Oxidative stress contributes to the enhanced expression of Gqα/PLCβ1 proteins and hypertrophy of VSMC from SHR: role of growth factor receptor transactivation

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Atef ME, Anand-Srivastava MB. Oxidative stress contributes to the enhanced expression of Gqα/PLCβ1 proteins and hypertrophy of VSMC from SHR: role of growth factor receptor transactivation. Am J Physiol Heart Circ Physiol 310: H608–H618, 2016. First published January 4, 2016; doi:10.1152/ajpheart.00659.2015 —We showed previously that vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHRs) exhibit overexpression of Gqα/PLCβ1 proteins, which contribute to increased protein synthesis through the activation of MAP kinase signaling. Because oxidative stress has been shown to be increased in hypertension, the present study was undertaken to examine the role of oxidative stress and underlying mechanisms in enhanced expression of Gqα/PLCβ1 proteins and VSMC hypertrophy. Protein expression was determined by Western blotting, whereas protein synthesis and cell volume, markers for VSMC hypertrophy, were determined by [3H]-leucine incorporation and three-dimensional confocal imaging, respectively. The increased expression of Gqα/PLCβ1 proteins, increased protein synthesis, and augmented cell volume exhibited by VSMCs from SHRs were significantly attenuated by antioxidants N-acetyl-cysteine (NAC), a scavenger of superoxide anion, DPI, an inhibitor of NAD(P)H oxidase. In addition, PP2, AG1024, AG1478, and AG1295, inhibitors of c-Src, insulin-like growth factor receptor (IGFR), epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor (PDGFR), respectively, also attenuated the enhanced expression of Gqα/PLCβ1 proteins and enhanced protein synthesis in VSMCs from SHRs toward control levels. Furthermore, the levels of IGF-1R and EGFR proteins and not of PDGFR were also enhanced in VSMCs from SHRs, which were attenuated significantly by NAC, DPI, and PP2. In addition, NAC, DPI, and PP2 also attenuated the enhanced phosphorylation of IGF-1R, PDGFR, EGFR, c-Src, and EKR1/2 in VSMCs from SHRs. These data suggest that enhanced oxidative stress in VSMCs from SHRs activates c-Src, which through the transactivation of growth factor receptors and MAPK signaling contributes to enhanced expression of Gqα/PLCβ1 proteins and resultant VSMC hypertrophy.

oxidative stress; Gqα/PLCβ1; VSMC; hypertrophy; SHR

NEW & NOTEWORTHY
The present study reports for the first time that enhanced oxidative stress exhibited by vascular smooth muscle cells from spontaneously hypertensive rats through the activation of c-Src and growth factor receptors increases the activity of MAP kinase and contributes to enhanced expression of Gqα and PLCβ1 proteins and resultant vascular smooth muscle cell hypertrophy.

HYPERTENSION IS ASSOCIATED with vascular remodeling characterized by enhanced media-to-lumen ratio in arteries (29) and is attributed to hyperproliferation and hypertrophy of vascular smooth muscle cells (VSMCs) (43). Several hormones including ANG II that increase blood pressure are also implicated in vascular remodeling by promoting VSMC proliferation and hypertrophy (4, 7, 10) through the activation of several signaling mechanisms including adenyl cyclase/cAMP, phosphatidylinositol 3-kinase, MAP kinase, and tyrosine kinase pathways (17, 19, 38). These signaling mechanisms are regulated by guanine nucleotide regulatory proteins (G proteins). The G proteins are heterotrimeric proteins composed of three subunits (α, β, and γ). Two G proteins, stimulatory (Gso) and inhibitory (Gio), regulate adenyl cyclase activity and cAMP levels, whereas the Gqα family of G proteins is subdivided into Gqα, G11α, G14α, and G16α. The activation of Gqα by a G protein-coupled receptor (GPCR) stimulates phospholipase C-β (PLC-β), which hydrolyzes inositol biphosphate (PIP2) and produces inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] (IP3) and diacylglycerol (DAG) (22) and activates PKC (8, 42).

The implication of Gqα and MAPK/phosphatidylinositol 3-kinase signaling has been shown in VSMC hypertrophy induced by vasoactive peptides in A10 VSMC (32). Gqα protein and associated signaling has also been shown to be involved in ANG II-induced VSMC hypertrophy (38). We have further shown that VSMCs from 16-wk-old and not from 12-wk-old SHRs exhibit enhanced expression of Gqα and PLCβ1 proteins, which contribute to VSMC hypertrophy through the activation of MAP kinase signaling pathway (4). The enhanced levels of Gqα and PLCβ1 proteins in VSMCs from SHRs were shown to be attributed to the enhanced levels of endogenous ANG II and endothelin-1 (ET-1) (4).

The generation of reactive oxygen species (ROS) that increases oxidative stress has been implicated in the pathogenesis of many vascular diseases, such as hypertension, diabetes, atherosclerosis, and vascular remodeling (6, 16, 23). The contribution of oxidative stress in hyperglycemia-induced enhanced expression of Gqα protein in VSMCs has been reported (14). VSMCs from SHRs have been shown to exhibit enhanced oxidative stress due to the overproduction of superoxide anion (O2−) and enhanced activity of NADPH oxidase as well as the enhanced expression of Nox1/Nox2/Nox4 and p47phox subunits of NADPH oxidase (19, 34). We previously showed the implication of enhanced oxidative stress in enhanced expression of Gqα proteins in VSMCs from SHRs (2). Taken together, it may be possible that the increased oxidative stress exhibited by VSMCs from SHRs may also be a contributing factor in enhanced expression of Gqα and PLCβ1 proteins and...
resultant hypertrophy. The present study is therefore undertaken to examine the role of oxidative stress in enhanced expression of Gqα and PLCβ1 proteins and hypertrophy in VSMCs from SHRs and to further explore the underlying mechanisms responsible for this response.

We showed that enhanced oxidative stress exhibited by VSMCs from SHRs activates c-Src, which through the transactivation of growth factor receptors and MAPK signaling contributes to enhanced expression of Gqα/PLCβ1 proteins and resultant enhanced protein synthesis.

Fig. 1. Effect of N-acetyl-cysteine (NAC) and DPI, an inhibitor of NAD(P)H oxidase, on protein synthesis and Gqα/PLCβ1 protein expression in vascular smooth muscle cells (VSMCs) from 16-wk-old spontaneously hypertensive rats (SHRs) and age-matched Wistar Kyoto (WKY) rats. VSMCs from 16-wk-old SHRs and age-matched WKY rats were incubated with NAC and DPI. The cell lysates were prepared and subjected to Western blotting using specific antibodies against Gqα and PLCβ1 (A and B, top) as described in MATERIALS AND METHODS. The β-actin was used as the loading control (CTL). The proteins were quantified by densitometric scanning (A and B, bottom), and the protein synthesis was determined by [3H]-leucine incorporation (C) as described in MATERIALS AND METHODS. The results are expressed as percentage of control, taken as 100%. Values are means ± SE of 4 separate experiments using different cell cultures. *P < 0.05, **P < 0.001 vs. WKY rats; #P < 0.05, ##P < 0.01 vs. SHRs.

We showed that enhanced oxidative stress exhibited by VSMCs from SHRs activates c-Src, which through the transactivation of growth factor receptors and MAPK signaling contributes to enhanced expression of Gqα/PLCβ1 proteins and resultant enhanced protein synthesis.

Fig. 2. Effect of NAC and DPI on cell volume of VSMCs from 16-wk-old SHRs and age-matched WKY rats. VSMCs from 16-wk-old SHRs and age-matched WKY rats were grown to 50% confluence in petri dishes. Cells were serum deprived for 24 h to induce cell quiescence and were incubated for 16 h in the absence or presence of NAC (in μM) and DPI (5 μM). The cells were then washed twice and fixed with 10% formalin for 1 h in 4°C and further incubated 45 min in the room temperature with whole cell stains reagent using Thermo Scientific Cellomics Whole Cell Stains (green). The volume of VSMCs was evaluated by 3-dimensional live cell microscopy imaging with inverse point scanning confocal microscope with 2 PMT channels: Objective Plan-Apochromat 63×/1.40 Oil differential interference contrast (DIC) and 40×/1.40 Oil DIC. Three-dimensional microscopy datasets interpretation was performed with the software Imaris (Bitplane). Values are means ± SE of 3 separate experiments using different cell cultures. **P < 0.01 vs. WKY rats; #P < 0.05 vs. SHRs.
MATERIALS AND METHODS

Materials. Epidermal growth factor receptor (EGFR) inhibitor AG1478, platelet-derived growth factor receptor (PDGFR) inhibitor AG1295, insulin-like growth factor receptor 1 (IGF-1R) inhibitor AG1028, free radicals scavenger N-acetyl-cysteine (NAC), c-Src inhibitor PP2, inactive analog for Src inhibitor PP3, and inhibitor of NADPH oxidase DPI were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Leucine, L-4,5,3H(N) was purchased from Perkin Elmer (Boston, MA). Monoclonal Gqα antibody (10), monoclonal PLC-β1 antibody (D-8), monoclonal (phospho)-ERK1/2 (phosphospecific-tyrosine204) antibody, polyclonal ERK1/2 antibody (C-14), monoclonal dynein IC1/2 antibody (74-1), and Western blotting reagents were from Calbiochem. Polyclonal (phospho)-PDGFR (phosphospecific-tyrosine 857) were from Calbiochem. Polyclonal EGFR, IGF-1R, (phospho)-c-Src (phosphospecific-tyrosine-419), PDGFR, and (phospho)-IGF-1R (phosphospecific-tyrosine1165/1166) antibodies were from St. Cruz Biotech. Antibodies against Gqα (12), and PLC-β1 were from Thermo Scientific Cello. (A5441) and all other chemicals used in these experiments were purchased from Sigma-Aldrich (St. Louis, MO). Thermo Scientific Cellomics Whole Cell Stains was from fisher scientific.

Animals, cell culture, and incubation. VSMCs from 16-wk-old SHRs and age-matched Wistar Kyoto (WKY) rats were cultured as described previously (3). These cells were plated in 75-cm² flasks and incubated at 37°C in 95% air-5% CO2 humidified atmosphere in DMEM (with glucose, L-glutamine, and sodium bicarbonate) containing 1% antibiotics (containing penicillin, streptomycin, and amphotericin B) and 10% heat-inactivated FBS. Cells were passaged upon reaching confluence with 0.5% trypsin and used between passages 2 and 8. Confluent cells were starved by incubation for 24 h. VSMCs were cell quiescent at 37°C to have cell quiescence. For the receptor antagonist and antioxidant studies, VSMCs from SHRs and WKY rats were incubated for 16 h in the absence or presence of NAC (10 mM), DPI (5 μM), AG1478 (5 μM), AG1295 (5 μM), AG1028 (5 μM), PP2 (1 μM), and PP3 (1 μM). After incubation, the cells were washed twice with ice-cold 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 μg/ml aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5 μg/ml leupeptin on ice. The cell lysates were centrifuged at 12,000 g for 15 min at 4°C, and the supernatants were used for Western blot analysis. Cell viability was checked by the trypan blue exclusion technique and indicated that >90–95% cells were viable. 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 (No. 99050). The investigation conforms to the Guide for the Care and Use of Laboratory.

Western blotting. The levels of protein expression and phosphorylation were determined by Western blotting as described previously (4). 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 (Gqα, c-Src, and ERK1/2) or a liquid transfer apparatus (Bio-Rad Laboratories) at 100 V for 1 h (PLCβ1, EGFR, IGF-1R, and PDGFR).

Membranes were blocked for 1 h at room temperature with 5% dry milk and incubated overnight with specific antibodies against different proteins: (10) against Gqα, (D-8) against PLCβ1, E-4 against (phospho)-ERK1/2 (phosphospecific-tyrosine204) antibody, C-14 against ERK1/2, phosphospecific Tyr1173 against p-EGFR, 1005 against EGFR, phosphospecific Tyr419 against pc-Src, and 74-1 against dynein.

Membranes were then washed three times with PBS before reaction with enhanced chemiluminescence (ECL). Quantitative analysis of the proteins was performed by densitometric scanning (A, H11021 cultures. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WKY rats; #P < 0.01 vs. SHRs.

Fig. 4. Effect of the knockdown of epidermal growth factor receptor (EGFR) on enhanced expression Gqα/PLCβ1 proteins and enhanced protein synthesis in VSMCs from 16-wk-old SHRs and age-matched WKY rats. Confluent VSMCs from 16-wk-old SHRs and age-matched WKY rats were incubated in the absence or presence of EGFR-small interfering RNA (siRNA) for 12 h. A scramble siRNA was used as a negative control. Cell lysates were prepared and subjected to Western blot analysis using specific antibody against EGFR (A), Gqα (B), and PLCβ1 (C). The β-actin and dynein were used as the loading control. The protein bands were quantified by densitometric scanning (A, B, and C, bottom), and protein synthesis was determined by [3H]-leucine incorporation (D) as described in MATERIALS AND METHODS. Results are expressed as a percentage of control taken as 100%. Values are means ± SE of 3 separate experiments using different cell cultures. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WKY rats; #P < 0.01 vs. SHRs.

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of NAC (10 mM), DPI (5 μM), AG1478 (5 μM), AG1295 (5 μM), AG1028 (5 μM), PP2 (1 μM), and PP3 (1 μM). [3H]-leucine (2μCi per well) was added at the same time as the inhibitors. For RNA interference studies, the cells were incubated in the absence or presence of siRNA against c-Src and EGFR. [3H]-leucine was added and further incubated for 24 h before the cells were harvested. The presence of siRNA against c-Src and EGFR. [3H]-leucine was added and further incubated for 24 h before the cells were harvested. The cells were rinsed twice with ice-cold 1× PBS and incubated with 5% TCA for 1 h at 4°C. After being washed twice with ice-cold 1× PBS, the cells were incubated with 0.4 N sodium hydroxide solution for 30 min at room temperature, and radioactivity was determined by liquid scintillation counter and adjusted by protein concentration.

Cell volume measurement. VSMCs from 16-wk-old SHRs and age-matched WKY rats were grown to 50% confluence in cell culture dish (35 × 10 mm). Cells were serum deprived for 24 h to induce cell quiescence and were incubated for 16 h in the absence or presence of NAC (10 μM) and DPI (5 μM). The cells were then washed twice and fixed with 10% formalin for 1 h in 4°C and further incubated for 45 min at room temperature with whole cell reagent using Thermo Scientific Cellomics Whole Cell Stains (green). The volume of VSMCs was evaluated by three-dimensional live cell microscopy imaging by using Zeiss LSM-T-PMT 700 (Zen 2012), Objective Plan-Apochromat 63x/1.40 Oil DIC, and 40×/1.40 Oil DIC. The three-dimensional microscopy datasets interpretation was performed with the software Imaris (Bitplane).

Statistical analysis. Results are expressed as means ± SE. Comparisons between groups were made with one-way ANOVA followed by Bonferroni’s post hoc test. A difference between groups was significant at $P < 0.05$.

RESULTS

Implication of oxidative stress in enhanced expression of Gqα/PLCβ1 proteins and enhanced protein synthesis. We have earlier shown that VSMC from SHR exhibit enhanced expression of Gqα and PLCβ1 proteins that contribute to VSMC hypertrophy (4). Because VSMCs from SHRs have been shown to exhibit enhanced oxidative stress due to the enhanced levels of superoxide anion (O$_2^-$) and NADPH oxidase activity (40), it was of interest to examine if the enhanced oxidative stress contributes to the enhanced expression of Gqα and PLCβ1 proteins and enhanced protein synthesis in VSMC from SHR. To test this, the effect of antioxidant DPI, an inhibitor of NADPH oxidase and N-acetyl-cysteine (NAC), free radical scavenger, on the expression of Gqα and PLCβ1 proteins and protein synthesis was examined. Results shown in Fig. 1 indicate that DPI (5 μM) and NAC (10 mM) decreased significantly the enhanced expression of Gqα by about 45% (Fig. 1A) and the enhanced expression of PLCβ1 protein by 30% and 40%, respectively (Fig. 1B). In addition, the enhanced protein synthesis was almost completely abol-
We also determined the effect of NAC and DPI on cell volume, another marker of VSMC hypertrophy and the results are shown in Fig. 2. VSMCs from SHRs exhibit enhanced cell volume compared with VSMCs from WKY rats, and this enhanced cell volume was significantly attenuated by about 40% and 45% by NAC and DPI, respectively.

Implication of growth factor receptors in enhanced expression of Gqα/PLCβ1 proteins and enhanced protein synthesis in VSMCs from 16-wk-old SHRs. Because the levels of growth factors have been shown to be enhanced in SHRs, it was of

Fig. 6. Effect of c-Src inhibitor (PP2) on enhanced expression of Gqα/PLCβ1 proteins and enhanced protein synthesis in VSMCs from 16-wk-old SHRs and age-matched WKY rats. VSMCs were incubated in the presence or absence of PP2 (5 μM) or PP3 (5 μM) for 16 h. Membranes were prepared and subjected to Western blotting using specific antibodies against Gqα/PLCβ1 (A and B, top) as described in MATERIALS AND METHODS. The proteins were quantified by densitometric scanning (A and B, bottom), and the protein synthesis was determined by [3H]-leucine incorporation (C) as described in MATERIALS AND METHODS. The results are expressed as a percentage of control taken as 100%. Values are means ± SE of 4 separate experiments using different cell cultures. *P < 0.05, ***P < 0.001 vs. WKY rats; ##P < 0.01, ###P < 0.001 vs. SHRs.

Fig. 7. Effect of the knockdown of c-Src on the expression of Gqα/PLCβ1 proteins and the expression of c-Src-siRNA for 12 h. A scramble siRNA was used as a negative control. Cell lysates were prepared and subjected to Western blot analysis using specific antibody against c-Src (A), Gqα (B), and PLCβ1 (C). β-Actin and dynein were used as the loading control. Protein synthesis was determined by [3H]-leucine incorporation (D). Results are expressed as a percentage of control taken as 100%; values are means ± SE of 3 separate experiments using different cell cultures. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WKY rats; #P < 0.05, ##P < 0.01 vs. SHRs.
interest to examine the contribution of growth factor receptor phosphorylation in enhanced protein synthesis and enhanced expression of Gqα and PLCβ1 proteins. To investigate this, the effects of AG1478, an inhibitor of EGFR; AG1295, an inhibitor of PDGFR; and AG1024, an inhibitor of IGF-1R were assessed on the expression of Gqα and PLCβ1 proteins in VSMCs from SHRs and WKY rats, and the results are shown in Fig. 3. The enhanced expression of Gqα proteins (Fig. 3A) in VSMCs from SHRs was completely restored to the control WKY levels by AG1478, AG1295, and AG1024, whereas these inhibitors attenuated the enhanced expression of PLCβ1 by about 70%, 75%, and 90%, respectively (Fig. 3B). On the other hand, these inhibitors did not have any significant effect on the phosphorylation of Gqα or PLCβ1 proteins in VSMC from WKY rats. In addition, AG1478, AG1295, and AG1028 also attenuated significantly the enhanced protein synthesis by about 45%, 60%, and 75%, respectively, in VSMCs from SHRs, whereas these inhibitors did not have any significant effect on the protein synthesis in VSMCs from WKY rats (Fig. 3C).

We also used siRNA of EGFR to confirm its role in enhanced expression of Gqα and PLCβ1 proteins and associated enhanced protein synthesis in VSMCs from SHRs, and the results are shown in Fig. 4. The expression of EGFR was significantly enhanced by about 125% in VSMCs from SHRs as compared with WKY rats. Treatment of VSMCs with EGFR-siRNA that attenuated the expression of EGFR in VSMCs from SHRs by about 85% (Fig. 4A) also resulted in a significant attenuation of enhanced expression of Gqα (Fig. 4B) and PLCβ1 (Fig. 4C) proteins by about 60% and 65%, respectively, and enhanced protein synthesis by about 45% in VSMCs from SHRs (Fig. 4D). In addition, EGFR-siRNA also attenuated the expression of Gqα by about 45% in VSMC from WKY rats (Fig. 4B).

**Implication of oxidative stress in enhanced expression and phosphorylation of growth factor receptors in VSMCs from SHRs.** Because growth factor receptors and enhanced oxidative stress contribute to the enhanced expression of Gqα and PLCβ1 proteins in VSMCs from SHRs, it was desirable to examine whether increased oxidative stress contributes to the enhanced expression and activation of growth factor receptor in VSMCs from SHRs. To test this, the effects of NAC and DPI were evaluated on the expression and phosphorylation of growth factor receptors in VSMCs from SHRs and WKY rats. As shown in Fig. 5, the increased phosphorylation of EGFR (Fig. 5A), IGF-1R (Fig. 5C), and PDGFR (Fig. 5E) was diminished by NAC and DPI toward WKY levels. Furthermore, the phosphorylation levels of IGF-1R and PDGFR were also significantly decreased by these antioxidants in VSMCs from WKY rats. In addition, the expression of EGFR (Fig. 5B) and IGF-1R (Fig. 5D) but not of PDGFR was increased in VSMCs from 16-wk-old SHRs as compared with WKY rats by about 200% and 150%, respectively; both these antioxidants NAC and DPI attenuated the enhanced expression of EGFR by about 65% and 80%, respectively, whereas the enhanced expression of IGF-1R was completely abolished by these antioxidants. Furthermore, the protein expression of IGF-1R in VSMCs from WKY rats was also significantly decreased (≈70%) by NAC.

**Implication of c-Src in enhanced expression of Gqα/PLCβ1 proteins and protein synthesis in VSMCs from SHRs.** Because c-Src has been reported to be activated by ROS in VSMCs (12), it was of interest to examine whether c-Src is also implicated in oxidative-stress-induced enhanced expression of Gqα/PLCβ1 proteins and enhanced protein synthesis in VSMCs from SHRs. To investigate this, we examined the effect of PP2, an inhibitor of c-Src on the enhanced expression of Gqα and PLCβ1 proteins and enhanced protein synthesis. Results shown in Fig. 6 indicate that treatment of cells with PP2 but not PP3, an inactive analog of PP2, almost completely attenuated the enhanced expression of Gqα protein (Fig. 6A), whereas the enhanced expression of PLCβ1 protein (Fig. 6B) and enhanced protein synthesis (Fig. 6C) were attenuated by about 75% and 80%, respectively, in VSMCs from SHRs. On the other hand, PP2 and PP3 did not have any effect on the expression of Gqα and PLCβ1 proteins in VSMCs from WKY rats; however, PP2 and not PP3 inhibited the protein synthesis in WKY rats by about 20%.

To further confirm the role of c-Src molecule in enhanced expression of Gqα and PLCβ1 proteins and enhanced protein synthesis in VSMCs from SHRs, we tested the effect of c-Src specific siRNA on the enhanced levels of Gqα and PLCβ1 protein and associated hypertrophy in VSMCs from SHRs and the results are shown in Fig. 7. Treatment of VSMCs with c-Src siRNA that attenuated significantly the expression of c-Src in both VSMCs from SHRs and WKY rats by about 35% and 50% (Fig. 7A), respectively, resulted in a significant attenuation of enhanced expression of Gqα (Fig. 7B) and PLCβ1 (Fig. 7C) proteins by about 75% and 90%, respectively, and enhanced protein synthesis by about 60% (Fig. 7D) in VSMCs from SHRs. In addition, the expression of Gqα protein and associated hypertrophy in VSMCs from SHRs and WKY rats were subjected to Western blotting using specific antibodies against (phospho)-c-Src (Fig. 8), as described in MATERIALS AND METHODS. The results are expressed as a percentage of control taken as 100%. Values are means ± SE of 3 separate experiments using different cell cultures.

**Fig. 8. Effect of NAC and DPI on enhanced c-Src phosphorylation in VSMCs from 16-wk-old SHRs and age-matched WKY rats.** VSMCs from 16-wk-old SHRs and age-matched WKY rats were incubated in the presence or absence of NAC (10 mM) or DPI (5 μM) for 16 h. Membranes were prepared and subjected to Western blotting using specific antibodies against (phospho)-c-Src and c-Src (top) as described in MATERIALS AND METHODS. The results are expressed as a percentage of control taken as 100%. Values are means ± SE of 3 separate experiments using different cell cultures.

***P < 0.001 vs. WKY rats; ###P < 0.001 vs. SHRs.
protein (Fig. 7B) and the protein synthesis (Fig. 7D) in VSMCs from WKY rats were also significantly decreased.

Implication of oxidative stress in enhanced phosphorylation of c-Src in VSMCs from SHRs. To determine whether the activation of growth factor receptors by oxidative stress occurs directly or through c-Src activation, the effect of NAC and DPI was assessed on the phosphorylation of c-Src at tyrosine 419 (Tyr419) in VSMCs from SHRs and WKY rats. As shown in Fig. 8, the increased phosphorylation of c-Src at Tyr419 (85%) was attenuated by NAC (10 mM) and DPI (5 μM) toward WKY levels, whereas it did not have any significant effect in VSMCs from WKY rats. Furthermore, the expression level of total c-Src molecule was not altered in VSMCs from SHRs as compared with WKY rats, and NAC and DPI did not affect the expression level of c-Src.

Implication of c-Src in enhanced expression and phosphorylation of growth factor receptors in VSMCs from SHRs. To determine whether c-Src is the upstream signaling molecule and contributes to the activation of growth factor receptors, the effect of c-Src inhibitor PP2 and PP3, an inactive analog of PP2, was assessed on the phosphorylation of growth factor receptors in VSMCs from SHRs and WKY rats. As illustrated in Fig. 9, PP2, but not PP3, attenuated the enhanced phosphorylation of EGFR (Fig. 9A), IGF-1R (Fig. 9C), and PDGFR (Fig. 9E) in VSMCs from SHRs to WKY control levels. In addition, the enhanced expression of EGFR (Fig. 9B) and IGF-1R (Fig. 9D) in VSMCs from SHRs was also attenuated by PP2 by about 40% and 90%, respectively; however, PP2 or PP3 did not have any effect on the phosphorylation and expression levels of growth factor receptors in VSMCs from WKY rats.

Implication of oxidative stress and c-Src in MAP kinase signaling in VSMCs from SHRs. We earlier showed the implication of MAP kinase in enhanced protein synthesis in VSMCs from SHRs (4) as well as in ANG II-induced enhanced protein synthesis in A10 VSMCs (32). Growth factor receptors signal through MAP kinase pathway (26, 36). Because oxidative stress through c-Src transactivate growth factor receptors, it was desirable to examine the implication of ROS and c-Src in the activation of MAPK signaling. To test this, we examined the effect of NAC, DPI, and PP2 on the ERK1/2 phosphorylation in VSMCs from SHRs and WKY rats. Results shown in Fig. 10A indicate that ERK1/2 phosphorylation was significantly enhanced in VSMCs from SHRs as compared with WKY rats. Treatment with DPI attenuates the ERK1/2 phosphorylation in both VSMCs from SHRs and WKY rats; however, the enhanced phosphorylation of ERK1/2 was completely abolished in VSMCs from SHRs, whereas about 50% inhibi-

![Graphs and images showing the phosphorylation levels of various proteins in VSMCs from SHRs and WKY rats, with statistical analysis indicating significant differences.](http://ajpheart.physiology.org/)
ion was observed in WKY rats. On the other hand, NAC attenuated only slightly the enhanced ERK1/2 phosphorylation in VSMCs from SHRs. In addition, PP2 but not PP3 attenuated completely the enhanced phosphorylation of ERK1/2 in VSMCs from SHRs, whereas about 35% inhibition was observed in WKY rats (Fig. 10B).

DISCUSSION

The implication of ROS in cardiomyocytes and VSMC hypertrophy has been shown by several studies (20, 39, 45). In addition, a role of oxidative stress in the enhanced expression of Gqα and PLCβ proteins in A10 VSMC exposed to high glucose and aortic VSMCs from STZ-diabetic rats has been shown (14). We earlier showed that the enhanced levels of endogenous ANG II and ET-1 in VSMCs from 16-wk-old SHRs contribute to the enhanced expression of Gqα and PLCβ1 proteins and resultant increased protein synthesis through the activation of MAP kinase signaling (4). In the present study, we report that enhanced oxidative stress contributes to the enhanced expression of Gqα and PLCβ1 proteins and VSMC hypertrophy in VSMCs from SHRs.

The fact that NAC and DPI that have been shown to restore the enhanced levels of ROS production (30, 44) also restored the enhanced expression of Gqα, PLCβ1 proteins, enhanced protein synthesis, and increased cell volume, markers of VSMC hypertrophy in SHRs suggests the implication of ROS in enhanced expression of Gqα and PLCβ proteins as well as VSMC hypertrophy in SHRs. In this regard, VSMCs from SHRs have been shown to exhibit enhanced oxidative stress due to the overproduction of \( \text{O}_2^- \), enhanced activity, and overexpression of Nox1/Nox2/Nox4 and \( \text{p47}^\text{phox} \) subunits of NADPH oxidase (19, 40). However, Nox5 that has been shown to be expressed in several tissues including human endocardial cells, human VSMCs, and human endothelial cells (1) does not appear to contribute to enhanced oxidative stress in VSMCs from SHRs because Nax5 is expressed only in primates and not in rodents (28). In addition, a role of NAD(P)H oxidase-derived ROS in potentiating Ang II-induced VSMC hypertrophy and in exacerbating large arteries remodeling has also been shown (45). In addition, NAC was shown to reduce pulmonary vascular remodeling and left ventricular hypertrophy induced by monocrotaline (11). Furthermore, NOX2 was also implicated in ANG II-induced cardiomyocyte hypertrophy (21).

The underlying mechanism by which ROS enhanced the expression of Gqα and PLCβ proteins in VSMCs from SHRs appears to involve the transactivation of growth factor receptors, because the enhanced phosphorylation of growth

![Fig. 10. Effect of reactive oxygen species (ROS) inhibition and c-Src inhibition on p42/44MAPK signaling in VSMCs from 16-wk-old SHR and age-matched WKY rats. VSMCs were incubated in the presence or absence of NAC (10 mM), DPI (5 µM), and PP2/PP3 (5 µM) for 16 h. Membranes were prepared and subjected to Western blotting using specific antibodies against pERK1/2 and ERK1/2 (A and B, top) as described in MATERIALS AND METHODS. The proteins were quantified by densitometric scanning (A and B, bottom). The results are expressed as a percentage of control taken as 100%. Values are means ± SE of 3 separate experiments using different cell cultures. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WKY rats; ###P < 0.001 vs. SHRs.](image)

![Fig. 11. The possible intracellular signaling mechanisms implicated in the oxidative stress-induced overexpression of Gqα/PLCβ1 proteins and resultant VSMC hypertrophy. ROS, through the activation of c-Src, transactivate receptor tyrosine kinases and MAPK signaling, which increases the protein expression of Gqα/PLC-β1 and results in VSMC hypertrophy.](image)
factor receptors exhibited by VSMCs from SHRs was attenuated by both NAC and DPI. In this regard, a role of oxidative stress in the transactivation of growth factor receptors has been reported (5, 37).

Our results showing that the inhibition of growth factor receptor tyrosine kinase that phosphorylate the receptors by specific inhibitors AG1478, AG1295, and AG1024 and the downregulation of EGFR by siRNA that attenuated significantly the enhanced expression of GqGα and PLCβ1 proteins and enhanced protein synthesis in VSMCs from SHRs suggest the implication of both the expression and activation of growth factor receptors in enhanced expression of GqGα and PLCβ1 proteins and enhanced protein synthesis in these cells. These results are consistent with our earlier studies showing a role of growth factor receptors in the enhanced expression of GqGα proteins and hyperproliferation of VSMCs from SHRs (17, 33). In addition, ET-1 was also shown to enhance the expression of GqGα proteins and proliferation through the transactivation of EGFR in A10 VSMC (18). Furthermore, a role of growth factor receptors in VSMC hypertrophy has also been shown by several studies (7, 9, 10). Bouallegue et al. (10) demonstrated the implication of IGF-1R in ET-1 and ANG II-induced VSMC hypertrophy. In addition, in vivo treatment with AG1478 was also shown to attenuate ANG II-induced vascular hypertrophy (7). Furthermore, Kagiyma et al. (24, 25) have shown the implication of EGFR in ANG II and hypertension (SHR)-induced left ventricular hypertrophy by using antisense oligodeoxynucleotide to EGFR (EGFR-AS).

We demonstrate for the first time an enhanced expression of EGFR and IGF-1R in VSMCs from SHRs in 16-wk-old SHRs, which may be linked to VSMC dedifferentiation in this animal model of essential hypertension. The enhanced levels of EGFR protein were shown in several diseases such as ovarian carcinomas and primary human brain tumor (27, 35, 46). ANG II that induces VSMC hypertrophy was also reported to increase the expression of IGF-1R (15, 41).

We also demonstrate that c-Src inhibitor PP2 and siRNA attenuated the enhanced expression of GqGα and PLCβ1 proteins and enhanced protein synthesis to WKY levels suggest the role of oxidative stress is the upstream signaling molecule of c-Src because of the fact that NAC and DPI attenuated the enhanced phosphorylation of c-Src in VSMCs from SHRs. In this regard, the contribution of oxidative stress in c-Src activation has also been shown previously in VSMCs from SHRs (17, 33). Furthermore, the activation of c-Src by H2O2 in VSMCs has also been reported (5, 9, 37). We also report that PP2, a c-Src inhibitor, attenuated the enhanced expression and phosphorylation of growth factor receptors as well as enhanced phosphorylation of ERK1/2 to control WKY levels and suggests that c-Src is upstream of growth factor receptor activation. Taken together, it may be suggested that oxidative stress, through the activation of c-Src, transactivate growth factor receptors and results in the activation of ERK1/2, which contributes to the enhanced expression of GqGα and PLCβ1 proteins and resultant VSMC hypertrophy. In this regard, a role of MAPK in enhanced expression of GqGα/PLCβ1 proteins and enhanced protein synthesis in VSMCs from SHRs has been shown (4). The role of oxidative stress in enhanced activation of ERK1/2 in VSMCs from SHRs has also been shown (31). In addition, H2O2 was shown to activate ERK1/2 in an EGF-R/c-Src-dependent fashion (37).

In summary, we provide the first direct evidence that the enhanced oxidative stress exhibited by VSMCs from SHRs activates c-Src, which through the activation of growth factor receptor and MAP kinase signaling increases the expression of GqGα and PLCβ1 proteins and results in hypertrophy (Fig. 11). From these studies, it may be suggested that GqGα and PLCβ1 proteins may be used as the potential targets for the development of new therapies for the treatment of cardiovascular diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.E.A. and M.B.A.-S. conceived and designed research; M.E.A. and M.B.A.-S. analyzed data; M.E.A. and M.B.A.-S. interpreted results of experiments; M.E.A. performed experiments; M.E.A. and M.B.A.-S. approved final version of manuscript; M.B.A.-S. edited and revised manuscript.

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