Role of EGFR Transactivation in Angiotensin II Signaling to Extracellular Regulated Kinase in Preglomerular Smooth Muscle Cells

Bradley T. Andresen, Jenny J. Linnoila, Edwin K. Jackson, Guillermo G. Romero

Abstract—Angiotensin (Ang) II promotes the phosphorylation of extracellular regulated kinase (ERK); however, the mechanisms leading to Ang II–induced ERK phosphorylation are debated. The currently accepted theory involves transactivation of epidermal growth factor receptor (EGFR). We have shown that generation of phosphatidic acid (PA) is required for the recruitment of Raf to membranes and the activation of ERK by multiple agonists, including Ang II. In the present report, we confirm that phospholipase D–dependent generation of PA is required for Ang II–mediated phosphorylation of ERK in Wistar-Kyoto and spontaneously hypertensive rat preglomerular smooth muscle cells (PGSMCs). However, EGF stimulation does not activate phospholipase D or generate PA. These observations indicate that EGF recruits Raf to membranes via a mechanism that does not involve PA, and thus, Ang II–mediated phosphorylation of ERK is partially independent of EGFR-mediated signaling cascades. We hypothesized that phosphoinositide-3-kinase (PI3K) can also act to recruit Raf to membranes; therefore, inhibition of PI3K should inhibit EGF signaling to ERK. Wortmannin, a PI3K inhibitor, inhibited EGF-mediated phosphorylation of ERK (IC_{50}, ≈14 nmol/L). To examine the role of the EGFR in Ang II–mediated phosphorylation of ERK we utilized 100 nmol/L wortmannin to inhibit EGFR signaling to ERK and T19N RhoA to block Ang II–mediated ERK phosphorylation. Wortmannin treatment inhibited EGF-mediated but not Ang II–mediated phosphorylation of ERK. Furthermore, T19N RhoA inhibited Ang II–mediated ERK phosphorylation, whereas T19N RhoA had significantly less effect on EGF-mediated ERK phosphorylation. We conclude that transactivation of the EGFR is not primarily responsible for Ang II–mediated activation of ERK in PGSMCs. **(*Hypertension*. 2003;41[part 2]:781-786.)**

Key Words: angiotensin II ■ epidermal growth factors ■ extracellular regulated kinase ■ phospholipase D ■ phosphoinositide-3-kinase ■ transactivation

Angiotensin (Ang) II is known to stimulate p42 and p44 extracellular regulated kinase (ERK), and this pathway has recently been shown to be involved in Ang II–dependent regulation of mean arterial blood pressure.[1] However, the mechanism for Ang II–mediated activation of p42 and p44 ERK remains poorly understood. Currently accepted models involve transactivation of receptor tyrosine kinases.[2] In smooth muscle cells transactivation has been proposed to occur via 2 distinct mechanisms: (1) Ang II stimulation results in the release of a matrix metalloproteinase that cleaves matrix-bound pro-heparin–bound epidermal growth factor (HB-EGF), thus releasing HB-EGF and stimulating the epidermal growth factor receptor (EGFR)[3,4]; and (2) Ang II activates c-Src through an unknown mechanism that then phosphorylates the EGFR,[5] most likely at tyrosine 854.[6] In either case, the prescribed role of Ang II in activation of p42 and p44 ERK is apparently only through transactivation of the EGFR.

We have previously demonstrated, in A10 smooth muscle cells, that phospholipase D2 (PLD)-dependent generation of phosphatidic acid (PA) is required for Ang II–mediated activation of p42 and p44 ERK.[7] PA plays a key role in activation of p42 and p44 ERK by mediating Raf-1 translocation to membranes, where Raf-1 is then activated by Ras.[8,9] Inhibition of this translocation event by mutating the PA binding domain on Raf-1 is sufficient to significantly abrogate agonist-mediated activation of p42 and p44 ERK.[10]

In the spontaneously hypertensive rat (SHR), the kidney[11] and Ang II[12–14] are responsible for hypertension. In smooth muscle cells from SHR, Ang II–mediated PLD activity[15,16] and p42 and p44 ERK phosphorylation,[17] which is involved in contraction,[18] is increased compared with their normotensive controls, the Wistar-Kyoto rat (WKY). Additionally, we have previously indicated that in preglomerular smooth muscle cells (PGSMCs) Ang II stimulates PLD more potently in SHR compared with WKY. Therefore, the increase in Ang

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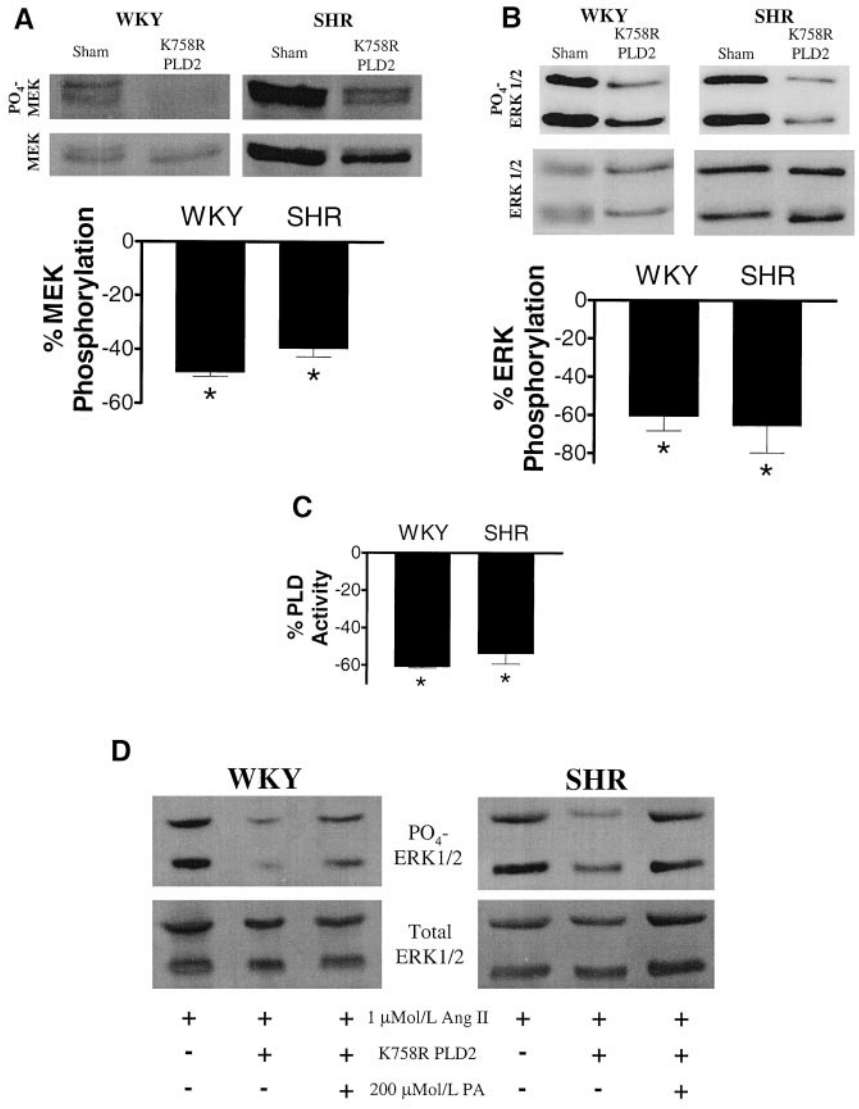

Figure 1. Role of PLD in Ang II–mediated MEK and p42 and p44 ERK phosphorylation. PGSMCs were transfected with K758R PLD2 and assayed for phosphorylated MEK (A) and p42 and p44 ERK (ERK 2 and ERK 1, respectively; B). A, 1 μmol/L Ang II–mediated phosphorylation of MEK was decreased in the presence of K758R PLD2 (top); replication of the experiment indicates that phospho-MEK was significantly reduced in both SHR and WKY PGSMCs by K758R PLD2 (bottom) (n=3). B, Similarly, 1 μmol/L Ang II–mediated phosphorylation of p42 and p44 ERK was also decreased in the presence of K758R PLD2 (top); replication of the experiment indicates that ERK phosphorylation was significantly reduced in both SHR and WKY PGSMCs by K758R PLD2 (bottom) (n=3); *$P<0.05$, control vs K758R PLD2. C, K758R PLD2 decreased 1 μmol/L Ang II–mediated PLD activity in WKY and SHR PGSMCs, similar to the levels of inhibition seen in ERK phosphorylation (n ≥6); *$P<0.05$, control vs K758R PLD2. D, In the presence of K758R PLD2, addition of 200 μmol/L PA, the product of PLD, partially recovers 1 μmol/L Ang II–mediated phosphorylation of p42 and p44 ERK.

II–mediated PLD activity in SHR may contribute to the increase in Ang II–mediated phosphorylation of p42 and p44 ERK and, thus, potentially play a role in vascular contraction. In the present study, we have (1) determined if PLD is required for Ang II–mediated phosphorylation of p42 and p44 ERK in WKY and SHR PGSMCs, (2) determined if epidermal growth factor (EGF) signals to PLD in PGSMCs, and (3) examined the role of RhoA and phosphoinositide-3-kinase (PI3K) in Ang II and EGFR–mediated and in Ang II–mediated phosphorylation of p42 and p44 ERK.

Methods

Materials

Most cell culture products were purchased from Invitrogen/GIBCO. FBS was obtained from Atlanta Biologicals. Ang II was obtained from Sigma Chemical Co., and EGF was obtained from Calbiochem. E10, total ERK1/2, phospho-MEK, and total MEK antibodies were obtained from Cell Signaling, and secondary antibodies were obtained from Jackson Immuno Laboratories. PA was obtained from Advanti Polar Lipis. PD 153,035, wortmannin, and LY 294002 were obtained from Calbiochem. All constructs were described previously.[7,9,16]

Cell Culture

Six 13- to 15-week-old SHR and WKY from Taconic Farms (Germantown, NY) were used to acquire the PGSMCs as previously described.[16] All experiments were conducted on confluent cells in 60-mm dishes between passage 3 and 10. Lipofectamine 2000 (Invitrogen/GIBCO) was used according to manufacturer's instructions to transfect the PGSMCs.

Measurement of PA Production and PLD Activity

The PGSMCs were serum starved in 2 mL DMEM/F12 with [^{3}H]palmitate (5 μCi/mL)for ≥15 hours. The PGSMCs were then stimulated with 100 nmol/L Ang II or 100 ng/mL EGF for the desired time, and the cells were washed with ice-cold water. The lipids were then separated as previously described,[7] and the spots corresponding to PA were analyzed in a scintillation counter. PLD activity was accessed as described previously.[7,16]

Phospho-ERK/MEK Assay

PGSMCs were serum-starved for ≥15 hours and stimulated with Ang II or EGF for 5 minutes, and Western blots were run as previously described.[9] Quantitative measurements of ERK and MEK phosphorylation were obtained by stripping the phospho-specific antibody and reprobing with the antibody that recognizes total ERK and MEK, respectively. A Molecular Dynamics densitometer was

used to measure the intensities of the bands, and the phospho-protein signal was divided by the signal of the total protein.

Statistical Analysis

For all mathematical operations containing 2 independent data sets with a measurable error, the following error propagation formulas were applied. If f and g are 2 means and fe and ge are their respective error, then the error for f/g is $[fe\times g - f\times ge]/g^2$, and the error for $f\pm g$ is $fe+ge$. For multiple comparisons, the data were analyzed by ANOVA with the Fisher least significant difference post hoc test. Data points are indicated to be significant only if $P<0.05$. Statistical analysis was conducted by using the NCSS 2000 software package. Curves were analyzed using the curve-fit routines of GraphPad Prism.

Results

Previously, we reported that Ang II stimulates PLD2 in WKY and SHR PGSMCs[16]; therefore, we used the catalytically inactive mutant of PLD2, K758R PLD2, to determine the role of PLD-dependent generation of PA in Ang II–mediated phosphorylation of MEK and p42 and p44 ERK in WKY and SHR PGSMCs (Figure 1). K758R PLD2 significantly inhibited 1 μmol/L Ang II–mediated MEK and ERK phosphorylation and Ang II–mediated PLD activity in both WKY and SHR PGSMCs (Figures 1A through 1C). Because PLD catalyzes the production of PA from phosphatidylcholine, we added 200 μmol/L PA for 5 minutes before addition of 1 μmol/L Ang II in an attempt to rescue the cells from K758R PLD2 (Figure 1D). PA partially rescued both WKY and SHR PGSMCs from the effects of K758R PLD2, indicating that PLD2 generation of PA is required for Ang II–mediated phosphorylation of p42 and p44 ERK.

We next examined the ability of Ang II and EGF to generate PA (Figure 2A). In both WKY and SHR PGSMCs, Ang II rapidly induced PA formation, whereas EGF did not generate PA. Although the EGF-treated cells resulted in a slight decrease in PA levels, linear regression indicated that the slope obtained with EGF was not significant from 0. Furthermore, 1 nmol/L PD 153,035, an EGFR kinase inhibitor with a reported K_i of 5 pmol/L,[19] failed to inhibit Ang II–mediated PLD activity (Figure 2B). Thus, the activation of PLD by Ang II is totally independent of the EGFR kinase in WKY and SHR PGSMCs.

We previously indicated that the generation of PA is essential for activation of ERK;[7] however, EGF stimulates ERK but not PLD. Because PA is a negatively charged phospholipid, we hypothesized that PI3K-dependent generation of phosphatidyl inositol phosphate may play a role in EGFR recruitment of Raf to membranes; 100 nmol/L of the PI3K inhibitor wortmannin inhibited EGF-mediated phosphorylation of p42 and p44 ERK by ≈90%, whereas 7 μmol/L of the synthetic PI3K inhibitor LY 294002 had significantly less effect on EGF-mediated phosphorylation of p42 and p44 ERK, resulting in ≈35% inhibition. To verify that wortmannin indeed inhibits EGF-mediated phosphorylation of p42 and p44 ERK, a dose-response curve was generated, resulting in an IC_{50} of 15.48±1.49 and 13.74±1.29 nmol/L for WKY and SHR PGSMCs, respectively (Figure 3).

Because PI3K inhibition inhibits EGFR-dependent ERK phosphorylation, the transactivation mechanism implies that

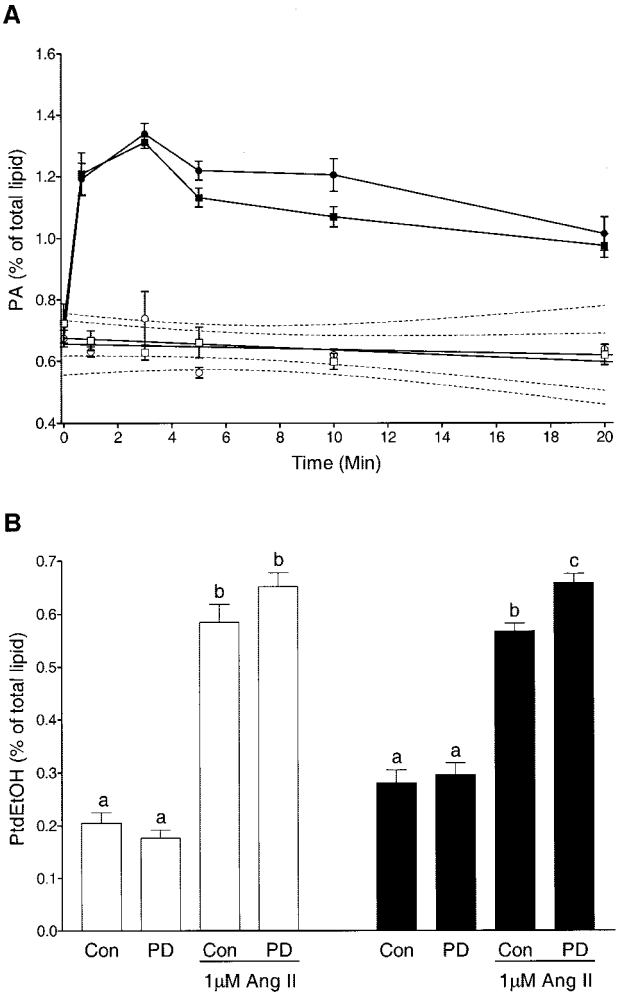

Figure 2. A, Ang II, but not EGF, activates PLD: 1 μmol/L Ang II (solid symbols) leads to time-dependent PA accumulation, whereas linear regression analysis indicates that 100 ng/mL EGF (open symbols) fails to generate any PA accumulation in WKY (circles) and SHR (squares) PGSMCs (n=3). Dotted lines indicate the 95% confidence intervals obtained from linear regression. B, Ang II–mediated PLD activity is independent of EGFR kinase activity. 1 nmol/L PD 153,035 (PD), an EGFR kinase inhibitor, failed to inhibit Ang II–mediated PLD activity in WKY (open bars) and SHR (solid bars) PGSMCs. Data represents mean±SEM, n=3; bars with different letters are significantly different ($P<0.05$). PtdEtOH indicates phosphatidylethanol.

Ang II–mediated phosphorylation of p42 and p44 ERK should also be wortmannin-sensitive. However, 100 nmol/L wortmannin nearly abolished EGF-mediated phosphorylation of p42 and p44 ERK in both WKY and SHR PGSMCs but had no significant effect on Ang II–mediated phosphorylation of p42 and p44 ERK (Figure 4A). On the other hand, we have previously shown that RhoA is responsible for transmitting the signal from the angiotensin type 1 receptor to PLD2.[16] Thus, T19N RhoA, a RhoA mutant defective in GDP exchange that acts as a dominant-negative, should inhibit Ang II (PLD-dependent) but not EGF (PLD-independent) signaling to ERK. As shown, transfection of T19N RhoA resulted in a much greater degree of inhibition of Ang II–mediated phosphorylation of p42 and p44 ERK compared with EGF (Figure

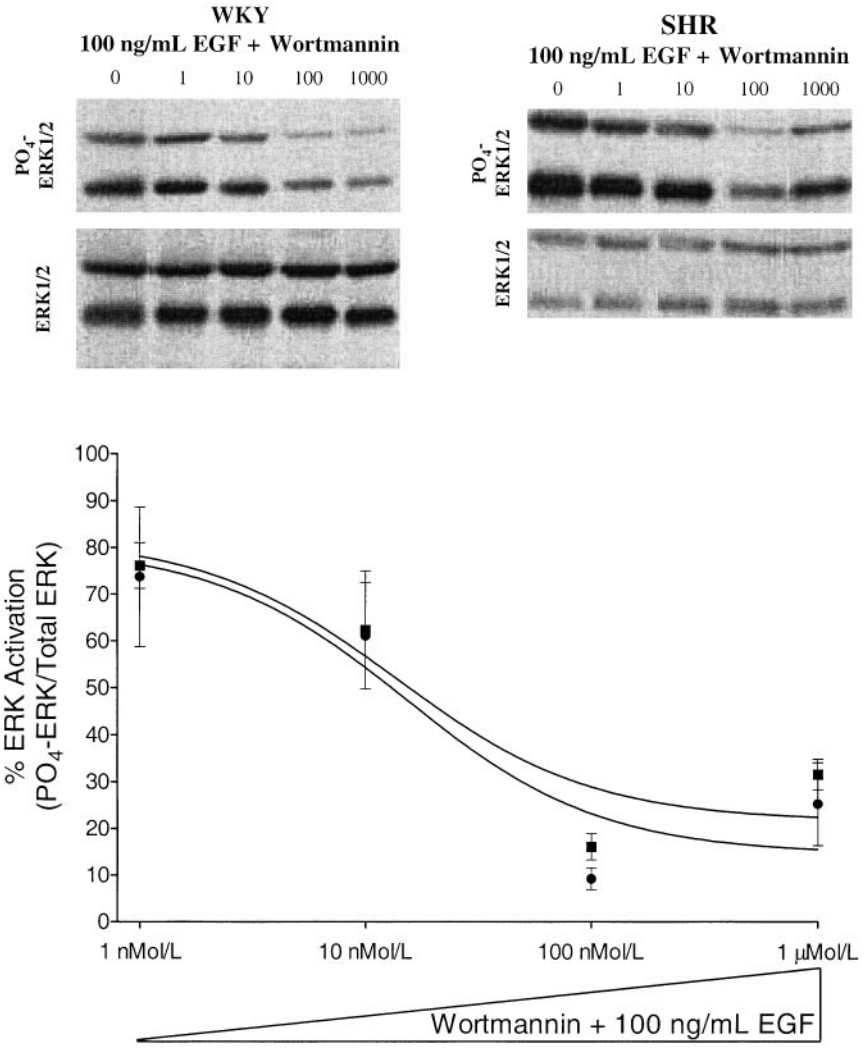

Figure 3. Top, Wortmannin inhibits EGF-mediated phosphorylation of p42 and p44 ERK; 100 ng/mL EGF robustly phosphorylates p42 and p44 ERK, and this is inhibited in a dose-dependent fashion by wortmannin in both WKY and SHR PGSMCs (numbers above the blots indicate wortmannin in nanomoles per liter). Bottom, Analysis of the dose-response curve indicates that wortmannin inhibits EGF signaling to p42 and p44 ERK with an IC_{50} of 15.48±1.49 and 13.74±1.29 nmol/L for WKY (●) and SHR (■) PGSMCs, respectively. Data represents mean±SEM, n=3.

4B). However, contrary to our expectations, the effects of EGF on ERK phosphorylation were significantly inhibited by T19N RhoA (Figure 4B). ANOVA analysis indicates that there is a significant interaction between T19N RhoA and Ang II and EGF, but that EGF-dependent ERK phosphorylation is significantly less sensitive to T19N RhoA than the effects of Ang II. In summary, wortmannin inhibits EGF, but not Ang II, signaling to ERK, whereas RhoA activity is essential for Ang II–mediated phosphorylation of p42 and p44 ERK and plays a secondary role in EGF signaling to p42 and p44 ERK.

Discussion

Raf-1 translocation to membranes is essential for Raf-1–mediated activation of the MEK/ERK pathway.[9,10] We previously demonstrated, in A10 smooth muscle cells, that PLD-dependent generation of PA is required for Ang II–mediated phosphorylation of p42 and p44 ERK.[7] The data presented here indicates that the generation of PA is also crucial for Ang II–mediated phosphorylation of MEK and p42 and p44 ERK in WKY and SHR PGSMCs. K758R PLD2 inhibits Ang II–mediated activation of PLD and phosphorylation of ERK. In addition, stimulation with Ang II results in a rapid accumulation and slow degradation of PA. Finally, exogenously added PA partially reversed the inhibition Ang II–mediated ERK phosphorylation caused by the expression of K758R PLD2. Therefore, primary PGSMCs require PLD-dependent generation of PA for proper Ang II signaling to ERK. It should be noted that the dependence of ERK phosphorylation on the generation of PA has now been examined and confirmed in 4 different cell types with identical results, strongly suggesting that this model is general.[7,9]

However, EGF is a potent stimulant of p42 and p44 ERK but a poor activator of PLD (data not shown). Therefore, this model cannot explain EGF signaling unless EGF can generate PA through a PLD-independent mechanism. As shown in Figure 2, EGF does not generate PA within 20 minutes, which is well beyond the initiation of EGF-mediated ERK phosphorylation, which is seen within 1 minute (data not shown). Therefore, Raf must translocate to the membrane in a PLD- and PA-independent fashion. Because PI3K has been implicated in EGF-mediated ERK activation[20] and because PA is a negatively charged phospholipid, we hypothesized that phosphatidylinositol-3-phosphate, which can be generated by PI3Ks, may substitute for PA in the recruitment of

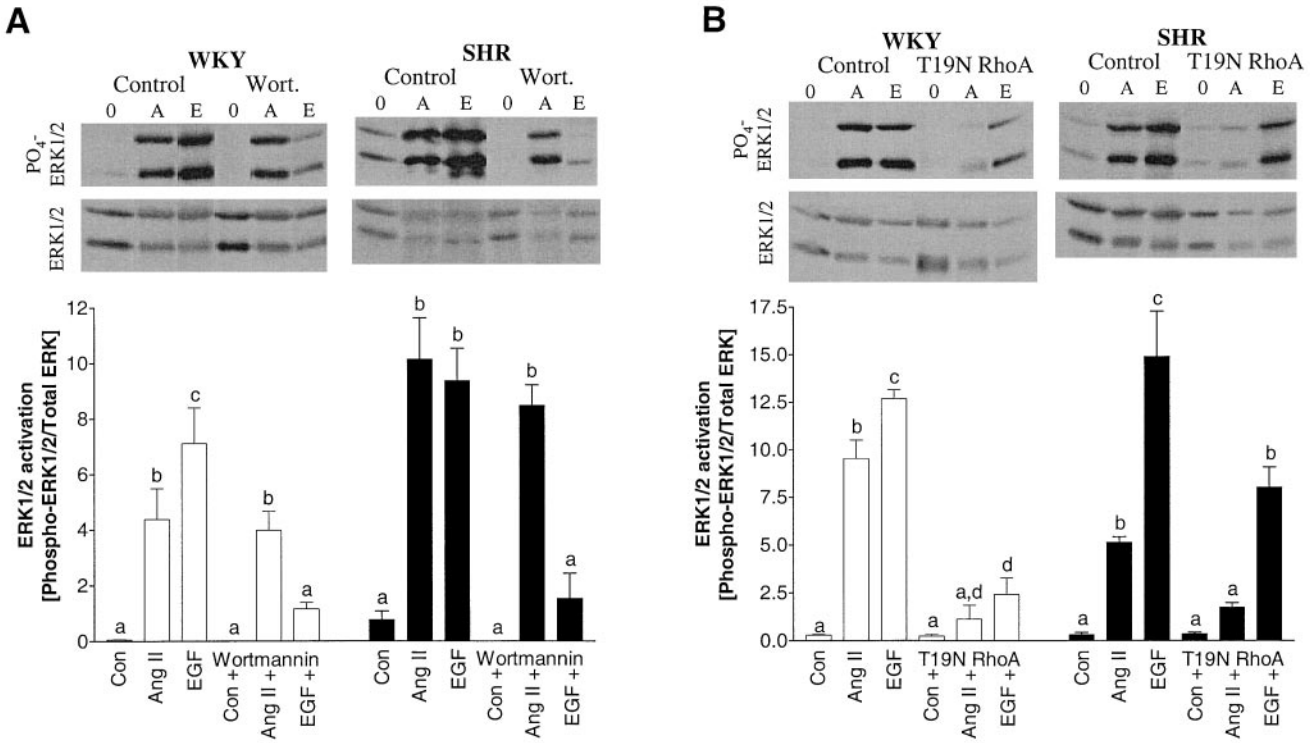

Figure 4. A, Pharmacological separation of Ang II and EGF signaling to ERK; 100 nmol/L wortmannin inhibits 100 ng/mL EGF (E), but not 1 μmol/L Ang II (A), and mediates phosphorylation of p42 and p44 ERK (top). Replication of the experiment indicates that there is no significant effect of 100 nmol/L wortmannin on 1 μmol/L Ang II–mediated phosphorylation of p42 and p44 ERK; however, wortmannin abolishes basal p42 and p44 ERK phosphorylation and nearly abolishes 100 ng/mL EGF-mediated phosphorylation of p42 and p44 ERK (bottom). Data represents mean±SEM, n=3; bars with different letters are significantly different ($P<0.05$). B, Transfection of T19N RhoA inhibits 1 μmol/L Ang II–mediated, more so than 100 ng/mL EGF-mediated, phosphorylation of p42 and p44 ERK (top). Replication of the experiment indicates that T19N RhoA abolishes 1 μmol/L Ang II–mediated phosphorylation of ERK and significantly reduces 100 ng/mL EGF-mediated phosphorylation of p42 and p44 ERK, but not to the same extent as Ang II (bottom). Data represents mean±SEM, n=3; bars with different letters are significantly different ($P<0.05$).

Raf to membranes. To determine if a PI3K is involved in EGF-mediated phosphorylation of ERK, we utilized the PI3K inhibitors wortmannin and LY 294002. Surprisingly, only wortmannin inhibited EGF signaling to ERK in a manner consistent with PI3K involvement in EGF-mediated phosphorylation of ERK. Wortmannin is a metabolite from *Penicillium funiculosum* that inhibits all type I PI3K family members as well as some type II PI3K at higher concentrations; whereas LY 294002 is a synthetic PI3K inhibitor that is more specific to type I PI3Ks and does not significantly inhibit type II PI3Ks at the concentrations used in these experiments.[21–23] Therefore, our data suggest that a type II PI3K is most likely involved in EGF-mediated phosphorylation of ERK. Additionally, PI3K has been implicated in EGF-mediated activation of ERK through recruitment of Gab1/SHP2 to the EGFR in monkey Vero cells.[20] Because our experiments were conducted under similar conditions to those reported by Yart et al, who found that wortmannin and LY 294002 similarly inhibited EGF signaling to ERK, it is unlikely that wortmannin but not LY 294002 is blocking Gab1/SHP2 recruitment in PGSMCs. However, further experiments are required to verify which PI3K family member is involved in EGF signaling to ERK and PI3K's precise role in the EGF-mediated signaling pathway.

Although we have not definitively elucidated the role of PI3K in EGF-mediated phosphorylation of ERK, our data show that wortmannin can be used as a tool to examine the role of the EGFR in Ang II–mediated phosphorylation of p42 and p44 ERK. Given that Ang II is proposed to signal to ERK via the transactivation of the EGFR, either by release of a matrix metalloproteinase that cleaves matrix bound proHB-EGF, releasing HB-EGF[3,4] and consequently activating the EGFR, or via the activation of c-Src and consequent phosphorylation of the EGFR.[5] If this is true, then wortmannin should also inhibit Ang II–mediated phosphorylation of ERK. However, our data indicate that wortmannin does not block Ang II–mediated phosphorylation of p42 and p44 ERK, while potently inhibiting EGF-mediated phosphorylation of p42 and p44 ERK. Therefore, in WKY and SHR PGSMCs, transactivation of the EGFR is not essential for Ang II–induced ERK phosphorylation. Furthermore, similar experiments from Yart et al[24] indicate that a PI3K is involved in G$\beta\gamma$ signaling to ERK; thus, our data also suggests the Ang II is not signaling to ERK through G$\beta\gamma$ subunits.

RhoA is required for Ang II signaling to PLD in PGSMCs,[16] and consequently, as shown in Figure 1, RhoA is involved in Ang II–mediated activation of ERK in PGSMCs. However, it is not known whether RhoA plays a role in EGF signaling to ERK. We used a mutant of RhoA, T19N RhoA, which is defective in GDP exchange and thus acts as a dominant-negative to address this issue. The data indicate that

the expression of T19N RhoA has significantly greater inhibitory effects on Ang II–mediated rather than EGF-mediated phosphorylation of p42 and p44 ERK. Thus, we conclude that Ang II–mediated phosphorylation of ERK goes through RhoA and PLD, but not the EGFR. The mechanism underlying the significant reduction in EGF signaling to ERK in the presence of T19N RhoA is unknown, yet indicates that RhoA may also play a role in EGF-mediated phosphorylation of p42 and p44 ERK.

Perspectives

Angiotensin II signal transduction is clearly involved in the pathophysiology of hypertension. Thus, clear elucidation of the signal transduction machinery activated by Ang II in physiologically relevant cells may provide insight into a subset of the molecular mechanisms underlying hypertension and, ultimately, lead to novel therapies for hypertension. In the present study, we clearly separate Ang II from EGF signaling to p42 and p44 ERK in WKY and SHR PGSMCs. This does not indicate that transactivation of the EGFR does not occur. However, it does indicate that Ang II–mediated phosphorylation of p42 and p44 ERK requires EGFR-independent signal transduction pathways to phosphorylate p42 and p44 ERK. Our data show that RhoA-dependent activation of PLD2 is required for the coupling of the ERK kinase cascade in Ang II–dependent pathways, whereas PI3K-dependent mechanisms are required for the coupling of the EGF-dependent pathways.

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