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**Resveratrol prevents angiotensin II-induced hypertrophy of vascular smooth muscle cells
through the transactivation of growth factor receptors**

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Abstract

We previously showed that the augmented levels of endogenous angiotensin II (Ang II) contribute to vascular smooth muscle cell (VSMC) hypertrophy through the transactivation of growth factor receptors in spontaneously hypertensive rats. Resveratrol (RV), a polyphenolic component of red wine, has also been shown to attenuate Ang II-evoked VSMC hypertrophy, however, the molecular mechanism mediating this response is obscure. The present study is therefore undertaken to examine if RV could prevent Ang II-induced VSMC hypertrophy through the transactivation of growth factor receptor and associated signaling pathways. Ang II treatment of VSMC enhanced the protein synthesis which was attenuated towards control levels by RV pre-treatment as well as by the inhibitors of NADPH oxidase, c-Src, and growth factor receptors. Furthermore, RV pre-treatment also inhibited the enhanced levels of superoxide anion, NADPH oxidase activity, the increased expression of NADPH oxidase subunits and phosphorylation of c-Src, EGF-R, PDGE-R, ERK1/2, and AKT1/2. In conclusion, these results indicate that RV attenuates Ang II-induced VSMC hypertrophy through the inhibition of enhanced oxidative stress, activation of c-Src, growth factor receptors and MAPK/AKT signaling. It may be suggested that RV could be used as a therapeutic agent in the treatment of vascular complications associated with hypertension and hypertrophy.

Keywords: Resveratrol, Angiotensin II, Oxidative stress, c-Src, Growth factor receptor

Introduction

Hypertrophy and hyperproliferation of vascular smooth muscle cells (VSMCs) contribute to vascular remodeling that is attributed to several vascular diseases such as atherosclerosis, restenosis, and hypertension (Touyz 2005). Angiotensin II (Ang II), the main vasoactive peptide of the renin-angiotensin-aldosterone system (RAAS), regulates a variety of pathophysiological functions including high blood pressure, VSMC hyperproliferation, and hypertrophy (Atef and Anand-Srivastava 2014; Beaucage and Moreau 2004; Bouallegue et al. 2009) by the activation of various signaling mechanisms including adenylyl cyclase/cAMP, phosphatidyl inositide 3-kinases (PI3K), mitogen-activated protein kinase (MAPK), and tyrosine kinase pathways (Gomez Sandoval and Anand-Srivastava 2011; Gusan and Anand-Srivastava 2013; Nakashima et al. 2008). At the cellular level, Ang II elicits these molecular signals by interacting with two distinct subtypes of receptor, designated AT₁ and AT₂ (Timmermans et al. 1993). Both AT₁ and AT₂ receptors are present in heart muscle as well as in cardiomyocytes and mediate the physiological effects of Ang II (Bkaily et al. 2005; Booz and Baker 1996). In addition, AT₁ receptor subtype is also present in rat vascular tissues, however, a small proportion of AT₂ receptors are present in rat aorta, (Chang and Lotti 1991). Most of the pathophysiological effects of Ang II are mediated by the activation of AT₁ receptors. Ang II has also been shown to increase the oxidative stress due to the production of reactive oxygen species (ROS) through the activation of NADPH oxidase (Griendling et al. 1994; Seshiah et al. 2002). ROS through the activation of various downstream kinases, including MAPK and the serine/threonine kinase AKT (also known as protein kinase B or PKB), are implicated in Ang II-induced hypertrophic response of VSMCs (Ushio-Fukai et al. 1998; Ushio-Fukai et al. 1999). We also reported that enhanced levels of endogenous Ang II in VSMC from spontaneously hypertensive rat (SHR)

through oxidative stress, transactivation of growth factor receptors; insulin-like growth factor receptor (IGF-R), epidermal growth factor receptor (EGF-R), and platelet-derived growth factor receptor (PDGF-R) and associated MAPK signaling, contributes to VSMC hypertrophy (Atef and Anand-Srivastava 2016a).

Resveratrol (RV), an antioxidant and an important polyphenolic component of red wine has been shown to attenuate the development of hypertension in SHR and other experimental models of hypertensive rats (Bhatt et al. 2011) as well as cardiac hypertrophy (Petrovski et al. 2011). In addition, RV has also been shown to attenuate Ang II-evoked VSMC hypertrophy through the inhibition of AKT/protein kinase B and p70 S6 kinase phosphorylation (Haider et al. 2002). However, whether RV inhibits Ang II-induced VSMC hypertrophy through the attenuation of growth factor receptor transactivation and associated signaling pathways that have been implicated in hypertrophy of VSMC has not been explored. Therefore, the present study was undertaken to investigate the effect of RV on Ang II-induced VSMC hypertrophy and to delineate the role of the transactivation of growth factor receptor and associated signaling in the antihypertrophic effect of RV.

We showed that RV inhibits Ang II-induced VSMC hypertrophy through its ability to attenuate oxidative stress, activation of c-Src, growth factor receptors and MAPK/AKT signalling pathways.

Materials and Methods

Materials

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene), Angiotensin II (human), epidermal growth factor receptor (EGF-R) inhibitor AG1478, platelet-derived growth factor receptor (PDGF-R) inhibitor AG1295, c-Src inhibitor PP2, inactive analog for Src inhibitor PP3 and inhibitor of NADPH oxidase diphenyleneiodonium (DPI) were purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA). Western blotting primary antibodies against EGF-R (sc-373746), p-EGF-R (sc-101668, phosphospecific-tyrosine¹¹⁷³), PDGFR- β (sc-432), p-PDGFR- β (sc-12909-R, phosphospecific-tyrosine¹⁰²¹), c-Src (sc-18), p-c-Src (sc-166860, phosphospecific-tyrosine⁴¹⁹), ERK1/2 (sc-135900), p-ERK1/2 (sc-7383, phosphospecific-tyrosine²⁰⁴), AKT1/2/3 (sc-8312), p-AKT1/2/3 (sc-7985-R, phosphospecific-serine⁴⁷³), Nox-1 (sc-5821), dynein IC1/2 (sc-13524), secondary antibodies goat anti-mouse IgG horseradish-peroxidase (HRP) conjugate (sc-2005), donkey anti-goat IgG horseradish-peroxidase (HRP) conjugate (sc-2020) and enhanced chemiluminescence (ECL) detection system kits were purchased from Santa-Cruz Biotechnologies (Santa Cruz, CA, USA). Anti-p47-phox (07-497) and Nox-4 (ABC271) primary antibodies were purchased from EMD Millipore (USA). Anti-Nox-2/gp91phox (ab129068) primary antibody was purchased from Abcam Inc (ON, Canada). Secondary antibody Goat Anti-Rabbit IgG (H+L)-HRP conjugate was purchased from BIO RAD (USA). The L-[¹⁴C(U)]-Leucine was from PerkinElmer Inc. (Waltham, Massachusetts, USA). All other chemicals used in the experiments were purchased from Sigma-Aldrich.

Methods

Animal preparation

Male Sprague Dawley (SD) rats (225 to 230 g, ten-week-old) purchased from Charles River (Saint-Constant, Québec, Canada) were maintained on a standard rat chow diet and tap water *ad libitum* with a 12:12 h light:dark cycle in a quiet environment for two weeks (acclimatization). The rats were anesthetized by CO₂ exposure and then euthanized by decapitation. The aortas were dissected out and used for cell culture as described previously (Anand-Srivastava et al. 1982). All the animal procedures used in the present study were approved by the Comité de Déontologie de l'Expérimentation sur les Animaux (CDEA) of the University of Montreal (Approval no. 99050). The investigation conforms to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH) (Guide, NRC 2011).

Cell culture, incubation, and cell lysis

Aortic VSMCs from twelve-week-old SD rats were cultured as described previously (Anand-Srivastava et al. 1982). The cell purity was determined by immunofluorescence using α -actin as described previously (Liau and Chan 1989). The cells were found to contain high levels of smooth muscle-specific α -actin. Cells were plated in 100 mm culture dishes and incubated at 37°C in 95% air, 5% CO₂, humidified atmosphere in Dulbecco's Modified Eagle's Medium (DMEM, with glucose, L-glutamine, and sodium bicarbonate) containing 1% antibiotics (containing penicillin, streptomycin, and amphotrecin B) and 10% heat-inactivated fetal bovine serum (FBS). Cells were passaged upon reaching confluence with 0.5% trypsin and used between passages two and ten. Subconfluent cell cultures were inactivated by incubation for 12 h in DMEM without FBS at 37°C to reduce the interference by growth factors present in the

serum. After inactivation, aortic VSMCs were pre-incubated in the absence or presence of different concentrations of RV (10, 30 and 50 μM) or 30 μM for 1 h before stimulation with 10^{-6} M Ang II, a concentration previously shown to be effective in VSMC (Li and Anand-Srivastava 2012; Li et al. 2007). After 24 h incubation, the cells were washed twice with ice-cold phosphate buffer saline (PBS) and lysed in a 200 μl buffer containing 25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{mL}$ aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5 $\mu\text{g}/\text{mL}$ leupeptin on ice. The cell lysates were centrifuged at 12000 g for 15 min at 4°C, and the supernatants were used for Western blot analysis. Protein concentrations were measured with a Bradford assay (Bradford 1976).

Western blotting

The levels of protein expression and phosphorylation were determined by Western blotting as described previously (Atef and Anand-Srivastava 2016a). After SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane with a semi-dry transblot apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada) at 15 V for 45 min (Nox-1, gp91^{phox}, Nox-4, p47^{phox}, c-Src, AKT1/2, and ERK1/2) or a liquid transfer apparatus (Bio-Rad Laboratories) at 100 V for 1 h (EGF-R, and PDGF-R). After transfer, membranes were blocked for 1 h at room temperature with 5% dry milk and incubated overnight with specific antibodies against different proteins. Dynein was used for loading controls. The antibody-antigen complexes were detected by incubating the membranes with HRP-conjugated antibodies for 1 h at room temperature. The blots were then washed three times with PBS before reaction with enhanced chemiluminescence (ECL). Quantitative analysis of the proteins was performed by densitometric scanning of the autoradiographs using the enhanced laser densitometer LKB

Ultrosan XL and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia (Baie d'Urfé, Québec, Canada).

[³H]-Leucine incorporation assays

Cell hypertrophy was quantified by protein biosynthesis. Protein biosynthesis was evaluated by [³H]-Leucine incorporation into cells as described previously (Atef and Anand-Srivastava 2016a). Subconfluent VSMCs from twelve-week-old SD rats were inactivated by incubation for 12 h in serum-free DMEM at 37°C. The inactivated VSMCs were pre-incubated in the absence or presence of 30 µM or different concentrations of RV (10-50 µM), DPI (10 µM), AG1478 (10 µM), AG1295 (10 µM), PP2 (1 µM), and PP3 (1 µM) for 1 h before stimulation with or without 10⁻⁶ M Ang II. These concentrations of different inhibitors/antagonists have been used in earlier studies (Gomez Sandoval and Anand-Srivastava 2011; Ichiki et al. 2004; Li et al. 2010). After 12 h, [³H]-Leucine (2 µCi per well) was added and further incubated for 12 h before cells were harvested. Cells were rinsed twice with ice-cold PBS and incubated with 5% trichloroacetic acid (TCA) for 1 h at 4 °C. After being washed twice with ice-cold water, cells were incubated with 0.4 N sodium hydroxide (NaOH) solution for 30 min at room temperature, and radioactivity was determined with a liquid scintillation counter and adjusted by protein concentration. Cell viability was checked by the trypan blue exclusion technique and indicated that >90 ~ 95% cells were viable.

Protein/DNA ratio measurements

Cell hypertrophy was also assessed by calculating the ratio of total protein to genomic DNA (gDNA) content as described previously (McKay et al. 1998). For this assessment, VSMCs were plated in six-well plates at a seeding density of 2 to 3 × 10⁴ cells/well. After starvation by incubation in DMEM without FBS at 37°C as described above, VSMCs were pre-incubated in

the absence or presence of different concentrations of RV for 1 h and subsequently stimulated with or without 10^{-6} M Ang II for 24 h. After stimulation, cells were washed in ice-cold PBS and gDNA extraction from each sample was carried out by using PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. DNA concentration was determined by NanoDrop ND-2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The total protein content was estimated colorimetrically using the method described by Bradford (Bradford 1976). Serial concentrations of calf thymus DNA and bovine serum albumin (BSA) were used for the calibration curves.

Superoxide anion (O_2^-) measurements

Basal O_2^- production in VSMCs was measured by the lucigenin-enhanced chemiluminescence method with a low concentration (5 μ M/L) of lucigenin as described previously (Atef and Anand-Srivastava 2016b). The VSMCs were pre-incubated in the absence or presence of RV (30 μ M) for 1 h and subsequently stimulated with or without 10^{-6} M of Ang II for 24 h. After treatment with Ang II, VSMCs were washed in oxygenated Krebs-HEPES buffer, scraped, and placed in scintillation vials containing lucigenin solution, and the emitted luminescence was measured with a liquid scintillation counter (Wallac 1409; Perkin Elmer Life Science, St Laurent, QC, Canada) for 5 min. The average luminescence value was estimated and the background value subtracted, and the result was divided by the total weight of proteins in each sample.

NADPH oxidase activity determination

The activation of NADPH oxidase activity in the samples was assessed by adding 10^{-4} M/L NADH (Sigma) in the vials before counting. Basal O_2^- -induced luminescence was then subtracted from the luminescence value induced by NADH (Atef and Anand-Srivastava 2016b).

Statistical analysis

Results are expressed as means $\pm$ SE (Standard Error). Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test using GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, California, USA). A difference between groups was significant at $p < 0.05$.

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Results

RV inhibits Ang II-induced VSMC hypertrophy

Figure 1 shows the effect of different concentrations of RV on Ang II-induced hypertrophy of aortic VSMC. As reported earlier (Haider et al. 2002), Ang II enhanced the [^3H]-Leucine incorporation (Figure 1A) and protein/DNA ratio (Figure 1B) (markers of cell hypertrophy) in VSMC by about 120% and 70% respectively and RV pre-treatment attenuated the hypertrophy in a concentration-dependent manner. At 30 μM , RV attenuated Ang II-induced [^3H]-Leucine incorporation and protein/DNA ratio to almost control level whereas at 50 μM RV, the Ang II-induced [^3H]-Leucine incorporation was inhibited below control levels. In addition, RV at 30 and 50 μM also reduced the [^3H]-Leucine incorporation and protein/DNA ratio by approximately 20 to 30% in control cells (Figure 1A). Therefore, 30 μM RV was used for further studies.

RV inhibits Ang II-induced oxidative stress

Ang II has been shown to stimulate NADPH oxidases and produce reactive oxygen species (ROS) resulting in increased oxidative stress (Seshiah et al. 2002). To investigate if Ang II-induced enhanced protein synthesis in VSMC is due to enhanced oxidative stress, the effect of DPI, an inhibitor of NADPH oxidase on protein synthesis was examined. As shown in Figure 2A, the protein synthesis in VSMC that was significantly augmented by Ang II treatment by about 70% was completely attenuated to control level by DPI pre-treatment. In addition, DPI also inhibited the protein synthesis in control cells by about 50%. To further examine if RV-inhibits Ang II-induced hypertrophy through its ability to attenuate the enhanced oxidative stress, the effect of RV on the Ang II-mediated production of superoxide anion (O_2^-) and NADPH oxidase activity was examined. As reported earlier (Li et al. 2007), Ang II enhanced the

production of O_2^- (Figure 2B) and NADPH oxidase activity (Figure 2C) in VSMCs by about 100% and 75% respectively and RV pre-treatment attenuated completely the enhanced levels of O_2^- and NADPH oxidase activity to control levels. In addition, RV treatment decreased the basal levels of O_2^- and NADPH oxidase activity by about 25% and 40% respectively in control cells.

To further investigate if the inhibition of enhanced activity of NADPH oxidase induced by Ang II by RV is attributed to its ability to decrease the expression of different subunits of NADPH oxidase, the effect of RV pre-treatment on Ang II-stimulated enhanced expression of Nox-1, gp91^{phox}, Nox-4, and p47^{phox} were examined in VSMC. As shown in Figure 3A-D, Ang II treatment significantly enhanced the levels of Nox-1, gp91^{phox}, Nox-4, and p47^{phox} subunits by about 80%, 40%, 60% and 130% respectively which were almost completely attenuated by RV pre-treatment. On the other hand, the expression of Nox-1, gp91^{phox}, Nox-4, and p47^{phox} subunits were not altered by RV treatment alone.

RV inhibits Ang II-induced c-Src activation

We previously showed the implication of c-Src activation in oxidative stress-induced enhanced protein synthesis in VSMCs from SHR (Atef and Anand-Srivastava 2014). To investigate the implication of c-Src in Ang II-induced VSMC hypertrophy, the effect of PP2, a specific inhibitor of c-Src on Ang II-induced protein synthesis in VSMC was examined. Results shown in Figure 4A indicate that the enhanced protein synthesis induced by Ang II was attenuated to control levels by PP2. In addition, PP2 also decreased the protein synthesis by about 65% in control cells. Furthermore, treatment of VSMCs with PP3, an inactive structural analog of PP2, did not have any significant effect on Ang II-enhanced protein synthesis (data not shown).

To further investigate if RV- induced attenuation of Ang II-stimulated protein synthesis is also attributed to its ability to inhibit the enhanced activation of c-Src, the effect of RV on Ang II-induced c-Src phosphorylation in VSMC was examined. Results shown in Figure 4B indicate that Ang II significantly augmented by about 70%, the phosphorylation of Tyr⁴¹⁸ on c-Src in VSMC as compared to control cells and RV pre-treatment completely attenuated the enhanced phosphorylation of c-Src to control level. On the other hand, the expression of total c-Src was not altered by these treatments.

RV inhibits Ang II-induced growth factor receptor activation

Ang II-stimulated transactivation of EGF-R was shown to play a crucial role in cardiovascular remodeling including cardiac hypertrophy (Peng et al. 2016). We have shown recently that augmented levels of endogenous Ang II through the transactivation of growth factor receptors EGF-R and PDGF-R contribute to VSMC hypertrophy in SHR (Atef and Anand-Srivastava 2014). Therefore, it was also important to examine the role of EGF-R and PDGF-R in Ang II-induced VSMC hypertrophy. To test these, the effects of AG1478, a specific inhibitor of EGF-R and AG1295, a specific inhibitor of PDGF-R on Ang II-induced enhanced protein synthesis were examined in VSMC. Figure 5A shows that Ang II-induced enhanced ($\approx 60\%$) protein synthesis was completely attenuated to control levels by AG1478 and AG1295. In addition, both these inhibitors also inhibited protein synthesis in control cells by about 40-50%.

To gain insight into whether RV attenuates Ang II-mediated hypertrophy via inhibition of transactivation of EGF-R and PDGF-R in VSMC, we examined the effect of RV pre-treatment on Ang II-induced phosphorylation of EGF-R and PDGF-R in VSMC. Results shown in Figure 5B and C indicate that Ang II enhanced the phosphorylation of EGF-R and PDGF-R by about 200% and 70% respectively which were almost completely attenuated to control levels by RV

pre-treatment. In addition, RV also decreased the phosphorylation of EGF-R and PDGF-R by about 35% and 65% respectively in control cells. On the other hand, the expressions of total EGF-R and PDGF-R was not altered by these treatments.

RV inhibits Ang II-induced ERK1/2 and AKT/PKB activation

Growth factor receptors (GFRs) have been shown to signal through MAPK and AKT pathways (Oda et al. 2005). Since RV inhibits Ang II-induced activation of growth factor receptors, it was of interest to examine the effect of RV pre-treatment on Ang II-stimulated ERK1/2 and AKT1/2 activation. As reported earlier (Haider et al. 2002; Zhang et al. 2012), Ang II significantly enhanced the phosphorylation of ERK1/2 (Figure 6A) and AKT1/2 (Figure 6B) by about 100% and 200% respectively and RV pre-treatment completely attenuated the Ang II-induced enhanced activation of ERK1/2 as well as of AKT1/2. In addition, RV treatment also decreased the basal phosphorylation level of ERK1/2 and not of AKT1/2 by about 40% in control cells.

Discussion

The present study provides insight into the molecular mechanisms through which RV attenuates Ang II-induced hypertrophy of VSMC. Although, the inhibition of Ang II-induced AKT/protein kinase B (PKB) and p70 S6 kinase phosphorylation by RV, and the subsequent attenuation of hypertrophy of rat aortic VSMC has been shown earlier (Haider et al. 2002), we show for the first time that RV attenuates Ang II-stimulated VSMC hypertrophy through the inhibition of c-Src and growth factor receptor activation, the upstream signaling molecules of AKT pathway.

Growth factor receptor transactivation by G protein coupled receptor (GPCR) including AT₁ receptor is well established (Daub et al. 1996; Gomez Sandoval et al. 2009; Itoh et al. 1993). In addition, the enhanced activation of growth factor receptors EGF-R and PDGF-R exhibited by VSMC from SHR was also shown to be attributed to the enhanced levels of endogenous Ang II (Atef and Anand-Srivastava 2016a; Li et al. 2010; Sandoval et al. 2011). Furthermore, the implication of EGF-R transactivation induced by endogenous Ang II has been shown to mediate VSMC hypertrophy in SHR (Atef and Anand-Srivastava 2014). Consistent with results reported by others (Beaucage and Moreau 2004; Bokemeyer et al. 2000; Eguchi et al. 1998; Gao et al. 2006), we also show that Ang II enhanced the phosphorylation (activation) of both EGF-R and PDGF-R in VSMC and the activation of both EGF-R and PDGF-R contributes to Ang II-induced VSMC hypertrophy because the inhibition of EGF-R and PDGF-R by inhibitors AG1478 and AG1295 attenuated the hypertrophy of VSMC. Furthermore, the fact that pre-treatment of VSMC with RV inhibited Ang II-induced activation of EGF-R and PDGF-R strongly suggests that RV inhibits Ang II-evoked VSMC hypertrophy through the inhibition of growth factor receptor activation. Our results are in contrast with the studies of Haider et al. (Haider et al.

2005) who did not observe the inhibitory effect of RV on Ang II-induced enhanced phosphorylation of EGF-R in VSMC. The observed discrepancy may be attributed to the different experimental conditions used (30 μ M RV vs 100 μ M RV).

Our results showing that Ang II augments the production of O_2^- , NADPH oxidase activity and expression of Nox-1, gp91^{phox}, Nox-4, and p47^{phox} in VSMC are consistent with the studies reported earlier (Lassegue et al. 2001; Li et al. 2010; Touyz et al. 2002; Wingler et al. 2001). A role of oxidative stress in VSMC hypertrophy in SHR has been reported (Atef and Anand-Srivastava 2014, 2016a, b). Furthermore, the fact that DPI attenuates Ang II-induced VSMC hypertrophy further suggests the contribution of oxidative stress in Ang II-induced VSMC hypertrophy. In addition, our results showing that RV inhibits the Ang II-induced enhanced production of O_2^- , NADPH oxidase activity and enhanced expression of different subunits of NADPH oxidase together with the fact that it inhibits VSMC hypertrophy suggests that RV-induced inhibition of Ang II-stimulated VSMC hypertrophy is attributed to its ability to inhibit oxidative stress. In this regard, the inhibition of vascular NADPH oxidase by RV has been shown (Orallo et al. 2002). In addition, RV has also been shown to markedly suppress the increased activity of NADPH oxidase, as well as the enhanced expressions of gp91^{phox}, p47^{phox}, and Nox-4 induced by high glucose in cardiomyocytes (Guo et al. 2015).

c-Src, a downstream molecule of oxidative stress, through the activation of growth factor receptors has been shown to contribute to VSMC hypertrophy in SHR (Atef and Anand-Srivastava 2016a). Our results showing that PP2, an inhibitor of c-Src attenuates Ang II-stimulated VSMC hypertrophy suggests the implication of c-Src in Ang II-induced hypertrophy of VSMC. In addition, the fact that RV inhibits Ang II-induced enhanced phosphorylation of c-Src strongly supports that RV attenuates Ang II-evoked VSMC hypertrophy through the

inhibition of c-Src activation. In this regard, RV has also been shown to inhibit c-Src tyrosine kinase in Hela cells (Yu et al. 2001).

RV has been reported to suppress Ang II-induced AKT/protein kinase B and subsequent hypertrophy in rat aortic VSMCs (Haider et al. 2002). We also report that RV inhibits the enhanced phosphorylation of ERK1/2 and AKT1/2 induced by Ang II in VSMC. Ang II-induced activation of MAPK/ERK as well as AKT/PKB has been shown to mediate VSMC hypertrophy (Eguchi et al. 1998). In addition, the role of these kinases in hypertrophy of VSMC from SHR has also been shown (Atef and Anand-Srivastava 2014; Servant et al. 1996). We earlier showed that enhanced levels of endogenous Ang II in VSMC from SHR increase oxidative stress which through c-Src and growth factor receptor activation enhance MAPK and AKT activities and result in VSMC hypertrophy (Atef and Anand-Srivastava 2016a). Taken together, it may be suggested that RV attenuates Ang II-induced VSMC hypertrophy through the inhibition of oxidative stress, c-Src, growth factor receptor activation and associated MAPK and AKT signaling pathways.

In conclusion, we provide evidence that RV-induced inhibition of c-Src and growth factor receptor activation may be the important signaling pathways through which it exhibits antihypertrophic effect. It may be suggested that RV could be used as a therapeutic agent in the treatment of vascular complications associated with hypertension and hypertrophy.

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Conflicts of interest

There is no conflict of interest associated with this work.

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Figure legends

Figure 1. Dose-dependent effects of RV on Ang II-induced enhanced [³H]-Leucine incorporation and protein/DNA ratio in aortic vascular smooth muscle cells (VSMCs).

Subconfluent VSMCs were pre-incubated in the absence or presence of different concentration (10 to 50 μ M) of RV for 1 h and subsequently stimulated with or without Ang II (10^{-6} M) for 24 h. After 24 h, [³H]-Leucine incorporation (Figure A), and protein/DNA ratio (Figure B) were determined as described in the Methods section. Data are expressed as % of the control group (CTL, taken as 100%). Values are the mean $\pm$ SE of three separate independent experiments performed in triplicate. * p < 0.01, ** p < 0.001, *** p < 0.0001 vs. the control (CTL) group, $^{\dagger\dagger}p$ < 0.001, $^{\dagger\dagger\dagger}p$ < 0.001 vs. Ang II group.

Figure 2. Effects of RV and DPI on Ang II-induced enhanced oxidative stress and protein synthesis in aortic vascular smooth muscle cells (VSMCs).

Subconfluent VSMCs were pre-incubated in the absence or presence of RV (30 μ M) or DPI (10 μ M) for 1 h and subsequently stimulated with or without Ang II (10^{-6} M) for 24 h. After 24 h, [³H]-Leucine incorporation (Figure 2A), superoxide anion (O_2^-) (Figure 2B), and NADPH oxidase activity (Figure 2C) were determined as described in the Methods section. Data are expressed as % of the control group (CTL, taken as 100%). Values are the mean $\pm$ SE of three separate and independent experiments performed in triplicate. * p < 0.01, ** p < 0.001, *** p < 0.0001 vs. control (CTL) group; $^{\dagger\dagger\dagger}p$ < 0.0001 vs. Ang II group.

Figure 3. Effects of RV on Ang II-induced enhanced expression of NADPH oxidase subunits in aortic vascular smooth muscle cells (VSMCs).

Subconfluent VSMCs were pre-incubated in the absence or presence of RV (30 μ M) for 1 h and subsequently stimulated with or without Ang II (10^{-6} M) for 24 h. After 24 h, Nox-1 (Figure 3A), gp91^{phox} (Figure 3B), Nox-4

(Figure 3C), and p47^{phox} (Figure 3D) protein expression levels were determined by Western blot as described in the Methods section. In each subfigure (3A-D), the upper panel is a Western blot image. Data are expressed as % of the control group (CTL, taken as 100%). Values are the mean $\pm$ SE of three separate and independent experiments performed in triplicate. * $p < 0.01$, *** $p < 0.0001$ vs. control (CTL) group; $^{\dagger}p < 0.01$, $^{\dagger\dagger}p < 0.001$, vs. Ang II group.

Figure 4. Effects of RV and PP2 on Ang II-induced enhanced phosphorylation of c-Src and protein synthesis in aortic vascular smooth muscle cells (VSMCs). Subconfluent VSMCs were pre-incubated in the absence or presence of RV (30 μ M) or PP2 (1 μ M) for 1 h and subsequently stimulated with or without Ang II (10^{-6} M) for 24 h. After 24 h, [³H]-Leucine incorporation (Figure 4A), and c-Src phosphorylation (Figure 4B) were determined as described in the Methods section. In Figure 4B, the upper panels are Western blot images. Data are expressed as % of the control group (CTL, taken as 100%). Values are the mean $\pm$ SE of three separate and independent experiments performed in triplicate. ** $p < 0.001$, *** $p < 0.0001$ vs. control (CTL) group; $^{\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger}p < 0.0001$ vs. Ang II group.

Figure 5. Effects of RV, AG1478, and AG1295 on Ang II-induced enhanced phosphorylation of EGF-R and PDGF-R and protein synthesis in aortic vascular smooth muscle cells (VSMCs). Subconfluent VSMCs were pre-incubated in the absence or presence of RV (30 μ M), AG1478 (10 μ M), and AG1295 (10 μ M) for 1 h and subsequently stimulated with or without Ang II (10^{-6} M) for 24 h. After 24 h, [³H]-Leucine incorporation (Figure 5A), EGF-R phosphorylation (Figure 5B), and PDGF-R phosphorylation (Figure 5C) were determined as described in the Methods section. In Figure 5B and Figure 5C, the upper panels are Western blot images. Data are expressed as % of the control group (CTL, taken as 100%). Values are the

mean $\pm$ SE of three separate and independent experiments performed in triplicate. $**p < 0.001$, $***p < 0.0001$ vs. control (CTL) group; $^{\dagger\dagger}p < 0.001$, $^{\dagger\dagger\dagger}p < 0.0001$ vs. Ang II group.

Figure 6. Effects of RV on Ang II-induced enhanced phosphorylation of ERK1/2 and AKT1/2 in aortic vascular smooth muscle cells (VSMCs). Subconfluent VSMCs were pre-incubated in the absence or presence of RV (30 μ M) for 1 h and subsequently stimulated with or without Ang II (10^{-6} M) for 24 h. After 24 h, ERK1/2 phosphorylation (Figure 6A), and AKT1/2 phosphorylation (Figure 6B) were determined as described in the Methods section. In Figure 6A and Figure 6B, the upper panels are Western blot images. Data are expressed as % of the control group (CTL, taken as 100%). Values are the mean $\pm$ SE of three separate and independent experiments performed in triplicate. $*p < 0.01$, $***p < 0.0001$ vs. control (CTL) group; $^{\dagger\dagger}p < 0.001$ vs. Ang II group.