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Resveratrol inhibits angiotensin II- and EGF-mediated Akt activation – role of Gab1 and Shp2

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Impact of Resveratrol on Angiotensin II- and EGF-signaling

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Abbreviations: VSMC, vascular smooth muscle cells; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco`s modified Eagle`s medium; ROS, reactive oxygen species; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; Gab1, Grb2 associated binder 1; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PI3K, phosphoinositide-3-kinase; Shp2, scr homology 2-domain-containing tyrosine phosphatase 2; Erk1/2, extracellular signal-regulated kinase1/2; PYK2, proline rich tyrosine kinase 2; PDGF, platelet derived growth factor; IGF-1, insuline like growth factor 1; EDTA, ethylene diamine tetra-acetic acid; PMSF, phenyl methyl sulfonyl fluoride; PIP_3, phosphatidyl inositol-3,4,5-triphosphate; TRIS, tris(hydroxymethyl)aminomethane

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Abstract

Trans-Resveratrol (RV), a polyphenolic stilbene derivate found in grape skin and other food products, has been proposed to exert beneficial effects in cardiovascular disease. Our group has previously shown that RV inhibits angiotensin II (Ang II)-induced Akt activation and, consequently, vascular smooth muscle cell (VSMC) hypertrophy. In this work, in order to identify the molecular target of RV, we investigated the impact of RV on early, Ang II- and epidermal growth factor (EGF)-triggered signaling cascades in rat aortic VSMCs. 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, are 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.

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Introduction

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_1 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;Ushio-Fukai *et al.*, 1999). Additionally, it has become evident that G protein-coupled receptors such as the AT_1 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 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;Kalmes *et al.*, 2001;Ushio-Fukai *et al.*, 2001b;Ushio-Fukai *et al.*, 2001a;Wang *et al.*, 2000). Regarding the mechanisms responsible for Ang II-mediated EGF-R transactivation, an impact of ROS (Ushio-Fukai *et al.*, 2001a), Ca^{2+}, proline rich kinase 2 (PYK2) (Eguchi *et al.*, 1998;Eguchi *et al.*, 1999a), c-Src kinase (Bokemeyer *et al.*, 2000) and heparin-binding EGF-like growth factor (HB-EGF) (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 the Schlessinger group 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

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various signaling molecules containing phospho-tyrosine 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 (PIP_3) at the plasma membrane, which recruits additional Gab1 *via* its pleckstrin homology domain and subsequently recruits PI3K. Subsequently, Zhang *et al.* established an important modulatiory role for the protein-tyrosine phosphatase Shp2 in this process: Shp2 attenuates PI3K activation by specifically dephosphorylating the p85 binding sites on Gab1. Most interestingly, this is specific for EGF and not other growth factors such as PDGF and IGF-1 (Zhang *et al.*, 2002).

Resveratrol (RV), a polyphenolic phytoalexin found in grape skin, has been proposed to be, at least in part, responsible 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 presents evidence that RV is able to inhibit vascular NADPH oxidase (Orallo *et al.*, 2002). Wallerath *et al.* demonstrated that RV enhances expression and activity of endothelial nitric oxide synthase (Wallerath *et al.*, 2002). 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^{473}), EGF-receptor, phospho-EGF-receptor (Tyr^{845}), phospho-tyrosine (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 (Chicago, USA). The inhibitors AG 1478 and PP1 were from QBiogene-Alexis (Grünberg, Germany). EGF was obtained from Upstate Biotechnology (Lake Placid, USA). RV, NAC, HOECHST 33342 as well as Ang II were from Sigma (Taufkirchen, Germany). Complete™ was from Roche (Mannheim, Germany). Calf serum was from Life Technologies (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 µg/ml streptomycin (growth medium) and passaged twice a week by harvesting with trypsin:EDTA and seeding into 75-cm^2 flasks. For experiments, cells between passage 7 and 15 were used at 70-95% confluence. 3T3 immortalized fibroblasts from Shp2 exon 3$^{-/-}$ (Shp2$^{\Delta 46\text{-}110}$) mice were described previously (Saxton *et al.*, 1997; named Shp2$^{-/-}$). Shp2$^{-/-}$ cells which contain a stably reconstituted Shp2 protein *via* retroviral transfection (Zhang *et al.*, 2002) were used as control (Shp2 rescue 3T3 fibroblasts). 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 µM RV or vehicle for 30 min prior to stimulation with 100 ng/ml of EGF for 4 min.

Immunoprecipitation and immunoblotting. Cells grown in 60 mm dishes were serum-starved over night 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 prior to 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 PMSF, 1xComplete™). Western blot analysis was performed as described previously (Ushio-Fukai *et al.*, 1999). For immunoprecipitation, the lysates were cleared by centrifugation (10 min, 14,000xg), and protein concentrations were determined by the BCA method (Pierce). 5 µl of anti-EGF-R antibody were added to a 500 µg aliquot (1 µg protein/µl) and mixed over night at 4°C. Protein A-agarose beads (50 µl) washed with lysis buffer, were added for an additional 2 hours and subsequently collected by centrifugation. The beads were washed three times with 500 µl lysis buffer and resuspended in 25 µl 3x sample buffer containing 1.5% β-mercaptoethanol. After addition of 25 µl 1x sample buffer, beads were boiled for 5 min at 95°C and afterwards removed by centrifugation. 30 µl (for anti-phospho-tyrosine) or 10 µl (for anti-EGF-R) of the lysate were 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% non-fat dry milk in Tris-buffered saline containing 1% tween-20. Anti-EGF-R antibody (1:1000) or anti-phospho-tyrosine antibody (1:2000) were added over night 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 ScienceTM image station 440 cf (NEN, 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% BSA in PBS for one hour 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 one hour. After

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washing twice with PBS, HOECHST 33342 (5 µg/ml) was added for 5 min and then rinsed once with PBS. VSMCs were mounted on microscope slides with DAKO® fluorescent mounting medium and visualized by confocal laser scanning microscopy (CLSM 510 META, Zeiss, Jena, Germany).

Statistical analysis. Results are expressed as mean ±SE. Statistical analysis was performed by ANOVA followed by a Dunnett multiple comparison test or by a one-sample t-test using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California).

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) as an artificial substrate. Briefly, for immune complexes, Shp2 rescue 3T3 fibroblasts were starved over night, 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 X NP-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% NP-40 lysis buffer (without sodium orthovanadate) and once in phosphatase buffer without DTT. 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^{o} C for 10 min in phosphatase buffer containing 30 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM NaCl, 5 mM dithiothreitol (DTT), 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 minute.

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 previously described to act as inhibitors of transactivation (the antioxidant NAC, PP1, an inhibitor of c-Src kinase and the tyrphostin AG 1478, an inhibitor of EGF-R kinase) were used as positive controls. Fig. 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, and 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 transactivation is indeed implicated in Akt phosphorylation in our cells, we treated VSMCs with AG 1478 prior to 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, to a smaller extent, parallel pathways should be considered.

RV inhibits EGF-induced Akt phosphorylation. Since EGF-R transactivation is not impeded by RV, we assumed this substance to influence pathways downstream of the EGF-R. Consequently, we tested whether RV was also effective in blocking Akt phosphorylation when EGF instead of Ang II is used for stimulation. EGF rapidly stimulated Akt phosphorylation with a peak at 2 min (Fig. 3A). Fig. 3B 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 both receptor tyrosine kinase- as well as G protein-coupled receptor-triggered pathways (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 previously shown that RV attenuates Ang II-stimulated tyrosine phosphorylation of the p85 subunit (Haider *et al.*, 2002). In contrast to wortmannin, however (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.*, 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_3. PIP_3 recruits additional Gab1 and, subsequently, PI3K to the plasma membrane (Rodrigues *et al.*, 2000). In VSMCs, however, this link has never been established. We therefore investigated whether EGF-stimulation a) enhances Gab1 tyrosine phosphorylation (Fig 5A) and b) leads to the association of Gab1 with the PI3K regulatory p85 subunit (Fig 5B). As 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). Fig 6 shows that upon stimulation with EGF, both Gab1 and p85 translocate to the membrane. This process is potently inhibited when cells are preincubated with RV. Importantly, RV alone does not alter the distribution pattern of Gab1 and p85 compared to vehicle-treated control cells.

RV decreases Ang II-induced Gab1 phosphorylation and PI3K p85 recruitment to Gab1. Next, we sought to clarify whether also in response to Ang II Gab1 tyrosine phosphorylation is increased and the PI3K p85 subunit is recruited to Gab1 in VSMC. Fig. 7A/B clearly shows that both, Gab1 phosphorylation as well as PI3K p85-Gab1 association is stimulated in response to Ang II. RV inhibits both signaling events, although Gab1 phosphorylation to a lesser extend than p85-Gab1 association (Fig. 7A/B).

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RV does not inhibit Akt activation in Shp2$^{-/-}$-cells. Recent evidence in fibroblasts revealed a major role for the phospho-tyrosine 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$^{\Delta 46\text{-}110}$, hereafter Shp2$^{-/-}$) and Shp2$^{-/-}$ fibroblasts where wild-type Shp2 expression was restored by retroviral gene transfer at a level comparable to that found in wild-type 3T3 fibroblasts (thereafter, controls) (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$^{-/-}$ cells, RV failed to diminish EGF-induced Akt activation (Fig 8A). Stimulation of Shp2$^{-/-}$ 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. RV-treated cells showed a dose-dependent (50 nM-5 µM) increase in Shp2 activity in the presence of EGF, with a maximal stimulation of about 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, 32P-RCM-Lysozyme (RCML) (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 due to 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_1 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^{2+}, PYK 2, metalloproteinases as well as ROS and c-Src kinase are involved in EGF-R transactivation ((Bokemeyer *et al.*, 2000;Eguchi *et al.*, 1998;Eguchi *et al.*, 1999a;Kalmes *et al.*, 2001;Ushio-Fukai *et al.*, 2001a), and 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. Consistently, Akt phosphorylation is 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). Interestingly, 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^{418} in the kinase domain is most important, as its autophosphorylation leads to increased kinase activity (Abram and Courtneidge, 2000). We found that RV does not alter phosphorylation of c-Src tyrosine^{418} (data not shown). We further found that EGF-induced phosphorylation of tyrosine^{845} 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

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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.

Most interestingly, 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, still very little is known 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. Consequently, in $Shp2^{-/-}$ cells, association between Gab1 and p85 is enhanced, while PI3K and Akt activity are increased (Zhang *et al.*, 2002). Most interestingly, this effect has been shown to be specific for EGF. Other growth factors such as PDGF or IGF-1 did not lead to enhanced Akt activation in $Shp2^{-/-}$ 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 RV`s mechanism of action. Therefore, we

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performed experiments using $Shp2^{-/-}$ fibroblasts and, as a control, $Shp2^{-/-}$ cells where WT-Shp2 expression was restored by retroviral gene transduction. Most interestingly, RV did not prevent EGF-induced Akt activation in $Shp2^{-/-}$ cells, while 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 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 following 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. Notably, 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;Haider *et al.*, 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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Footnotes

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Legends for Figures

Fig. 1. AG 1478, PP1 and NAC, but not RV, attenuate Ang II-induced EGF-R phosphorylation. VSMCs were preincubated with A, 250 nM AG 1478 (AG), B, 20 µM PP1, C, 10 mM NAC or D, 50 µM RV for 30 min before treatment with (+) or without (-) 100 nM Ang II for 2 min. Lysates were used as described in legend to Figure 1. Top panels show representative blots for tyrosine phosphorylated EGF-R. Bottom panels show representative blots for total levels of EGF-R. Panels show one representative western blot out of three.

Fig. 2. Effect of RV and AG 1478 on Ang II-induced Akt phosphorylation. VSMCs were preincubated with vehicle, 50 µM 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^{473}, top panels) or anti-Akt antibody (lower panels). Graphs at the bottom show mean ± SE from three independent experiments, expressed as inhibition of Ang II-induced Akt phosphorylation. ***P<0.01 vs Ang II; ***P<0.001 vs Ang II (one-sample t test).*

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^{473}, top panel) or anti-Akt antibody (lower panel). Graph at the bottom shows mean ± SE from three independent experiments, expressed as fold increase over control. **P<0.05 (ANOVA/Dunnett).* B, VSMCs were preincubated with vehicle, 50 µM RV, 20 µM 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^{473}, top panel) or anti-Akt antibody (lower panel). Graph at the bottom shows averaged data, expressed as inhibition of EGF-induced phosphorylation. Values are mean ± SE from four independent experiments. ***P<0.01 vs EGF; ***P<0.001 vs EGF (one-sample t test).*

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 µM RV for 30 min before stimulation with (+) or without (-)10% CS for 30 min. Lysates were immunoblotted with anti-phospho-Akt (Ser^{473}, top panels) or anti-Akt antibody (bottom panels).

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 pre-incubation with 50 µM 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-phospho-tyrosine antibody (upper panel) or anti-Gab1 antibody (lower panel). B, effect of RV on the binding of the regulatory subunit p85 of the PI3K to Gab1. VSMC were treated and cell lysates immunoprecipitated as described above. For Immunoblot analysis an anti-p85 antibody (top panel) or anti-Gab1 antibody (lower panel) was used. One representative western blot out of three is shown.

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

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 pre-incubation with 50 µM 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-phospho-tyrosine antibody (upper panel) or anti-

Gab1 antibody (lower panel). B, effect of RV on the binding of the regulatory subunit p85 of the PI3K to Gab1. VSMC were treated and cell lysates immunoprecipitated as described above. For Immunoblot analysis an anti-p85 antibody (top panel) or anti-Gab1 antibody (lower panel) was used. One representative western blot out 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 ($Shp2^{-/-}$) 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^{473}, top panel) or anti-Akt antibody (lower panel). B, 3T3 fibroblasts, reconstituted with wild-type Shp2 were treated as described in A. ***P<0.01 vs EGF; n.s., not significant (Anova/Dunnett).*

Fig 9. RV activates Shp2. A, Shp2 activity was measured in an immune complex phosphatase assay using Shp2 Rescue 3T3 fibroblasts. The amount of *p*NPP phosphate released was determined by measuring the absorbance at 410 nm. Results are means ± 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 *p*NPP by Shp2 was measured. Results are means ± 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.

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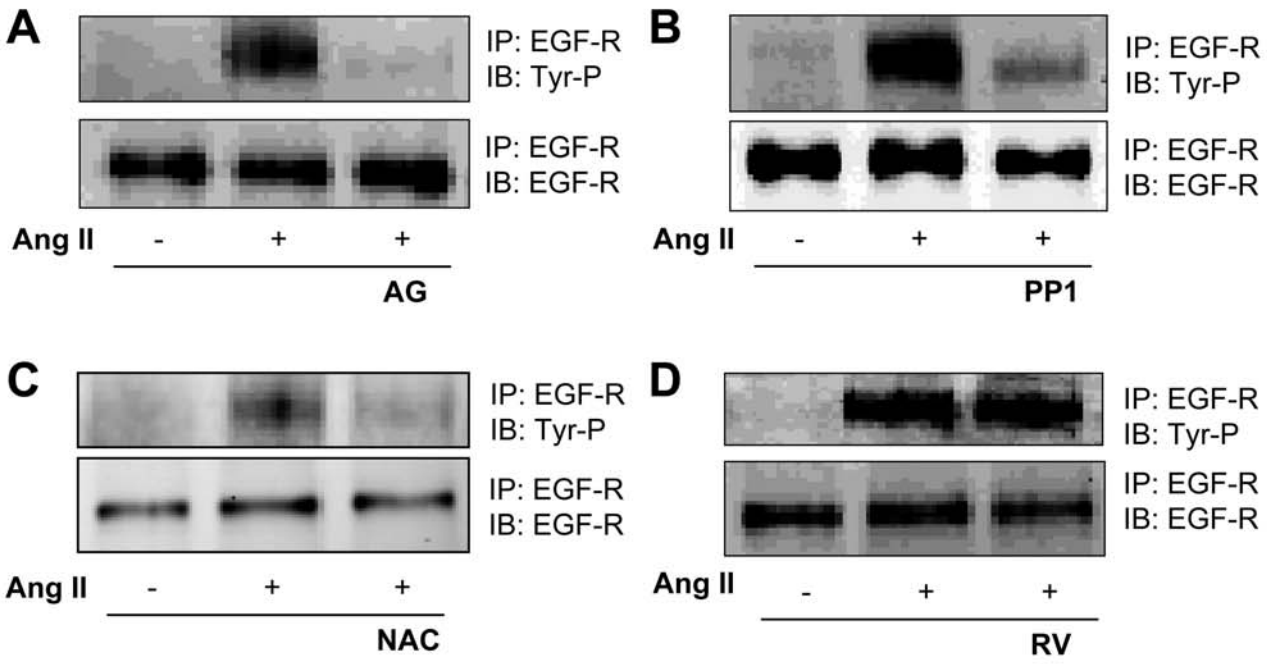

Figure 1

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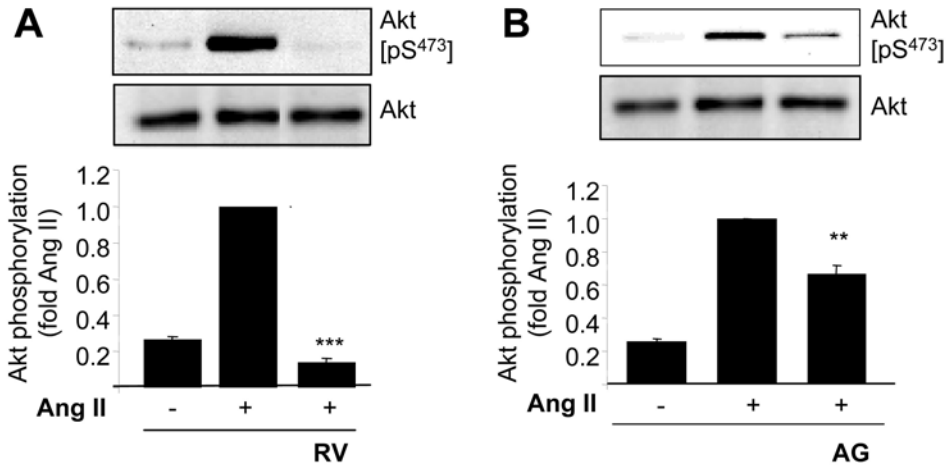

Figure 2

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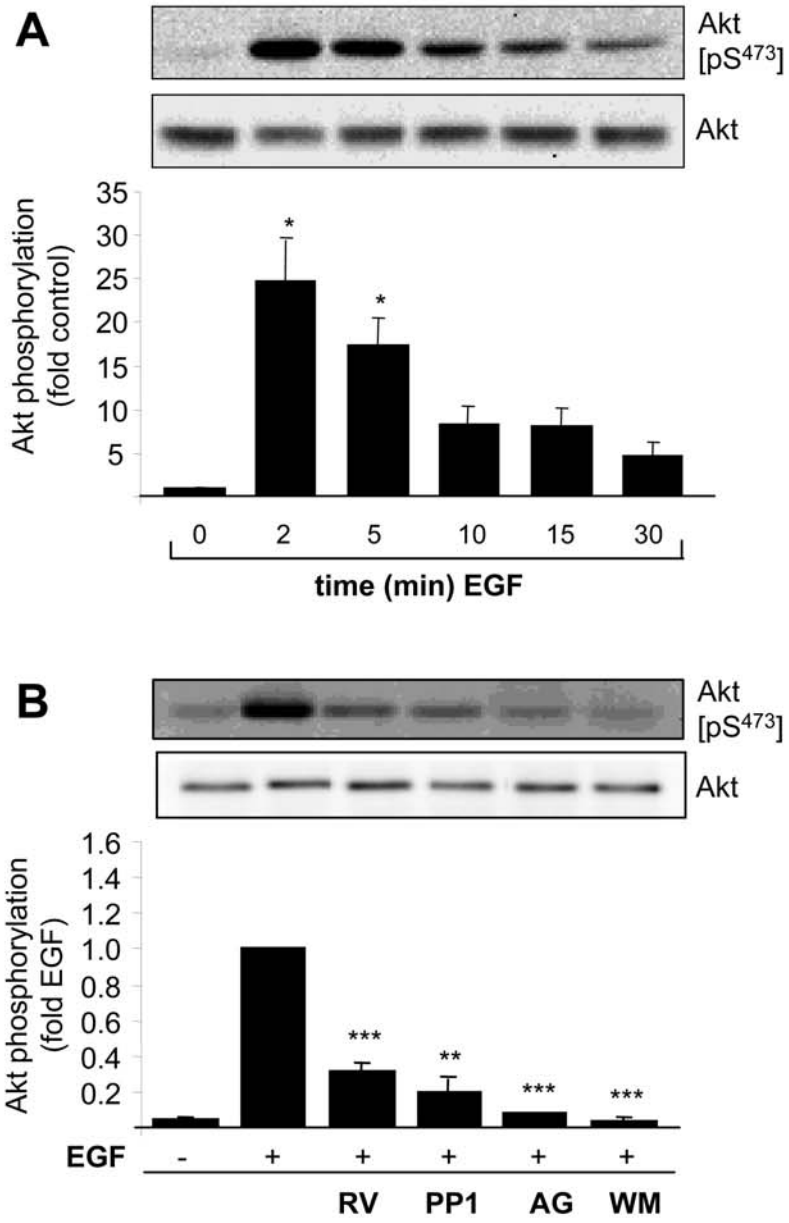

Figure 3

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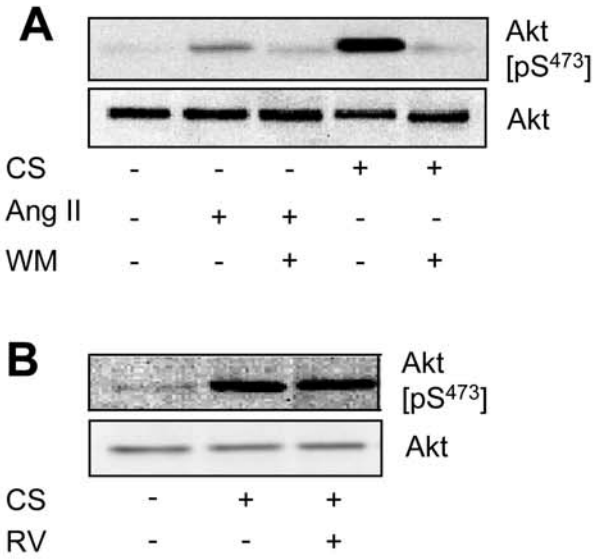

Figure 4

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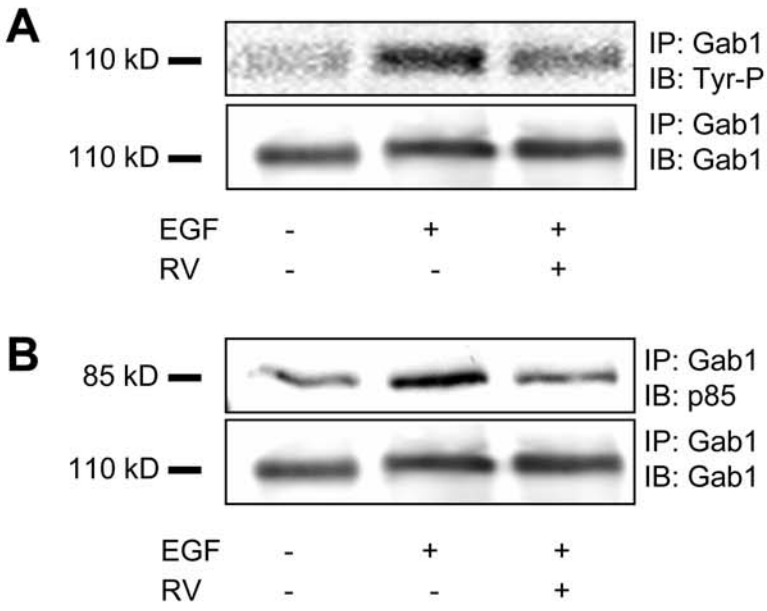

Figure 5

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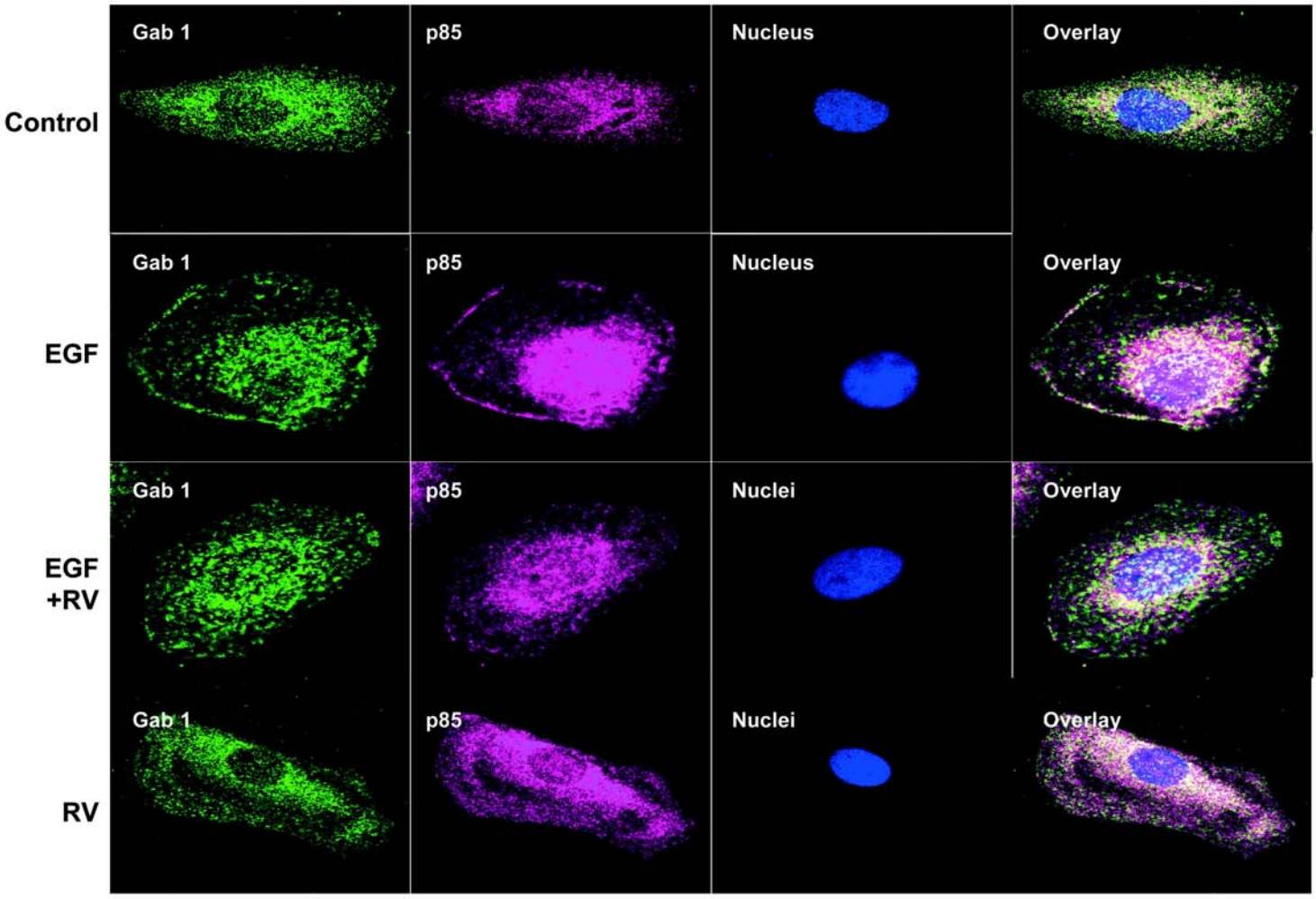

Figure 6

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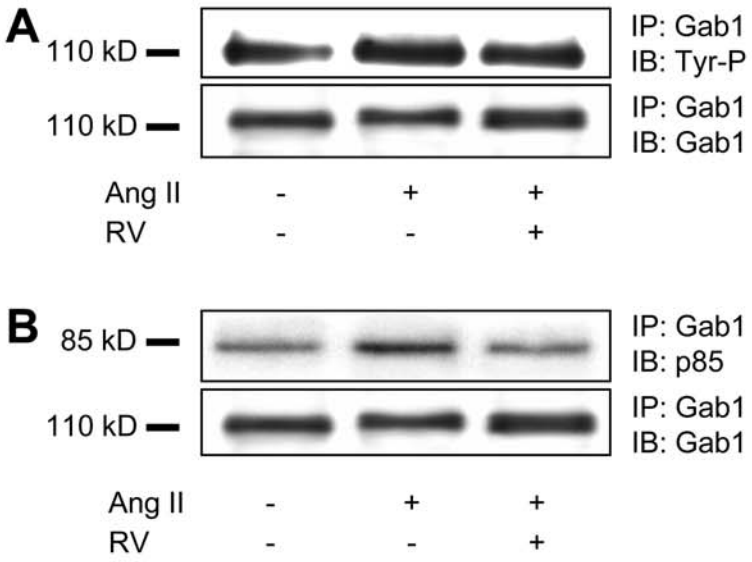

Figure 7

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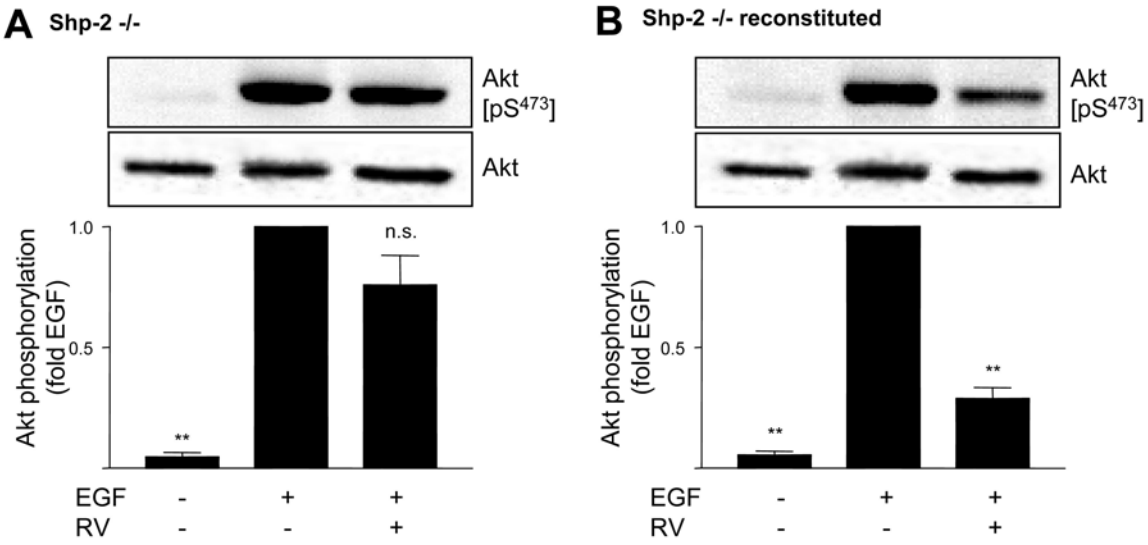

Figure 8

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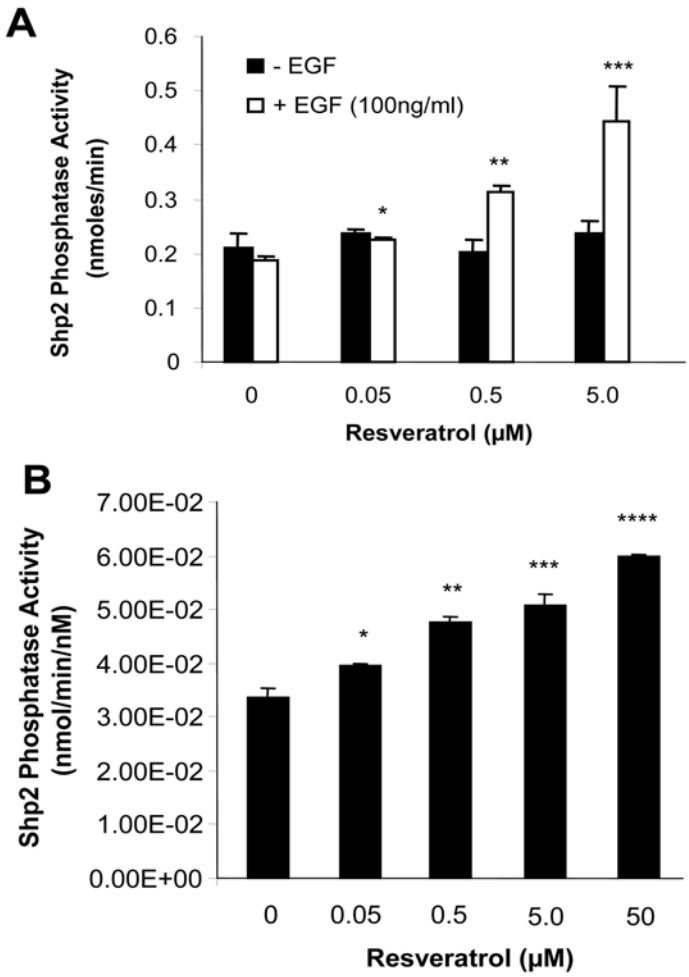

Figure 9