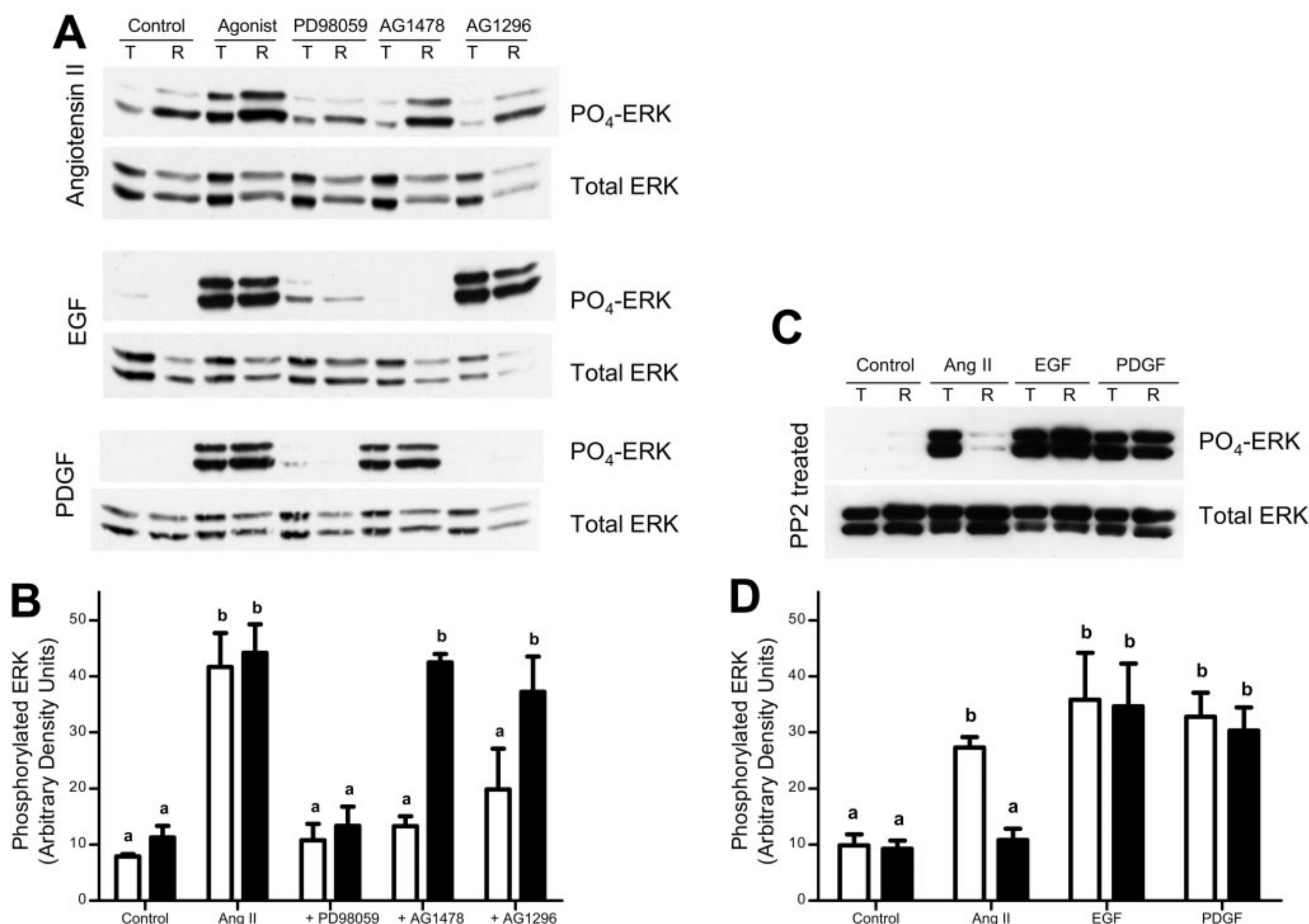

Fig. 2. Ang II signals through a nontransactivation, Src family kinase-dependent mechanism in RμVSMCs, but not in TASMCs. A, representative Western blots for phosphorylated and total ERK are shown. T represents TASMCs and R represents RμVSMCs. Cells were treated in the presence of DMSO control; the agonist- (listed at the left of each pair of blots) or agonist plus drug (listed above the first blot)-specific conditions are given in the text. Stimulation with EGF and PDGF demonstrate the specificity of the drugs and that the TASMCs and RμVSMCs respond to EGF and PDGF similarly. B, histogram of the Ang II-treated samples in A. Open bars represent TASMCs and closed bars represent RμVSMCs. Bars with different letters are significantly different ($p < 0.05$) by ANOVA with Tukey-Kramer multiple comparison post hoc test ($n = 3$). C, representative Western blots for phosphorylated and total ERK in the presence of 100 nM Src family kinase inhibitor PP2 are shown. Labeling of the blots is identical to A. D, histogram of C ($n = 3$); all labels and statistics are the same as in B.

microvasculature. The data consistently show differences in Ang II-mediated signaling between the thoracic and renal smooth muscle cells. Although, the physiological data do not specifically isolate the renal bed, they do indicate that vessels contributing to the transient increase in blood pressure are MEK inhibitor-sensitive but that they do not signal through a transactivation-mediated mechanism. Furthermore, the thoracic aorta, which does signal through transactivation, has no effect on Ang II-mediated alterations in blood pressure (Jackson and Herzer, 2001).

The role of ERK in vascular contraction has been described in multiple models (Touyz et al., 1999, 2002a; Muthalif et al., 2000a; Roberts, 2004), including a model of Ang II-dependent hypertension (Muthalif et al., 2000b). Using male Sprague-Dawley rats with induced hypertension via chronic Ang II infusion, Muthalif et al. (2000b) reported a decrease in mean arterial blood pressure of 20 mm Hg 30 min after a single injection of a comparable dose of PD98059. As shown in Table 1 and Fig. 1, injection of 15 mg/kg PD98059 significantly reduced transient Ang II-mediated increase in systolic blood pressure by 23 ± 8 mm Hg, which is drastically different from the DMSO-treated controls increase of 1 ± 5 mm Hg. Our method was designed specifically to isolate changes in systolic blood pressure derived from vasoconstriction, which is exemplified by the rapid increase in blood pressure followed by an equally rapid decline and return to baseline. This allowed us to conclude that the major cause of the increase in blood pressure is due to vasoconstriction and that MEK, and presumably ERK, provide for roughly 20% of the contractile response to Ang II in this model. Because of the similarity between our studies and the studies by Muthalif et al. (2000b), we suggest that MEK is involved in hypertension. However, both studies were conducted in anesthetized rats and remain unconfirmed in conscious animals.

The molecular mechanisms underlying Ang II-mediated phosphorylation of ERK do not fit into a unified mechanism. Most studies of smooth muscle cellular biology are conducted in TASMCs; however, these vessels are conduit vessels that have little physiological role in controlling blood pressure, especially Ang II-mediated changes in blood pressure (Jackson and Herzer, 2001). Through using TASMCs, and other cell lines, it has been established that Ang II signals to ERK through transactivation of receptor tyrosine kinases (Eguchi et al., 1998). This pathway relies on the ability of EGF and

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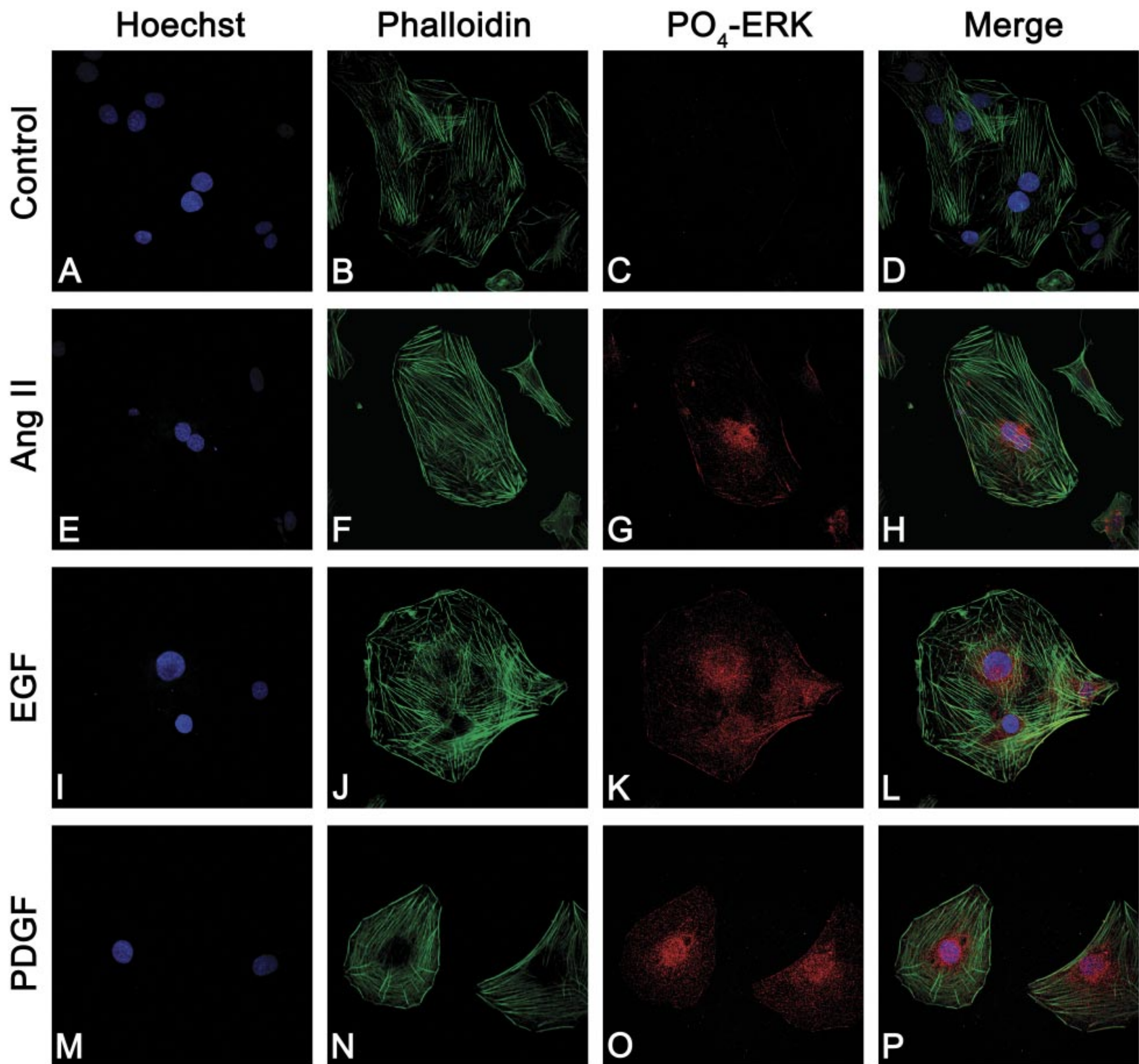

Fig. 3. Localization of phosphorylated ERK in TASMCs. Cells were treated with: control (A–D), 1 μM Ang II (E–H), 50 ng/ml EGF (I–L), or 50 ng/ml PDGF (M–P) for 5 min and stained with Hoechst (A, E, I, and M), Alexa 488-labeled phalloidin (B, F, J, and N), and antiphosphorylated ERK with Cy5 (C, G, K, and O). The merge panels are to the far right (D, H, L, and P). All settings are identical for each image, and the magnification is 600×.

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PDGF receptor inhibitors to block Ang II-mediated activation of ERK; yet, as shown in Fig. 2, this is not the case in RμVSMCs. Because the renal vasculature plays the largest role in modulating transient vascular changes in response to Ang II (Jackson and Herzer, 2001), the lack of transactivation in the RμVSMCs explains the lack of significant effect of AG1478 and AG1296 on transient Ang II-mediated changes in systolic blood pressure. It is important to note that these experiments examine transient increases in blood pressure; thus, these data do not preclude a role for EGF and PDGF receptors in the maintenance or progression of hypertension. Further studies in other models are required to definitively comment on the role of EGF and PDGF receptors in the maintenance or progression of hypertension.

Beyond transactivation, there exist multiple mechanisms that can mediate signaling from a GPCR, such as the angiotensin type 1 receptor, to ERK. Likely candidates are β-arrestin-mediated internalization of the receptor and scaffolding of signaling components (Luttrell et al., 2001), PLD generation of phosphatidic acid (Rizzo et al., 1999), and Src (Ettehadieh et al., 1992) and protein kinase C (Carroll and May, 1994). Previous studies in RμVSMCs indicate that PLD is involved in Ang II-mediated activation of ERK (Andresen et al., 2003), and as shown in Fig. 2, a Src family member plays a role in Ang II-mediated ERK activation. These data agree with reports that Src is involved in Ang II-mediated preglomerular vascular contraction (Che and Carmines, 2005). However, these data are contrary to multiple papers, indicating that transactivation is Src-dependent (Eguchi et al., 1998; Shah and Catt, 2002; Touyz et al., 2002b). This may be because the Wistar-Kyoto seems to be less sensitive to Src inhibition (Touyz et al., 2002b). In addition, multiple reports use concentrations of PP2 in the micromolar range. In addition to transactivation, Src can directly activate ERK (Ettehadieh et al., 1992) and may be involved in β-arrestin-mediated activation of ERK (Luttrell et al., 1999). Therefore, Fig.

Hoechst Phalloidin PO_4-ERK Merge

Control Ang II EGF PDGF

Fig. 4. Localization of phosphorylated ERK in RμVSMCs. Cells were treated with control (A–D), 1 μM Ang II (E–H), 50 ng/ml EGF (I–L), or 50 ng/ml PDGF (M–P) for 5 min and stained with Hoechst (A, E, I, and M), Alexa 488-labeled phalloidin (B, F, J, and N), and antiphosphorylated ERK with Cy5 (C, G, K, and O). The merged panels are to the far right (D, H, L, and P). All settings are identical for each image, and the magnification is 600×.

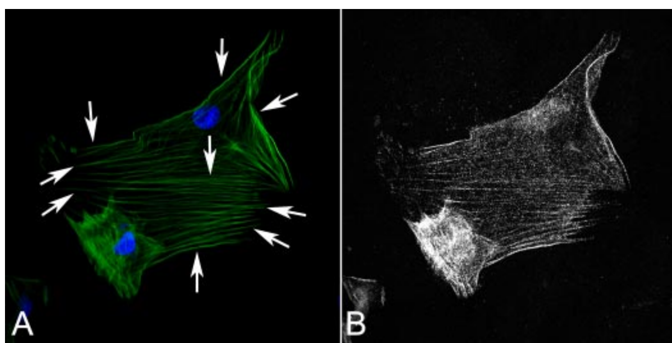

Fig. 5. Ang II directs ERK to actin filaments in RμVSMCs. Cells were treated and stained identically as in Figs. 4 and 5. A, merged frame of Hoechst and phalloidin staining, whereas B is an unmanipulated black-and-white image of phosphorylated ERK. The bright area on the lower left is a second, constricted, cell that is in the same plane. Arrows point to phosphorylated ERK-laden actin filaments that are not directed toward the nucleus. The magnification is 600×.

2 demonstrates that the transactivation pathway is Src-independent, but it does little to further elucidate the pathway used by Ang II in RμVSMCs. Overall, since the PLD/phosphatidic acid mechanism only provides for the recruitment and scaffolding of the ERK signaling cascade in RμVSMCs (for review, see Andresen et al., 2002), a Src family member most probably provides the mechanism to activate ERK.

The β-arrestin and PLD mechanisms are linked to internalization, where actin is known to play a role and internalization can occur on actin tracks (Engqvist-Goldstein and Drubin, 2003). Actin tracks are generally oriented in a pattern that point from the plasma membrane toward the center of the cell. These tracks allow for cargo to traffic to the nucleus or a recycling center. Although our data indicate that ERK is localized on actin filaments, they do not seem to be used to internalize cargo. This is concluded from the following observations. First, the colocalization of ERK with actin filaments in the RμVSMCs is not aligned in a pattern that

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resembles internalization (Fig. 5) in that the actin is not ordered from the membrane to a central point in the cell. Second, although a time course of ERK activation was not addressed in this study, Fig. 2B indicates that at 5 min the level of activated ERK is similar in TASMCs and RμVSMCs. Generally, ERK is internalized and within the nuclear/perinuclear region within 5 min. This is observed in TASMCs (Fig. 3), but not RμVSMCs (Fig. 4), indicating that either the RμVSMCs are "slow" traffickers of ERK, or that ERK is purposefully targeted to actin filaments. Therefore, the data suggest that phosphorylated ERK is targeted to actin filaments in RμVSMCs. Further time course and live cell experiments are needed to confirm this conclusion. Targeting phosphorylated ERK to actin filaments provides a potential mechanism for ERK-mediated phosphorylation of MLC20 (Roberts, 2004), and thus vascular contraction and increases in blood pressure.

Unexpectedly, stimulation with EGF or PDGF also placed phosphorylated ERK on actin filaments. This indicates that there is a general mechanism for trafficking ERK that is independent of the mechanism of ERK activation in RμVSMCs and that this mechanism is most probably different or at least slower than that in TASMCs. In addition, the data suggest that agonists that activate ERK, such as EGF, could also contribute to modulating blood pressure, as has been demonstrated previously (Carmines et al., 2001). Interestingly, infusion of EGF in rats slowly and marginally increases blood pressure, but decreases blood pressure in monkeys (Keiser and Ryan, 1996). However, a more physiological dose of EGF did not alter blood pressure in rats, but it did alter arteriolar constriction and cause renal alterations (Harris et al., 1988). In this study, we found that inhibition of EGF receptors is neither significantly different nor similar to the controls, suggesting that EGF receptors may have a role in blood pressure regulation, but they are not significantly involved in Ang II-mediated transient elevations of blood pressure. Further investigation is warranted to determine the role of EGF and EGF receptors in both transient and long-term blood pressure regulation. Inhibition of PDGF receptors has been shown to be a potent therapy for treatment of pulmonary hypertension, whereas no effect was observed on mean arterial pressure (Schermuly et al., 2005). Thus, PDGF has a negligible role, if any, in regulating systemic blood pressure. Importantly, the studies conducted here use only Ang II as an agonist in the physiological experiments, so the role of EGF or PDGF cannot be assessed. Only the role of the EGF and PDGF receptors in Ang II-mediated rapid changes in blood pressure can be discerned.

In conclusion, this is the first report to document that signaling to ERK in smooth muscle cells within resistance vessels from the kidney is different from that of the smooth muscle cells from the thoracic artery from the same strain of rats. Specifically, in RμVSMCs, Ang II-mediated activation of ERK is independent of receptor tyrosine kinases, but is dependent on a Src family kinase. In TASMCs, the transactivation pathway is dominant, but it has little to no role in regulating transient Ang II-mediated changes in blood pressure. A notable difference between TASMCs and RμVSMCs is that agonists that activate ERK direct phosphorylated ERK to actin filaments and the nuclear/perinuclear region in RμVSMCs, whereas in TASMCs phosphorylated ERK is directed to only the nuclear/perinuclear region. This may play a role in ERK-mediated increased blood pressure. Thus, the molecular mechanisms underlying Ang II-mediated activation of ERK and ERK trafficking to actin filaments in RμVSMCs should be investigated further.

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References

Adam LP and Hathaway DR (1993) Identification of mitogen-activated protein kinase phosphorylation sequences in mammalian h-Caldesmon. *FEBS Lett* **322:** 56–60.

Andresen BT, Linnoila JJ, Jackson EK, and Romero GG (2003) Role of EGFR transactivation in angiotensin II signaling to extracellular regulated kinase in preglomerular smooth muscle cells. *Hypertension* **41:**781–786.

Andresen BT, Rizzo MA, Shome K, and Romero G (2002) The role of phosphatidic acid in the regulation of the Ras/MEK/Erk signaling cascade. *FEBS Lett* **531:**65–68.

Carmines PK, Fallet RW, Che Q, and Fujiwara K (2001) Tyrosine kinase involvement in renal arteriolar constrictor responses to angiotensin II. *Hypertension* **37:**569–573.

Carroll MP and May WS (1994) Protein kinase C-mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells. *J Biol Chem* **269:**1249–1256.

Che Q and Carmines PK (2005) Src family kinase involvement in rat preglomerular microvascular contractile and [Ca^{2+}]i responses to ANG II. *Am J Physiol Renal Physiol* **288:**F658–F664.

Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, Kim HS, Smithies O, Le TH, and Coffman TM (2006) Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci U S A* **103:**17985–17990.

D'Angelo G and Adam LP (2002) Inhibition of ERK attenuates force development by lowering myosin light chain phosphorylation. *Am J Physiol* **282:**H602–H610.

D'Angelo G, Graceffa P, Wang CA, Wrangle J, and Adam LP (1999) Mammal-specific, ERK-dependent, caldesmon phosphorylation in smooth muscle. Quantitation using novel anti-phosphopeptide antibodies. *J Biol Chem* **274:**30115–30121.

Daub H, Weiss FU, Wallasch C, and Ullrich A (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379:**557–560.

Dell S, Peters S, Muther P, Kociok N, and Joussen AM (2006) The role of PDGF receptor inhibitors and PI3-kinase signaling in the pathogenesis of corneal neovascularization. *Invest Ophthalmol Vis Sci* **47:**1928–1937.

Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T, Utsunomiya H, Motley ED, Kawakatsu H, Owada KM, Hirata Y, et al. (1998) Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J Biol Chem* **273:**8890–8896.

Engqvist-Goldstein AE and Drubin DG (2003) Actin assembly and endocytosis: from yeast to mammals. *Annu Rev Cell Dev Biol* **19:**287–332.

Ettehadieh E, Sanghera JS, Pelech SL, Hess-Bienz D, Watts J, Shastri N, and Aebersold R (1992) Tyrosyl phosphorylation and activation of MAP kinases by p56lck. *Science* **255:**853–855.

Grisk O and Rettig R (2001) Renal transplantation studies in genetic hypertension. *News Physiol Sci* **16:**262–265.

Harris RC, Hoover RL, Jacobson HR, and Badr KF (1988) Evidence for glomerular actions of epidermal growth factor in the rat. *J Clin Invest* **82:**1028–1039.

He H, Hirokawa Y, Manser E, Lim L, Levitzki A, and Maruta H (2001) Signal therapy for RAS-induced cancers in combination of AG 879 and PP1, specific inhibitors for ErbB2 and Src family kinases, that block PAK activation. *Cancer J* **7:**191–202.

Hu WY, Han YJ, Gu LZ, Piano M, and de Lanerolle P (2007) Involvement of ras-regulated myosin light chain phosphorylation in the captopril effects in spontaneously hypertensive rats. *Am J Hypertens* **20:**53–61.

Jackson EK and Herzer WA (2001) Regional vascular selectivity of angiotensin II. *J Pharmacol Exp Ther* **297:**736–745.

Keiser JA and Ryan MJ (1996) Hemodynamic effects of epidermal growth factor in conscious rats and monkeys. *Proc Natl Acad Sci U S A* **93:**4957–4961.

Lew MJ (2006) Principles: when there should be no difference–how to fail to reject the null hypothesis. *Trends Pharmacol Sci* **27:**274–278.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, et al. (1999) beta-Arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283:** 655–661.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, and Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A* **98:**2449–2454.

Matrougui K, Eskildsen-Helmond YE, Fiebeler A, Henrion D, Levy BI, Tedgui A, and Mulvany MJ (2000) Angiotensin II stimulates extracellular signal-regulated kinase activity in intact pressurized rat mesenteric resistance arteries. *Hypertension* **36:**617–621.

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Muthalif MM, Benter IF, Khandekar Z, Gaber L, Estes A, Malik S, Parmentier JH, Manne V, and Malik KU (2000a) Contribution of Ras GTPase/MAP kinase and cytochrome P450 metabolites to deoxycorticosterone-salt-induced hypertension. *Hypertension* **35:**457–463.

Muthalif MM, Karzoun NA, Gaber L, Khandekar Z, Benter IF, Saeed AE, Parmentier JH, Estes A, and Malik KU (2000b) Angiotensin II-induced hypertension: contribution of Ras GTPase/Mitogen-activated protein kinase and cytochrome P450 metabolites. *Hypertension* **36:**604–609.

Rizzo MA, Shome K, Vasudevan C, Stolz DB, Sung TC, Frohman MA, Watkins SC, and Romero G (1999) Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J Biol Chem* **274:**1131–1139.

Roberts RE (2004) The role of Rho kinase and extracellular regulated kinase-mitogen-activated protein kinase in α2-adrenoceptor-mediated vasoconstriction in the porcine palmar lateral vein. *J Pharmacol Exp Ther* **311:**742–747.

Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, et al. (2005) Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* **115:**2811–2821.

Shah BH and Catt KJ (2002) Calcium-independent activation of extracellularly regulated kinases 1 and 2 by angiotensin II in hepatic C9 cells: roles of protein kinase Cδ, Src/proline-rich tyrosine kinase 2, and epidermal growth receptor trans-activation. *Mol Pharmacol* **61:**343–351.

Shah BH, Yesilkaya A, Olivares-Reyes JA, Chen HD, Hunyady L, and Catt KJ (2004) Differential pathways of angiotensin II-induced extracellularly regulated kinase 1/2 phosphorylation in specific cell types: role of heparin-binding epidermal growth factor. *Mol Endocrinol* **18:**2035–2048.

Touyz RM, Deschepper C, Park JB, He G, Chen X, Neves MF, Virdis A, and Schiffrin EL (2002a) Inhibition of mitogen-activated protein/extracellular signal-regulated kinase improves endothelial function and attenuates Ang II-induced contractility of mesenteric resistance arteries from spontaneously hypertensive rats. *J Hypertens* **20:**1127–1134.

Touyz RM, El Mabrouk M, He G, Wu XH, and Schiffrin EL (1999) Mitogen-activated protein/extracellular signal-regulated kinase inhibition attenuates angiotensin II-mediated signaling and contraction in spontaneously hypertensive rat vascular smooth muscle cells. *Circ Res* **84:**505–515.

Touyz RM, He G, Wu XH, Park JB, Mabrouk ME, and Schiffrin EL (2001) Src is an important mediator of extracellular signal-regulated kinase 1/2-dependent growth signaling by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients. *Hypertension* **38:**56–64.

Touyz RM, Wu XH, He G, Salomon S, and Schiffrin EL (2002b) Increased angiotensin II-mediated Src signaling via epidermal growth factor receptor transactivation is associated with decreased C-terminal Src kinase activity in vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertension* **39:**479–485.

Tsai MH and Jiang MJ (2005) Extracellular signal-regulated kinase1/2 in contraction of vascular smooth muscle. *Life Sci* **76:**877–888.

Watts SW (2000) 5-Hydroxytryptamine-induced potentiation of endothelin-1- and norepinephrine-induced contraction is mitogen-activated protein kinase pathway dependent. *Hypertension* **35:**244–248.

Watts SW, Florian JA, and Monroe KM (1998) Dissociation of angiotensin II-stimulated activation of mitogen-activated protein kinase kinase from vascular contraction. *J Pharmacol Exp Ther* **286:**1431–1438.

Webb RC (2003) Smooth muscle contraction and relaxation. *Adv Physiol Educ* **27:**201–206.

Wetzker R and Bohmer FD (2003) Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat Rev Mol Cell Biol* **4:**651–657.

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