

# Resveratrol Inhibits Angiotensin II- and Epidermal Growth Factor-Mediated Akt Activation: Role of Gab1 and Shp2

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## ABSTRACT

*trans*-Resveratrol (RV), a polyphenolic stilbene derivative found in grape skin and other food products, has been proposed to exert beneficial effects in cardiovascular disease. Our group has shown previously that RV inhibits angiotensin II (Ang II)-induced Akt activation and, consequently, vascular smooth muscle cell (VSMC) hypertrophy. In this work, to identify the molecular target of RV, we investigated the impact of RV on early signaling cascades in rat aortic VSMCs triggered by Ang II and epidermal growth factor (EGF). We show that RV does not influence Ang II-mediated transactivation of EGF-receptor but potently inhibits EGF-induced phosphorylation of Akt kinase, suggesting that RV acts downstream of EGF-receptor transactivation in VSMCs. Recent evidence indicates that the adapter molecule Gab1, together with the protein tyrosine phosphatase

Shp2, is critically involved in regulating the strength and duration of phosphatidylinositol-3-kinase (PI3K) and Akt activation upon EGF stimulation in fibroblasts. Our results show that stimulation of VSMCs with EGF as well as Ang II leads to a rapid tyrosine phosphorylation of Gab1 and its association with the p85 subunit of PI3K. RV attenuates these processes. Experiments performed in Shp2-deficient fibroblasts revealed that RV does not inhibit EGF-stimulated Akt activation in these cells, suggesting that Shp2 is necessary for the inhibitory effect of RV on the PI3K/Akt pathway. Furthermore, RV treatment activates Shp2. We therefore propose that RV blocks Akt activation in Ang II- and EGF-stimulated VSMCs by activating Shp2, thus preventing interaction between Gab1 and PI3K that is necessary for further signal transduction.

Angiotensin II (Ang II) is the main effector peptide of the renin-angiotensin-aldosterone system. In addition to modulating vasomotor tone, Ang II contributes to the genesis of atherosclerosis and hypertension. The importance of Ang II in the development of cardiovascular diseases is underlined by the efficacy of angiotensin-converting enzyme inhibitors and Ang II receptor blockers in the therapy of these disorders. In VSMCs, Ang II triggers proliferation, migration and

increased extracellular matrix deposition via the G protein-coupled AT<sub>1</sub> receptor (Schmidt-Ott et al., 2000; Touyz and Schiffrin, 2000). VSMC hypertrophy, another hallmark of cardiovascular disease, is induced by Ang II in the absence of other growth factors (Braun-Dullaeus et al., 1999; Yamakawa et al., 2000).

The mitogen-activated protein kinases Erk 1/2 and p38 as well as the serine/threonine kinase Akt (also termed protein kinase B) have been proposed to play key roles in Ang II-mediated hypertrophy (Servant et al., 1996; Ushio-Fukai et al., 1998, 1999). In addition, it has become evident that G protein-coupled receptors such as the AT<sub>1</sub> receptor transactivate receptor tyrosine kinases, which then serve as a scaffold for various signaling molecules and mediate further signal transduction. In VSMCs, transactivation of the epidermal growth factor receptor (EGF-R) has been

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**ABBREVIATIONS:** Ang II, angiotensin II; VSMC, vascular smooth muscle cell; EGF-R, epidermal growth factor receptor; ROS, reactive oxygen species; EGF, epidermal growth factor; PIP<sub>3</sub>, phosphatidylinositol 3,4,5-triphosphate; PDGF, platelet-derived growth factor; RV, resveratrol; Gab1, Grb2 associated binder 1; Shp2, src homology 2-domain-containing tyrosine phosphatase 2; Erk1/2, extracellular signal-regulated kinase1/2; PI3K, phosphatidylinositol-3-kinase; AG 1478, 4-(3'-chloroanilino)-6,7-dimethoxy-quinazoline; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine; NAC, *N*-acetyl cysteine; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ANOVA, analysis of variance; *p*NPP, *para*-nitrophenyl phosphate; NOX, NADPH oxidase.

shown to occur upon Ang II stimulation, and there is strong evidence that Ang II-dependent activation of Erk 1/2 and Akt occurs in an EGF-R–dependent manner (Daub et al., 1997; Eguchi et al., 1998; Wang et al., 2000; Kalmes et al., 2001; Ushio-Fukai et al., 2001a,b). Regarding the mechanisms responsible for Ang II-mediated EGF-R transactivation, an impact of ROS (Ushio-Fukai et al., 2001a),  $\text{Ca}^{2+}$ , proline rich kinase 2 (Eguchi et al., 1998, 1999a), c-Src kinase (Bokemeyer et al., 2000) and heparin-binding EGF-like growth factor (Kalmes et al., 2001) have been discussed. However, the subsequent signaling steps linking the EGF-R to the Akt pathway in VSMCs are less well defined.

A recent study by Rodrigues et al. (2000) addressing the EGF-R signaling in various cell lines proposed a positive feedback loop involving the adapter molecule Gab1 and PI3K: Gab1 is initially recruited to the activated EGF-R directly or indirectly via Grb2 and subsequently phosphorylated by the receptor. Phosphorylation of Gab1 enables binding of various signaling molecules containing phosphotyrosine binding sites (Rodrigues et al., 2000). Binding of the PI3K p85 subunit via its SH2 domains leads to generation of phosphatidylinositol 3,4,5-triphosphate ( $\text{PIP}_3$ ) at the plasma membrane, which recruits additional Gab1 via its pleckstrin homology domain and subsequently recruits PI3K. Thereafter, Zhang et al. (2002) established an important modulatory role for the protein-tyrosine phosphatase Shp2 in this process: Shp2 attenuates PI3K activation by specifically dephosphorylating the p85 binding sites on Gab1. It is interesting that this effect is specific for EGF and not other growth factors such as platelet-derived growth factor and insulin-like growth factor 1.

Resveratrol (RV), a polyphenolic phytoalexin found in grape skin, has been proposed to be responsible, at least in part, for the beneficial effects ascribed to red wine in cardiovascular disease. The proposed cardiovascular benefits of RV have been attributed to a variety of biological effects, including its antioxidative properties and interference with both lipoxygenase and cyclooxygenase pathways (for review, see Soleas et al., 2001). A recent study performed in endothelium-denuded rat aortic rings presented evidence that RV is able to inhibit vascular NADPH oxidase (Orallo et al., 2002). Wallerath et al. (2002) demonstrated that RV enhances expression and activity of endothelial nitric oxide synthase. Our group has recently shown that RV reversibly inhibits cell cycle progression in early S phase in calf serum-treated VSMC (Haider et al., 2003). In addition, an in vivo study revealed that RV attenuates intimal hyperplasia after endothelial denudation in an experimental rabbit model (Zou et al., 2000). Regarding the signaling pathways triggered by Ang II, our group has previously shown that RV suppresses Ang II-induced VSMC hypertrophy, most likely by interfering with Akt-governed pathways (Haider et al., 2002). However, the exact target of RV in VSMCs awaits identification. In this report, we show that RV most likely inhibits Ang II- and EGF-stimulated Akt phosphorylation via activation of Shp2, without affecting early events such as EGF-R transactivation. Furthermore, our report for the first time provides evidence for an important role of Gab1 and Shp2 in Ang II- and EGF-induced Akt activation in VSMCs.

## Materials and Methods

**Reagents.** Materials were obtained from the following suppliers: antibodies against Akt, phospho-Akt (Ser<sup>473</sup>), EGF-R, phospho-EGF-R (Tyr<sup>845</sup>), and phosphotyrosine (P-Tyr-100) were from Cell Signaling Technology (Frankfurt, Germany). Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 633 goat anti-mouse IgG (highly cross-absorbed) were obtained from Molecular Probes (Leiden, Netherlands). Anti-PI3K p85 N-SH2, clone UB93-3 (mouse monoclonal IgG; used for confocal microscopy), anti-PI3K p85 (rabbit antiserum; used for Western blot) and anti-Gab1, CT (rabbit polyclonal IgG) antibodies were received from Upstate (Lake Placid, NY). The inhibitors AG 1478 and PP1 were from QBiogene-Alexis (Grünberg, Germany). EGF was obtained from Upstate. RV, NAC, Hoechst 33342, and Ang II were from Sigma-Aldrich (Taufkirchen, Germany). Complete protease inhibitor cocktail was from Roche (Mannheim, Germany). Calf serum was from Invitrogen (Karlsruhe, Germany). Phenol red-free DMEM was obtained from Pan Biotech GmbH (Aidenbach, Germany).

**Cell Culture.** VSMCs were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion as described previously (Griendling et al., 1991). Cells were grown in phenol red-free DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (growth medium) and passaged twice a week by harvesting with trypsin/EDTA and seeding into 75- $\text{cm}^2$  flasks. For experiments, cells between passages 7 and 15 were used at 70 to 95% confluence. 3T3 immortalized fibroblasts from Shp2 exon 3–/– (Shp2 $\Delta^{46-110}$ ) mice were described previously (Saxton et al., 1997). Shp2–/– cells that contain a stably reconstituted Shp2 protein via retroviral transfection (Zhang et al., 2002) were used as control (Shp2 reconstituted control cells). The fibroblasts were cultured as described for VSMCs. For all growth factor stimulation experiments, cells were first starved overnight in DMEM containing 0.2% calf serum and then exposed to 100  $\mu\text{M}$  RV or vehicle for 30 min before stimulation with 100 ng/ml EGF for 4 min.

**Immunoprecipitation and Immunoblotting.** Cells grown in 60-mm dishes were serum-starved overnight in DMEM supplemented with 0.1% calf serum and treated with Ang II (100 nM) or EGF (100 ng/ml) for the indicated times. If inhibitors were used, they were added 30 min before stimulation. Cell lysates were prepared by the addition of lysis buffer (50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 $\times$  Complete protease inhibitor cocktail). Western blot analysis was performed as described previously (Ushio-Fukai et al., 1999). For immunoprecipitation, the lysates were cleared by centrifugation (10 min, 14,000g), and protein concentrations were determined by the BCA method (Pierce). Anti-EGF-R antibody (5  $\mu\text{l}$ ) was added to a 500- $\mu\text{g}$  aliquot (1  $\mu\text{g}$  of protein/ $\mu\text{l}$ ) and mixed overnight at 4°C. Protein A-agarose beads (50  $\mu\text{l}$ ) washed with lysis buffer were added for an additional 2 h and subsequently collected by centrifugation. The beads were washed three times with 500  $\mu\text{l}$  of lysis buffer and resuspended in 25  $\mu\text{l}$  of 3 $\times$  sample buffer containing 1.5%  $\beta$ -mercaptoethanol. After addition of 25  $\mu\text{l}$  of 1 $\times$  sample buffer, beads were boiled for 5 min at 95°C and afterward removed by centrifugation. The lysate (30  $\mu\text{l}$  for anti-phospho-tyrosine or 10  $\mu\text{l}$  for anti-EGF-R) was separated on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane (90 min, 100 V). Membranes were blocked for 60 min with 5% nonfat dry milk in Tris-buffered saline containing 1% Tween 20. Anti-EGF-R antibody (1:1000) or anti-phosphotyrosine antibody (1:2000) were added overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were added for 60 min at room temperature. Proteins were detected and quantified by enhanced chemiluminescence with a Kodak Digital Science image station 440 cf (PerkinElmer Life and Analytical Sciences, Köln, Germany).

**Confocal Microscopy.** VSMCs grown in 24-well plates on coverslips were serum-starved overnight in DMEM containing 0.1% calf

serum and treated with 100 ng/ml EGF for 4 min. RV or vehicle was added 30 min before stimulation. Cells were rinsed twice with ice-cold PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and incubated once with 50 mM ammonium chloride for 10 min to quench excessive paraformaldehyde. Cells were blocked with 8% bovine serum albumin in PBS for 1 h and then incubated for another hour with primary antibodies against Gab1 or the PI3K p85 subunit, 1:200 each. Thereafter, cells were washed three times with PBS for 5 min before adding the secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 688 goat anti-mouse IgG for 1 h. After washing twice with PBS, Hoechst 33342 (5  $\mu$ g/ml) was added for 5 min and then rinsed once with PBS. VSMCs were mounted on microscope slides with fluorescent mounting medium (DakoCytomation Denmark A/S, Glostrup, Denmark) and visualized by confocal laser scanning microscopy (CLSM 510 META; Carl Zeiss GmbH, Jena, Germany).

**Statistical Analysis.** Results are expressed as mean  $\pm$  S.E. Statistical analysis was performed by ANOVA followed by a Dunnett multiple comparison test or by a one-sample *t* test using Prism (ver. 3.00 for Windows; GraphPad Software, San Diego, CA).

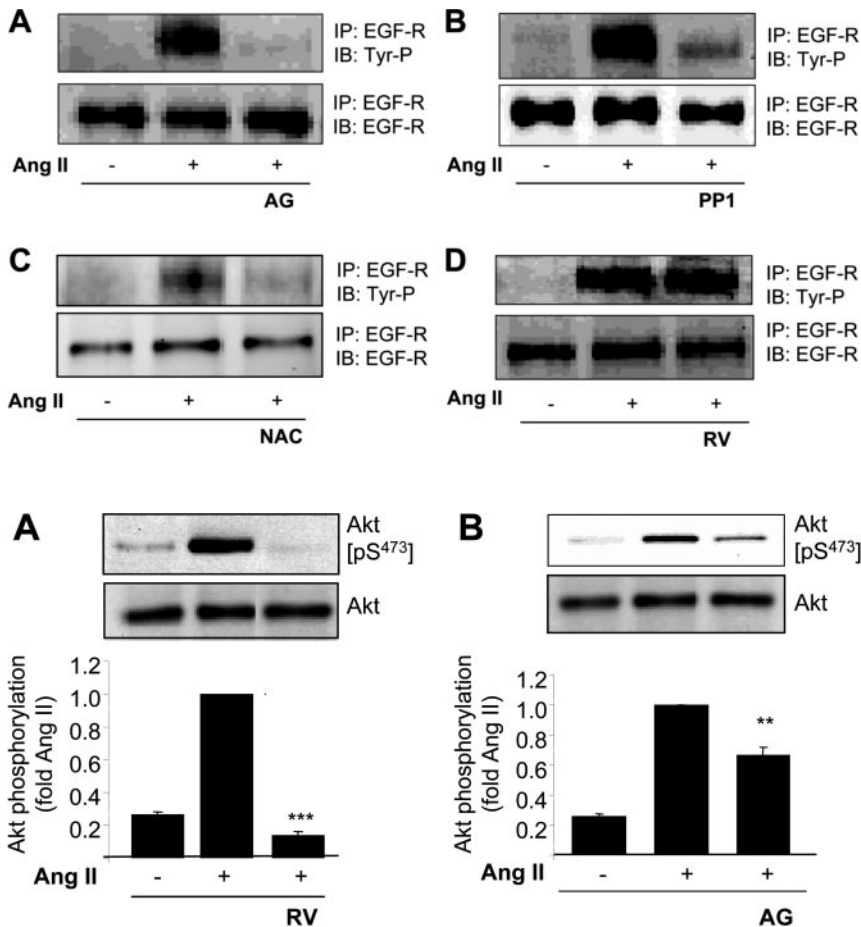
**Phosphatase Assays.** Shp2 activity was measured using either Shp2 immune complexes or in vitro using purified recombinant Shp2. The rate of hydrolysis was measured using *para*-nitrophenyl phosphate (*p*NPP; Sigma-Aldrich) as an artificial substrate. In brief, for immune complexes, Shp2 reconstituted control cells were starved overnight, treated with various concentrations of RV for 30 min, and then stimulated with EGF (100 ng/ml) for 5 min. Cells were harvested and lysed in 1 $\times$  Nonidet P-40 buffer containing protease inhibitors, but no sodium orthovanadate. Lysates were immunoprecipitated with Shp2 antibodies coupled to protein A-Sepharose beads. Immune complexes were washed three times in 1% Nonidet P-40 lysis buffer (without sodium orthovanadate) and once in phos-

phatase buffer without dithiothreitol. For recombinant protein experiments, *p*NPP phosphate release by Shp2 (10 nM) was measured in buffer containing various concentrations of RV. All experiments were performed in triplicate at 37°C for 10 min in phosphatase buffer containing 30 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM NaCl, 5 mM dithiothreitol, and 10 mM *p*NPP. Addition of 1.45 ml of 0.2 N NaOH stopped the reactions. The amount of phosphate released was determined by measuring the absorbance at 410 nm per min.

## Results

**Resveratrol Does Not Interfere with EGF-R Transactivation.** To confirm previous observations showing that Ang II transactivates the EGF-R, we treated VSMCs with Ang II for various times and determined EGF-R tyrosine phosphorylation. Ang II caused a rapid increase in EGF-R phosphorylation that remained above baseline for 10 min (data not shown). We hypothesized that RV may interfere with this process. Agents described previously to act as inhibitors of transactivation (the antioxidant NAC, the c-Src kinase inhibitor PP1, and an inhibitor of EGF-R kinase, the tyrphostin AG 1478) were used as positive controls. Figure 1 clearly shows that in contrast to NAC, PP1, and AG 1478, RV, at a concentration previously shown to inhibit Akt activation (Haider et al., 2002) (Fig. 2A), does not influence Ang II-induced EGF-R transactivation.

**Transactivation of EGF-R Is Important for Akt Phosphorylation.** Transactivation of EGF-R has been shown to be essential for Akt activation by Ang II (Eguchi et al., 1999b; Ushio-Fukai et al., 2001b). To confirm that trans-

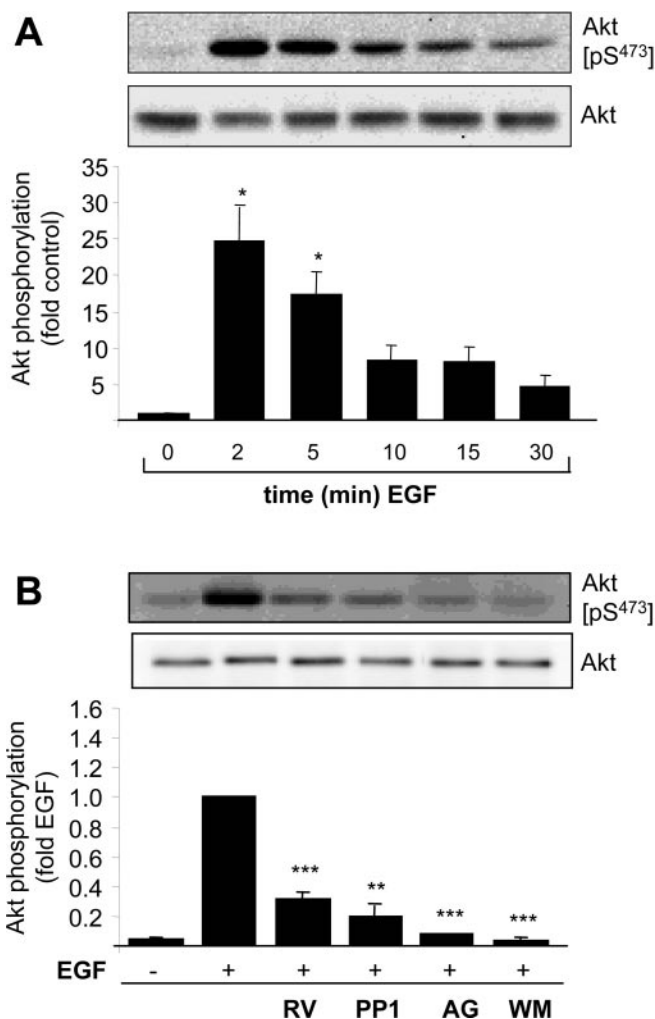


**Fig. 1.** AG 1478, PP1, and NAC, but not RV, attenuate Ang II-induced EGF-R phosphorylation. VSMCs were preincubated with 250 nM AG 1478 (AG; A), 20  $\mu$ M PP1 (B), 10 mM NAC (C), or 50  $\mu$ M RV (D) for 30 min before treatment with (+) or without (-) 100 nM Ang II for 2 min. Lysates were used as described under *Materials and Methods*. Top, representative blots for tyrosine-phosphorylated EGF-R. Bottom, representative blots for total levels of EGF-R show one representative Western blot of three.

**Fig. 2.** Effect of RV and AG 1478 on Ang II-induced Akt phosphorylation. VSMCs were preincubated with vehicle, 50  $\mu$ M RV or 250 nM AG 1478 (AG) for 30 min before treatment with (+) or without (-) 100 nM Ang II for 10 min. Lysates were immunoblotted with anti-phospho-Akt (Ser<sup>473</sup>; top) or anti-Akt antibody (bottom). Graphs at the bottom show mean  $\pm$  S.E. from three independent experiments, expressed as inhibition of Ang II-induced Akt phosphorylation. \*\*, *P* < 0.01 versus Ang II; \*\*\*, *P* < 0.001 versus Ang II (one-sample *t* test).

activation is indeed implicated in Akt phosphorylation in our cells, we treated VSMCs with AG 1478 before stimulation. The substance markedly decreased Akt phosphorylation (Fig. 2B) but failed to completely abolish the signal. These data indicate that EGF-R is indeed important for Ang II-induced Akt phosphorylation, although parallel pathways should be considered, but perhaps to a smaller extent.

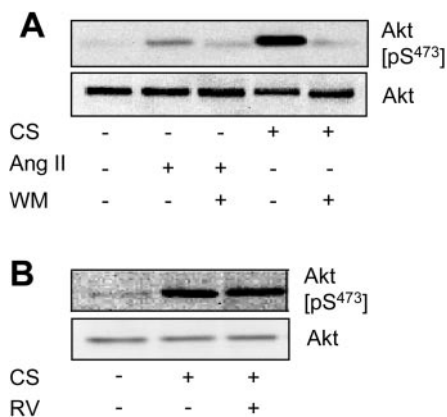
**RV Inhibits EGF-Induced Akt Phosphorylation.** Because EGF-R transactivation is not impeded by RV, we assumed this substance to influence pathways downstream of the EGF-R. Therefore, we tested whether RV was also effective in blocking Akt phosphorylation when EGF instead of Ang II was used for stimulation. EGF rapidly stimulated Akt phosphorylation with a peak at 2 min (Fig. 3A). Figure 3B



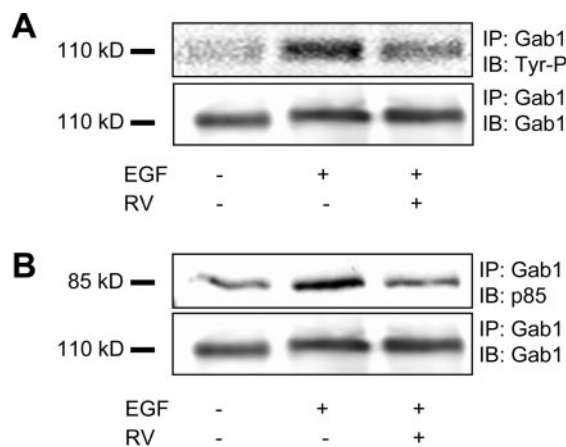
**Fig. 3.** Effect of RV, AG 1478, PP1, and wortmannin on EGF-induced Akt phosphorylation. A, time course of EGF-induced Akt phosphorylation. VSMCs were treated with 100 ng/ml EGF for the indicated times. Lysates were immunoblotted with anti-phospho-Akt (Ser<sup>473</sup>; top) or anti-Akt antibody (bottom). Graph at the bottom shows mean  $\pm$  S.E. from three independent experiments, expressed as fold increase over control. \*,  $P < 0.05$  (ANOVA/Dunnett). B, VSMCs were preincubated with vehicle, 50  $\mu$ M RV, 20  $\mu$ M PP1, 250 nM AG 1478 (AG), or 50 nM wortmannin (WM) for 30 min before treatment with (+) or without (-) 100 ng/ml EGF for 4 min. Lysates were immunoblotted with anti-phospho-Akt (Ser<sup>473</sup>; top) or anti-Akt antibody (bottom). Graph at the bottom shows averaged data, expressed as inhibition of EGF-induced phosphorylation. Values are mean  $\pm$  S.E. from four independent experiments. \*\*,  $P < 0.01$  versus EGF; \*\*\*,  $P < 0.001$  versus EGF (one-sample  $t$  test).

shows that RV, as well as PP1, AG 1478, and wortmannin used as positive controls, strongly inhibited EGF-induced Akt phosphorylation, suggesting that RV indeed interferes with pathways downstream of the EGF-R.

**RV Acts Upstream of PI3K Activation.** PI3K is essential for Akt activation in pathways triggered by either receptor tyrosine kinase or G protein-coupled receptor (Datta et al., 1999). Ang II strongly activates PI3K activity in VSMCs, with concomitant phosphorylation of the kinase's regulatory p85 subunit (Saward and Zahradka, 1997). Our group has shown previously that RV attenuates Ang II-stimulated tyrosine phosphorylation of the p85 subunit (Haider et al., 2002). In contrast to wortmannin, how-



**Fig. 4.** RV and wortmannin differently affect calf serum- and Ang II-induced Akt phosphorylation. A, impact of wortmannin (WM) on Ang II- and calf serum (CS)-induced Akt phosphorylation. Cells were treated with vehicle or 50 nM WM for 30 min and subsequently stimulated with (+) or without (-) CS (30 min) or Ang II (10 min). B, effect of RV on CS-induced Akt phosphorylation. Cells were treated with vehicle or 50  $\mu$ M RV for 30 min before stimulation with (+) or without (-) 10% CS for 30 min. Lysates were immunoblotted with anti-phospho-Akt (Ser<sup>473</sup>; top) or anti-Akt antibody (bottom).



**Fig. 5.** RV decreases phosphorylation of Gab1 and the binding of p85 to Gab1 in response to EGF. A, influence of RV on EGF-induced Gab1 phosphorylation. After preincubation with 50  $\mu$ M RV (+) or vehicle (-) for 30 min, cells were stimulated with (+) or without (-) 100 ng/ml EGF (4 min). Cell lysates were immunoprecipitated with an anti-Gab1 antibody and subsequently immunoblotted with an anti-phosphotyrosine antibody (top) or anti-Gab1 antibody (bottom). B, effect of RV on the binding of the regulatory subunit p85 of the PI3K to Gab1. VSMCs were treated and cell lysates immunoprecipitated as described under *Materials and Methods*. For immunoblot analysis an anti-p85 antibody (top) or anti-Gab1 antibody (lower) was used. One representative Western blot of three is shown.

ever (Fig. 4A), RV failed to inhibit Akt phosphorylation upon calf serum stimulation (Fig. 4B). Taken together, these results argue against direct inhibition of PI3K activity by RV as shown for wortmannin (Wymann et al., 1996).

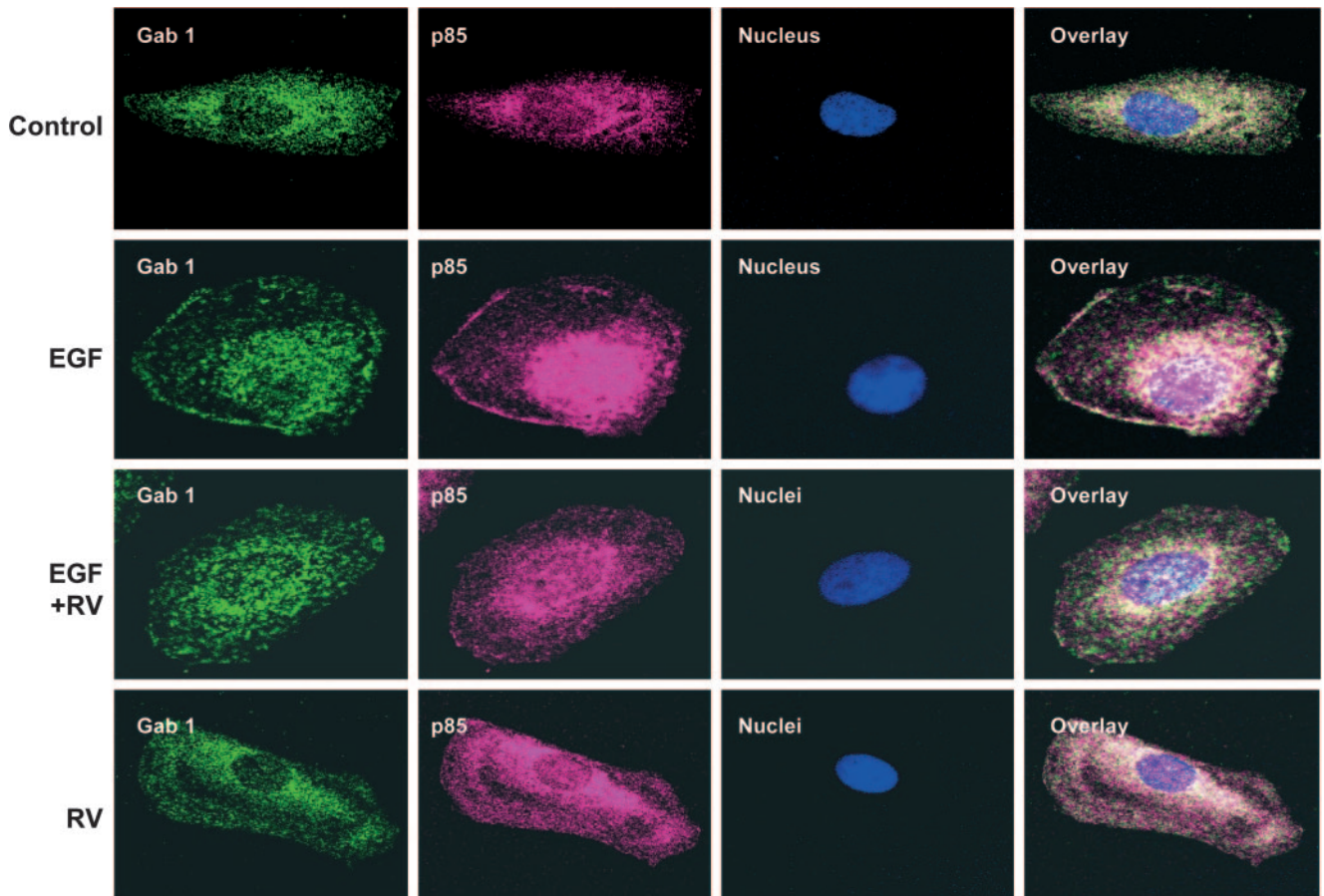
**RV Decreases Phosphorylation of Gab1 and Binding of the PI3K p85 Subunit to Gab1.** The adapter molecule Gab1 has recently been shown to be involved in signal transduction from the EGF-R to PI3K. In a model proposed by Rodrigues et al. (2000), Gab1 is first bound to and phosphorylated by the activated EGF-R, before its interaction with PI3K leads to signal amplification by generation of PIP<sub>3</sub>. PIP<sub>3</sub> recruits additional Gab1 and, subsequently, PI3K to the plasma membrane. In VSMCs, however, this link has never been established. We therefore investigated whether EGF-stimulation 1) enhances Gab1 tyrosine phosphorylation (Fig. 5A) and 2) leads to the association of Gab1 with the PI3K regulatory p85 subunit (Fig. 5B). Because both Gab1 tyrosine phosphorylation and p85-Gab1 association were strongly increased upon EGF stimulation, we tested a possible influence of RV on these processes. Most interestingly, RV reduced both overall Gab1 tyrosine phosphorylation (Fig. 5A) and association of Gab1 and p85 (Fig. 5B).

**RV Decreases Translocation of Gab1 and p85 to the Plasma Membrane.** To confirm the result obtained by immunoprecipitation, we performed experiments by confocal microscopy (Fig. 6). According to the model mentioned above,

EGF stimulation is associated with recruitment of Gab1 and p85 to the plasma membrane (Rodrigues et al., 2000). Figure 6 shows that upon stimulation with EGF, both Gab1 and p85 translocate to the membrane. This process is potentially inhibited when cells are preincubated with RV. It is noteworthy that RV alone does not alter the distribution pattern of Gab1 and p85 compared with vehicle-treated control cells.

**RV Decreases Ang II-Induced Gab1 Phosphorylation and PI3K p85 Recruitment to Gab1.** Next, we sought to clarify whether Gab1 tyrosine phosphorylation is also increased in response to Ang II and the PI3K p85 subunit is recruited to Gab1 in VSMC. Figure 7, A and B, clearly shows that both Gab1 phosphorylation and PI3K p85-Gab1 association are stimulated in response to Ang II. RV inhibits both signaling events, although Gab1 phosphorylation does so to a lesser extent than p85-Gab1 association (Fig. 7, A and B).

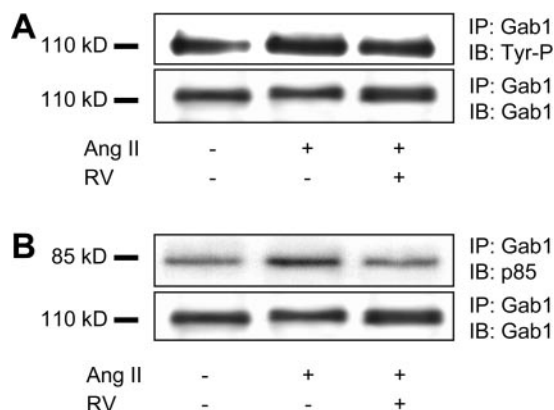
**RV Does Not Inhibit Akt Activation in Shp2<sup>-/-</sup> Cells.** Recent evidence in fibroblasts revealed a major role for the phosphotyrosine phosphatase Shp2 in regulating Gab1/PI3K interaction. In these cells, inactivation of Shp2 led to increased phosphorylation of the p85 binding sites on Gab1 and increased association of Gab1 and p85 upon EGF-stimulation with a subsequent increase in Akt activation (Zhang et al., 2002). We therefore hypothesized that RV may act via activation of Shp2. To verify this hypothesis, we used 3T3 immortalized fibroblasts from mice bearing a targeted mutation in Shp2 exon 3 (Shp2<sup>Δ46-110</sup>, hereafter Shp2<sup>-/-</sup>) and Shp2<sup>-/-</sup> fibro-



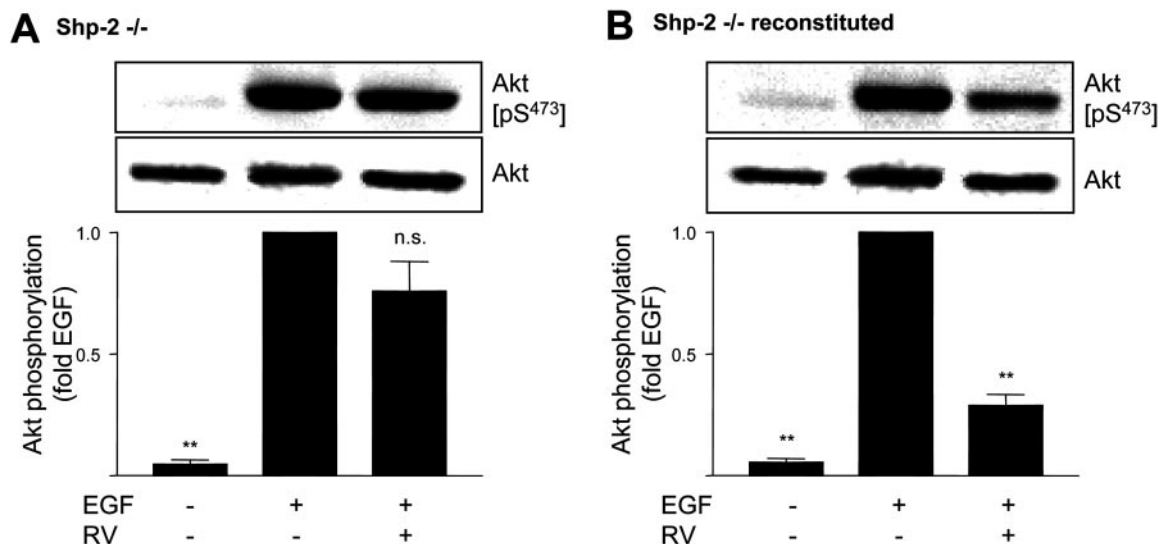
**Fig. 6.** RV inhibits EGF-induced translocation of Gab1 and p85 to the plasma membrane. VSMCs were preincubated with vehicle or 50  $\mu$ M RV for 30 min and subsequently stimulated with or without 100 ng/ml EGF for 4 min. After fixation with 4% paraformaldehyde, cells were costained with anti-Gab1 antibody (green channel), anti-p85 antibody (pink channel), and Hoechst 33342 (blue channel) and analyzed by confocal microscopy.

blasts where wild-type Shp2 expression was restored by retroviral gene transfer at a level comparable with that found in wild-type 3T3 fibroblasts (hereafter, control cells) (Zhang et al., 2002). Western blot analysis revealed that RV did inhibit Akt activation upon EGF stimulation in restored control cells (Fig. 8B). However, in Shp2<sup>-/-</sup> cells, RV failed to diminish EGF-induced Akt activation (Fig. 8A). Stimulation of Shp2<sup>-/-</sup> fibroblasts and reconstituted control cells with Ang II and treatment with RV provided virtually the same result (data not shown). These data strongly indicate that RV action depends on the presence of functional Shp2.

**RV Activates Shp2.** To gain further insight into the mechanism by which RV treatment affects Shp2, we assayed Shp2 activity in immune complexes from RV-treated cells as well as the direct effects of RV on recombinant Shp2 activity.



**Fig. 7.** RV decreases phosphorylation of Gab1 and the binding of p85 to Gab1 in response to Ang II. A, influence of RV on Ang II-induced Gab1 phosphorylation. After preincubation with 50  $\mu$ M RV (+) or vehicle (-) for 30 min, cells were stimulated with (+) or without (-) 100 nM Ang II (4 min). Cell lysates were immunoprecipitated with an anti-Gab1 antibody and subsequently immunoblotted with an anti-phosphotyrosine antibody (top) or anti-Gab1 antibody (bottom). B, effect of RV on the binding of the regulatory subunit p85 of the PI3K to Gab1. VSMCs were treated and cell lysates immunoprecipitated as described above. For immunoblot analysis an anti-p85 antibody (top) or anti-Gab1 antibody (bottom) was used. One representative Western blot of three is shown.



**Fig. 8.** RV does not inhibit Akt phosphorylation in a hypomorphic Shp2 mutant. A, 3T3 fibroblasts expressing hypomorphic mutant of Shp2 (Shp2<sup>-/-</sup>) were treated with vehicle (-) or RV (+) for 30 min before stimulation with (+) or without (-) 100 ng/ml EGF for 2 min. Lysates were immunoblotted with anti-phospho-Akt (Ser<sup>473</sup>; top) or anti-Akt antibody (bottom). B, 3T3 fibroblasts, reconstituted with wild-type Shp2 were treated as described in A. \*\*,  $P < 0.01$  versus EGF; n.s., not significant (ANOVA/Dunnett).

RV-treated cells showed a dose-dependent (50 nM-5  $\mu$ M) increase in Shp2 activity in the presence of EGF, with a maximal stimulation of approximately 2.5-fold. In the absence of EGF, RV had no statistical effect on Shp2 activity (Fig. 9A). RV also induced a smaller (maximum <2-fold), but significant, direct dose-dependent increase in Shp2 phosphatase activity (Fig. 9B). Similar results were obtained using another artificial substrate, <sup>32</sup>P-labeled reduced carboxymethyl lysozyme (data not shown). These data indicate that RV can activate Shp2 phosphatase activity in a dose-dependent manner, both in cell culture and in vitro.

## Discussion

The present study provides valuable new insight into the molecular mechanisms of RV to interfere with Ang II- and EGF-mediated signaling pathways in VSMCs. We show that RV does not impede EGF-R transactivation upon Ang II stimulation but potently inhibits EGF- as well as Ang II-stimulated Akt phosphorylation. Our data strongly suggest that this is caused by activation of Shp2, a protein tyrosine phosphatase previously shown to be involved in EGF-induced PI3K activation in fibroblasts. Moreover, our data provide evidence that the important role for Gab1 and Shp2 shown for EGF signaling to the PI3K/Akt-pathway in fibroblasts is also applicable for VSMCs.

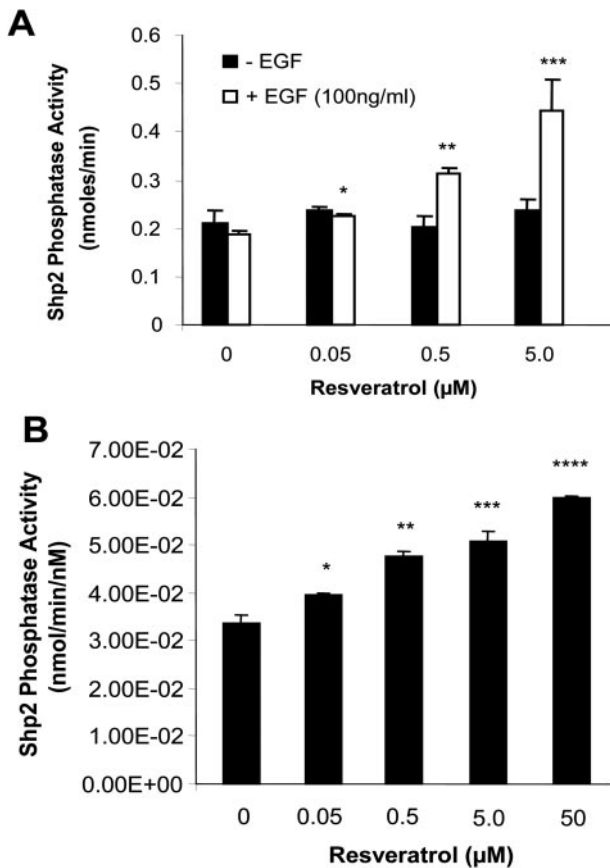
Ang II has emerged as a major player in the pathogenesis of atherosclerosis. It has been implicated in hypertrophy, proliferation, and migration of VSMCs as well as in inflammation and extracellular matrix deposition, underlining its importance for vascular remodeling (Schmidt-Ott et al., 2000). In this regard, transactivation of EGF-R has recently been shown to be important for early Ang II signaling through the AT<sub>1</sub> receptor (Kalmes et al., 2001). Consistent with results reported by others (Ushio-Fukai et al., 2001a), we show a rapid increase in overall EGF-R phosphorylation upon Ang II stimulation. Our results confirm, as far as addressed, that various signaling molecules including Ca<sup>2+</sup>, proline rich kinase 2, and metalloproteinases, as well as ROS

and c-Src kinase, are involved in EGF-R transactivation (Eguchi et al., 1998, 1999a; Bokemeyer et al., 2000; Kalmes et al., 2001; Ushio-Fukai et al., 2001a) (Fig. 2B). RV, however, was found not to interfere with Ang II-induced EGF-R phosphorylation, indicating that RV acts downstream of the EGF-R. Akt phosphorylation is consistently inhibited even when VSMCs are stimulated with EGF instead of Ang II.

PP1 completely inhibits EGF-induced Akt phosphorylation, and studies performed in other cell systems suggest that c-Src kinase is important for signaling downstream of EGF-R (Sato et al., 1995; Stover et al., 1995). It is interesting that in HeLa cells, RV has been shown to act as an inhibitor of c-Src tyrosine kinase (Yu et al., 2001). Although c-Src is phosphorylated at various sites, phosphorylation of tyrosine<sup>418</sup> in the kinase domain is most important, in that its autophosphorylation leads to increased kinase activity (Abram and Courtneidge, 2000). We found that RV does not alter phosphorylation of c-Src tyrosine<sup>418</sup> (data not shown). We further found that EGF-induced phosphorylation of tyrosine<sup>845</sup> of the EGF-R, a highly conserved site previously shown to be phosphorylated in a c-Src dependent manner (Biscardi et al., 1999), is not altered by RV (data not shown). These observations, together with the fact that RV, in

contrast to the c-Src inhibitor PP1, does not prevent Ang II-induced EGF-R transactivation, strongly indicate that c-Src activity is not impaired in RV-treated VSMCs.

It is very interesting that, unlike the PI3K-inhibitor wortmannin that binds to and inactivates the catalytic subunit of PI3K, RV did not reduce serum-induced Akt activation, strongly suggesting that RV acts upstream of PI3K activation when pathways of serum- and Ang II-induced Akt activation have not yet converged. Considering that many growth factors share the same adapter molecules and downstream effectors, very little is known about how specificity is achieved by different stimuli. For the EGF pathway, a positive feedback loop between the adapter Gab1 and PI3K has been proposed, placing PI3K both upstream and downstream of Gab1 (Rodrigues et al., 2000). In this regard, recent work shows that Gab1 and Shp2 act together in regulating PI3K activity. Shp2 attenuates PI3K activation in response to EGF by dephosphorylating the p85 binding sites on Gab1, thereby interrupting the amplification loop. Thereafter, in Shp2<sup>-/-</sup> cells, association between Gab1 and p85 is enhanced, whereas PI3K and Akt activation are increased (Zhang et al., 2002). It is most interestingly that this effect has been shown to be specific for EGF. Other growth factors such as platelet-derived growth factor or insulin-like growth factor-1 did not lead to enhanced Akt activation in Shp2<sup>-/-</sup> cells (Zhang et al., 2002), indicating specificity for the EGF-governed pathways. We were able to show that in VSMCs, EGF as well as Ang II stimulation leads to a rapid tyrosine-phosphorylation of Gab1 as well as association of Gab1 and the PI3K p85 subunit. RV decreased overall Gab1 phosphorylation and also p85 association with Gab1. Inhibition of Gab1 phosphorylation was not as pronounced as the inhibition of p85 recruitment to Gab1, especially after stimulation with Ang II. This is in accordance with the observation that Shp2 specifically dephosphorylates only the p85 binding sites on Gab1 (Zhang et al., 2002). Moreover, confocal microscopy revealed that RV inhibits EGF-induced translocation of Gab1 and p85 to the plasma membrane. Taken together, these results strongly point to an interference of RV with the association of Gab1 and p85, most likely an early stage in the above-mentioned feedback loop. Our results strongly suggest that the underlying mechanism is inhibition of Gab1 phosphorylation, raising the possibility that Shp2 may be essential for the mechanism of action of RV. Therefore, we performed experiments using Shp2<sup>-/-</sup> fibroblasts and, as a control, Shp2<sup>-/-</sup> cells where WT-Shp2 expression was restored by retroviral gene transduction. Most interestingly, RV did not prevent EGF-induced Akt activation in Shp2<sup>-/-</sup> cells, whereas inhibition was highly significant in the reconstituted WT-Shp2 expressing control cells suggesting that RV may act via activation of Shp2. Indeed, RV activates Shp2 both, in RV-treated cells (in vivo) as monitored by immune complex phosphatase assays and in vitro. Maximal stimulation in vivo is more dramatic than in vitro, suggesting that at least some of the effects of RV are not mediated by direct action of RV on Shp2. In this regard, it is interesting that previous work has suggested that RV inhibits NADPH oxidase (NOX) enzymes (see Introduction). NADPH oxidases catalyze the production of hydrogen peroxide, and recent studies indicate that PTPs, including Shp2 (Meng et al., 2002), are



**Fig. 9.** RV activates Shp2. A, Shp2 activity was measured in an immune complex phosphatase assay using Shp2 reconstituted control cells. The amount of pNPP phosphate released was determined by measuring the absorbance at 410 nm. Results are means  $\pm$  S.E. of the means from triplicate, and data were analyzed by a one-tailed paired *t* test. \*, *P* = 0.04; \*\*, *P* = 0.002; \*\*\*, *P* = 0.03. Resveratrol had no statistical significance on Shp2 activity in the absence of EGF. B, Shp2 phosphatase activity was measured in an in vitro assay using purified recombinant Shp2 protein. The rate of hydrolysis of pNPP by Shp2 was measured. Results are means  $\pm$  S.E. of the means from triplicate, and data were analyzed by a one-tailed paired *t* test. \*, *P* = 0.045; \*\*, *P* = 0.02; \*\*\*, *P* = 0.0003; \*\*\*\*, *P* = 0.003.

inhibited by peroxide-mediated oxidation of the catalytic cysteine residue (reviewed by Finkel, 2003). Taken together, these observations suggest that at least part of the mechanism of RV action may be to inhibit the generation of hydrogen peroxide via NOX proteins, thereby maintaining a higher level of catalytically active Shp2. This model is consistent with the observation that RV only affects Shp2 activity after EGF stimulation (Fig. 9A). Localization of Shp2 to the membrane, which follows EGF stimulation (Fig. 6) may be required to bring Shp2 to the vicinity of a specific NOX enzyme. Furthermore, in the absence of stimulation, Shp2 exists in a closed form in which ROS probably cannot access the active site cysteine (reviewed by Barford and Neel, 1998). Further studies will be required to test this model and also to determine how RV acts directly to stimulate recombinant Shp2.

In summary, this study delivers important new details on the mechanisms used by RV to interfere with Ang II- and EGF-induced signaling pathways known to contribute to the pathogenesis of cardiovascular disease. It is noteworthy that we provide evidence, for the first time, that Shp2 and Gab1 play an important and specific role in EGF signaling to the PI3K/Akt pathway in VSMCs. Both Ang II and EGF are expressed in atherosclerotic lesions (Jiang et al., 2002). It is important to note that the concentrations of RV used to perform this study were not toxic for VSMCs (Haider et al., 2002, 2003). Although little information is available about in vivo bioavailability of RV, there is some evidence that the amount of RV absorbed from the intestine may be sufficient to elicit biologic responses (Wu et al., 2001). Our study provides new details in early signaling events elicited by EGF and Ang II with regard to the role of Shp2 and Gab1 and contributes to a better understanding of the molecular basis of the action of RV in VSMCs. It may, therefore, be helpful to evaluate its potential as a putative therapeutic or preventive agent in cardiovascular disease.

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