Enhanced expression of $G_{q}\alpha$ and PLC-$\beta$1 proteins contributes to vascular smooth muscle cell hypertrophy in SHR: role of endogenous angiotensin II and endothelin-1

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Atef ME, Anand-Srivastava MB. Enhanced expression of $G_{q}\alpha$ and PLC-$\beta$1 proteins contributes to vascular smooth muscle cell hypertrophy in SHR: role of endogenous angiotensin II and endothelin-1. Am J Physiol Cell Physiol 307: C97–C106, 2014. First published April 24, 2014; doi:10.1152/ajpcell.00337.2013.—Vascular $G_{q}\alpha$ signaling has been shown to contribute to cardiac hypertrophy. In addition, angiotensin II (ANG II) was shown to induce vascular smooth muscle cell (VSMC) hypertrophy through $G_{q}\alpha$ signaling; however, the studies on the role of $G_{q}\alpha$ and PLC-$\beta$1 proteins in VSMC hypertrophy in animal model are lacking. The present study was therefore undertaken to examine the role of $G_{q}\alpha$/PLC-$\beta$1 proteins and the signaling pathways in VSMC hypertrophy using spontaneously hypertensive rats (SHR). VSMC from 16-wk-old SHR and not from 12-wk-old SHR exhibited enhanced levels of $G_{q}\alpha$/PLC-$\beta$1 proteins compared with age-matched Wistar-Kyoto (WKY) rats as determined by Western blotting. However, protein synthesis as determined by $[^{3}H]$leucine incorporation was significantly enhanced in VSMC from both 12- and 16-wk-old SHR compared with VSMC from age-matched WKY rats. Furthermore, the knockdown of $G_{q11}$ and PLC-$\beta$1 in VSMC from 16-wk-old SHR by antisense and small interfering RNA resulted in attenuation of protein synthesis. In addition, the enhanced expression of $G_{q}\alpha$/PLC-$\beta$1 proteins, enhanced phosphorylation of ERK1/2, and enhanced protein synthesis in VSMC from SHR were attenuated by the ANG II AT1 receptor antagonists losartan and BQ123, respectively, but not by the ETB receptor antagonist BQ788. In addition, PD98059 decreased the enhanced expression of $G_{q}\alpha$/PLC-$\beta$1 protein and protein synthesis in VSMC from SHR. These results suggest that the enhanced levels of endogenous ANG II and ET-1 through the activation of AT1 and ETA receptors, respectively, and MAP kinase signaling, enhanced the expression of $G_{q}\alpha$/PLC-$\beta$1 proteins in VSMC from 16-wk-old SHR and result in VSMC hypertrophy.

$G_{q}\alpha$ protein; PLC-$\beta$1 protein; SHR; VSMC; hypertrophy

THE HETEROTRIMERIC GUANINE nucleotide regulatory protein (G protein), composed of three subunits ($\alpha$, $\beta$, and $\gamma$), plays a crucial role in the regulation of cardiovascular functions through the activation of several signal transduction systems including adenylyl cyclase and phosphatidyl inositide system (39). Based on $\alpha$-subunit sequence similarity, $G_{q}\alpha$ proteins are divided into four families $G_{q3}$, $G_{q9}$/ $G_{q11}$, and $G_{12}/G_{13}$. $G_{q3}$ and $G_{q9}$ proteins regulate the activity of adenylyl cyclase whereas the activation of $G_{q11}$ by a G protein-coupled receptor (GPCR) stimulates phospholipase C-$\beta$ (PLC-$\beta$), which hydrolyzes inositol bisphosphate (PIP2) and produces inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] (IP3) and diacylglycerol (DAG) (23) and activates protein kinase C (PKC) (7, 43).

Alterations in the levels of $G_{q}\alpha$ protein and associated signaling pathways appear to contribute to the impaired cellular functions in several pathological states including diabetes, hyperglycemia, and cardiac hypertrophy (1, 12, 17, 46). The $G_{q}\alpha$ protein and associated signaling pathway activated by several hormones such as angiotensin II (ANG II), endothelin-1 (ET-1), and phenylephrine has also been implicated in the development and progression of cardiac hypertrophy and heart failure (2, 8, 13, 20, 24, 38). Cardiac overexpression of $G_{q}\alpha$ in transgenic mice has also been reported to result in hypertrophy and induction of classic hypertrophy gene expression profile (41). In addition, the transgenic overexpression of a $G_{q}\alpha$ dominant negative minigene that resulted in the lack of hypertrophy response to transverse aortic constriction (TAC) (1) further supports the implication of $G_{q}\alpha$ in hypertrophy. In addition, the $G_{q}\alpha$ signaling components including IP3-Ca2+ and DAG-PKC have also been shown to play an important role in the development of cardiac hypertrophy in the stroke-prone spontaneously hypertensive rat (SHRSP) (27). Furthermore, the contribution of vascular $G_{q}\alpha$-coupled signaling in the development of cardiac hypertrophy has also been reported by using transgenic mice with vascular-specific $G_{q3}$ inhibitor expression (28). We have shown earlier the implication of $G_{q3}$ and MAPK/phosphatidylinositol 3-kinase signaling in vascular smooth muscle cell (VSMC) hypertrophy induced by vasoactive peptides in A10 VSMC (34). However, whether the expression of $G_{q}\alpha$ and PLC-$\beta$1 is enhanced and contributes to VSMC hypertrophy in rat models of hypertension such as SHR has not yet been examined.

The present study was therefore undertaken to investigate if VSMC from 12-wk-old SHR that do not express cardiac hypertrophy and 16-wk-old SHR that express cardiac hypertrophy exhibit enhanced expression of $G_{q11}$ and PLC-$\beta$1 proteins and if this upregulation is associated with VSMC hypertrophy and is attributed to the increased levels of endogenous vasoactive peptides. We provided the first evidence that the levels of $G_{q}\alpha$ as well as PLC-$\beta$1 proteins were enhanced in VSMC from 16-wk-old SHR exhibiting cardiac hypertrophy and not from 12-wk-old SHR and that the enhanced expression of $G_{q}\alpha$ and PLC-$\beta$1 may be attributed to the increased levels of endogenous ANG II and ET-1 which through the activation of AT1 and ETA receptors, respectively, and MAPK signaling contribute to VSMC hypertrophy.

MATERIALS AND METHODS

Materials. The ETA receptor antagonist BQ123, ETB receptor antagonist BQ788, AT1 receptor antagonist losartan, MAPK/ERK

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kinase inhibitor PD98059, and PLC- inhibitor U73122 were purchased from Sigma-Aldrich Chemical (St. Louis, MO). Curcumin was purchased from Calbiochem (San Diego, CA), and helenalin was from Sigma-Aldrich Chemical (St. Louis, MO). Monoclonal G_qα antibody (10), polyclonal G_11α antibody (D-17), monoclonal PLC-β1 antibody (D-8), monoclonal p-ERK1/2 (phosphospecific-tyrosine204) antibody, polyclonal ERK1/2 antibody (C-14), monoclonal dynen IIC1/2 antibody (74–1), Western blotting reagents, G_qα, PLC-β1 small interfering (si)RNA(r), siRNA transfection medium, and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals used in these experiments were purchased from Sigma-Aldrich Chemical. Leucine, l-[4,5–3H(N)] was purchased from Calbiochem (San Diego, CA), and helenalin was from Sigma-Aldrich Chemical (St. Louis, MO). G_qα antisense oligodeoxynucleotide (AS-ODN) sequence was obtained from Alpha DNA (Montreal, Canada). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA).

**Cell culture and incubation.** Aortic VSMC from 12- and 16-wk-old SHR and age-matched Wistar-Kyoto (WKY) rats were cultured as described previously (3). The purity of the cells was checked by immunofluorescence technique using α-actin as described previously (35). These cells were found to contain high levels of smooth muscle-specific actin. VSMC 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 amphotrecin B) and 10% heat-inactivated FBS. Cells were passed upon reaching confluence with 0.5% trypsin and utilized between passages 2 and 3. Confluent cells were starved by incubation for 24 h in DMEM without FBS at 37°C to have cell quiescence. For the receptor antagonist studies, VSMC from SHR and WKY were incubated for 16 h in the absence or presence of BQ123 (1 μM), BQ788 (1 μM), or losartan (10 μM). For RNA interference studies, cells were incubated with G_qα and PLC-β1 siRNA (for 12 h) and G_qα AS-ODN (for 24 h). 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. 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 with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996).

**Transfection of VSMC with siRNA.** For optimal siRNA transfection efficiency, the manufacturer’s protocol was followed. Briefly, VSMC were seeded in a 12-well plate or Petri dishes and cultured in antibiotic free normal growth medium supplemented with 10% FBS until the cells were 50–60% confluent. On the day of transfection, cells were washed and incubated with transfection medium (sc-36868) containing 60 pmol of either MOCK (scrambled, sc-37007) or rat-specific G_qα siRNA (sc-45998) or PLC-β1 siRNA (sc-270424) complexed with Lipofectamine 2000 reagent for 12 h. After transfection, the medium was replaced with normal DMEM (containing 10% FBS and 1% antibiotics) for an additional 24 h (80–90% confluence). To examine the effect of the PLC inhibitor U73122 on protein synthesis in PLC-β1 silenced VSMC, the cells were starved with FBS free DMEM supplemented with 1% antibiotics for 6 h and treated for an additional 24 h with the PLC inhibitor U73122.

**Transfection of VSMC with antisense oligonucleotides.** The sequence of G_qα oligonucleotides used in this study was as follows: G_qα AS-ODN (485): 5’-CTA-CAC-GGT-CCA-AGT-CAT-3’, and G_qα sense (G_qα S-ODN): 5’-ATG-ACT-TGG-ACC-GTG-TAG-3’. Confluent VSMC (60%) were transfected with antisense oligonucleotides (5 μM) complexed with Lipofectamine 2000 reagent and incubated for 24 h in the DMEM medium without antibiotics and FBS. The cells were washed three times with prewarmed 1× PBS and incubated

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**Fig. 1.** The expression of G_qα, G_11α, and PLC-β1 proteins and protein synthesis in aortic vascular smooth muscle cells (VSMC) from 12- and 16-wk-old spontaneously hypertensive rats (SHR) and age-matched Wistar-Kyoto (WKY) rats. Cell lysates were subjected to Western blot analysis using specific antibodies against G_qα (A, top), G_11α (B, top), and PLC-β1 (C, top) as described in MATERIALS AND METHODS. The protein bands were quantified by densitometric scanning (A, C, bottom). Values are means ± SE of 3 separate experiments. **P < 0.01. D: protein synthesis was determined by [3H]leucine incorporation to the cells as described in MATERIALS AND METHODS. Results are expressed as percentage of control, taken as 100%. Values are means ± SE of 5 separate experiments. *P < 0.05 vs. 12-wk-old WKY rats; ##P < 0.01 vs. 16-wk-old WKY.
for an additional 24 h with normal DMEM containing 10% FBS and 1% antibiotics.

Western blotting. The levels of Gqα, G1α, and PLC-β1 proteins and ERK1/2 phosphorylation were determined by Western blotting as described previously (31). After SDS-PAGE, separated proteins were electrophoretically transferred to a nitrocellulose membrane with a semidry transblot apparatus at 15 V for 45 min (Gqα, G1α, and p-ERK1/2) or a liquid transfer apparatus at 100 V for 1 h (PLC-β1). Membranes were blocked with 5% dry milk and incubated overnight with specific antibodies. Dynein was used as a loading control. The antibody-antigen complexes were detected by incubating the membranes with horseradish peroxidase-conjugated antibodies for 1 h at room temperature. The blots were washed three times with PBS before reaction with enhanced chemiluminescence Western blotting detection reagents from Santa Cruz Biotechnology. Quantitative analysis of the proteins was performed by densitometric scanning of the autoradiographs employing the Enhanced Laser Densitometer, LKB Ultrascan XL, No. 2000 and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia (Baie d’Urfe, Quebec, Canada).

**Determination of protein synthesis.** VSMC from 12- and 16-wk-old SHR and age-matched WKY were grown to confluence in 12-well culture plates. Protein synthesis (cell hypertrophy) was evaluated by [%H]leucine incorporation into cells as described previously (34). Confluent cells were serum deprived for 24 h to induce cell quiescence and were incubated in the absence or presence of losartan (10 μM), BQ123 (1 μM), BQ 788 (1 μM), PD98059 (10 μM and 20 μM), and U73122 (5 μM) for 24 h. [%H]leucine (2 μCi/well) was added at the same time as that of antagonists and inhibitors.

For RNA interference studies, the cells were incubated in the absence or presence of siRNA or AS-ODN against Gqα and PLC-β1. [%H]leucine (2 μCi) 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.

**Statistical analysis.** Results are expressed as means ± SE. Comparisons between groups were made with one-way ANOVA followed by Bonferroni’s post hoc test. Unpaired t-test was used to compare heart weight-to-body weight ratio (HW/BW) between SHR and WKY age-matched rats. A difference between groups was significant if P < 0.05.

**RESULTS**

The HW/BW was used as an index of cardiac hypertrophy. HW/BW (mg/g) in 12-wk-old SHR was not different from age-matched WKY rats (SHR: 4.02 ± 0.18 mg/g; WKY: 3.91 ± 0.29; n = 6); however, 16-wk-old SHR exhibit cardiac hypertrophy compared with age-matched WKY rats (SHR: 5.20 ± 0.13 mg/g, WKY: 3.98 ± 0.22 mg/g; P < 0.05; n = 6).

VSMC from SHR exhibit enhanced expression of Gqα/PLC-β1 proteins and enhanced protein synthesis. To investigate if VSMC from 16-wk-old SHR that exhibit cardiac hypertrophy also exhibit enhanced expression of Gqα/PLC-β proteins, we determined the levels of Gq11α as well as PLC-β, a downstream signaling molecule of Gqα in VSMC from 16-wk-old SHR. The results shown in Fig. 1A indicate that the expression of Gqα protein was significantly increased by ~60% in VSMC from 16-wk-old SHR, whereas the expression of G1α was not altered in VSMC from 12-wk-old SHR that do not exhibit cardiac hypertrophy compared with age-matched WKY rats. On the other hand, the expression of G1α protein, which shares 88% homology with Gqα, was not significantly different in both the groups (Fig. 1B). In addition, the expression of PLC-β1 protein was significantly (~85%) increased in VSMC from 16-wk-old and not in 12-wk-old SHR compared to age-matched WKY rats (SHR: 4.02 ± 0.18 mg/g; WKY: 3.91 ± 0.29 mg/g; n = 6). This was in line with our results shown in Fig. 1C. In addition, we also found that the expression of PLC-β1 protein was significantly (~85%) increased in VSMC from 16-wk-old and not in 12-wk-old SHR compared...
with VSMC from age-matched WKY rats (Fig. 1C). However, the expression of PLC-β2 was not altered (data not shown). The protein synthesis as determined by [%H]leucine incorporation was significantly enhanced in both VSMC from 12-wk-old (60%) and 16-wk-old (85%) SHR compared with VSMC from age-matched WKY rats (Fig. 1D).

To investigate if the enhanced levels of G_\alpha/PLC-β1 proteins were due to increased RNA synthesis, the effect of actinomycin D (AD), an inhibitor of RNA synthesis, was examined on the enhanced expression of G_\alpha and PLC-β1 proteins in VSMC from SHR. Results shown in Fig. 2, A and B, indicate that treatment of cells with AD attenuated the enhanced expression of G_\alpha and PLC-β1 proteins respectively to WKY control levels.

Role of AP-1 and NF-κB in enhanced expression of G_\alpha/PLC-β1. AP-1 and NF-κB are important transcription factors that play a crucial role in cardiovascular pathophysiology (16, 21). To investigate the implication of AP-1 and NF-κB in enhanced expression of G_\alpha/PLC-β1 proteins in VSMC from 16-wk-old SHR, we examined the effects of curcumin and helenalin, inhibitors of AP-1 and NF-κB, respectively (5, 22, 36), on the enhanced expression of G_\alpha and PLC-β1 in VSMC from 16-wk-old SHR. Results shown in Fig. 2, C and D, indicate that curcumin and helenalin attenuated significantly the enhanced expression of G_\alpha (Fig. 2C) by about 90 and 80%, respectively and the enhanced expression of PLC-β1 (Fig. 2D) by about 90 and 85%, respectively. These inhibitors had no effect on the expression of G_\alpha and PLC-β1 proteins in VSMC from WKY rats. These results suggest that the overexpression of G_α and PLC-β1 occurs via the activation of AP-1 and NF-κB.

Knockdown of G_\alpha decreases protein synthesis in VSMC from SHR. To investigate if the increased expression of G_\alpha contributes to the increased protein synthesis in VSMC from SHR, the effect of the knockdown of G_\alpha was examined on the protein synthesis in VSMC from SHR and WKY. Results shown in Fig. 3 indicate that the treatment of VSMC with 5 μM of G_\alpha AS-ODN for 24 h that completely attenuated the enhanced expression of G_\alpha protein (Fig. 3A) also attenuated the enhanced protein synthesis by ~80% in VSMC from SHR (Fig. 3B). On the other hand, a small but insignificant decrease in the expression of G_\alpha and protein synthesis was observed in VSMC from WKY rats by antisense treatment. In addition, silencing of G_\alpha by siRNA also inhibited the expression of G_\alpha by ~80% (Fig. 3C) and protein synthesis by ~80% in VSMC from SHR but not in VSMC from WKY (Fig. 3D).

Fig. 3. Effect of the knockdown of G_\alpha on protein synthesis in VSMC from 16-wk-old SHR and age-matched WKY rats. Confluent VSMC from 16-wk-old SHR and age-matched WKY rats were incubated in the absence or presence of G_\alpha antisense oligodeoxynucleotide (AS-ODN; 5 μM) for 24 h and G_\alpha-small interfering (si)RNA for 24 h. Cell lysates were prepared and subjected to Western blot analysis as described in MATERIALS AND METHODS (A and C, top). The protein bands were quantified by densitometric scanning (A and C, bottom) and protein synthesis was determined by [%H]leucine incorporation (B and D) as described in MATERIALS AND METHODS. Results are expressed as a percentage of control taken as 100%. Values are means ± SE of 4 separate experiments. **P < 0.01, ***P < 0.001 vs. WKY rats; #P < 0.05, ##P < 0.01 vs. SHR.
Implication of PLC and PLC-β1 protein in enhanced protein synthesis in VSMC from SHR. To investigate if PLC is also implicated in the enhanced protein synthesis in VSMC from SHR, the effect of U73122 (5 μM), an inhibitor of PLC, was examined on the enhanced protein synthesis in VSMC from SHR. Results shown in Fig. 4A indicate that the PLC inhibitor significantly attenuated the enhanced protein synthesis by ≈70% in VSMC from SHR. To further confirm the role of enhanced expression of PLC-β1 in enhanced protein synthesis in VSMC from SHR, we examined the effect of knockdown of PLC-β1 by PLC-β1siRNA on the enhanced protein synthesis in VSMC from SHR. Results shown in Fig. 4B and C, indicate that the treatment of VSMC from SHR with PLC-β1siRNA attenuated the enhanced expression of PLC-β1 protein by ≈70% (Fig. 4B) and enhanced protein synthesis by ≈60% (Fig. 4C). However, PLC inhibitor and PLC-β1siRNA did not have any significant effect on the expression of PLC-β1 protein and protein synthesis in control cells. In addition, the attenuation of protein synthesis by knockdown of PLC-β1 by PLC-β1siRNA was not further potentiated by the PLC inhibitor U73122 (Fig. 4D).

Implication of AT₁ and ETA receptor in enhanced expression of G,q/PLC-β1 proteins and enhanced protein synthesis in VSMC from SHR. Since VSMC from SHR have been shown to exhibit increased levels of vasoactive peptides such as ANG II and ET-1 (18, 37), it was of interest to investigate whether the enhanced levels of endogenous ET-1 and ANG II contribute to the increased expression of G,q/PLC-β1 proteins in VSMC from SHR. To test this, we examined the effects of the antagonists of the ETA and ETB receptors BQ123 and BQ788, respectively, as well as losartan, an AT₁ receptor antagonist, on the increased expression of G,q/PLC-β1 proteins in VSMC from SHR. The results shown in Fig. 5A indicate that the treatment of VSMC from SHR for 16 h with BQ123 significantly (≈60%) attenuated the enhanced expression of G,q, whereas it was completely inhibited by losartan. In addition, the increased expression of PLC-β1 protein in VSMC from SHR was also significantly inhibited by ≈60% and ≈70%, respectively, by 16-h treatment with BQ123 and losartan (Fig. 5B). On the other hand, BQ788 had no significant effect on the expression of G,q and PLC-β1 proteins. In addition, a shorter period of treatment (45 min) with antagonists did not have any significant effect on the expression of G,q and PLC-β1 proteins.****P < 0.001 vs. WKY rats; #P < 0.01 vs. SHR.

Fig. 4. A: effect of the inhibition of PLC on protein synthesis in VSMC from 16 wk-old SHR and age-matched WKY rats. Confluent VSMC from 16 wk-old SHR and age-matched WKY rats were incubated in the absence or presence of U73122 (5 μM) for 24 h. [3H]leucine incorporation was measured as described in MATERIALS AND METHODS. Results are expressed as percentage of WKY control (CTL; taken as 100%). Values are means ± SE of 3 separate experiments. **P < 0.01 vs. WKY rats; #P < 0.01 vs. SHR. B and C: effect of the knockdown of PLC-β1 on the expression of PLC-β1 protein and protein synthesis in VSMC from 16 wk-old SHR and age-matched WKY rats. Confluent VSMC from 16 wk-old SHR and age-matched WKY rats were incubated in the absence or presence of PLC-β1-siRNA for 12 h. A scramble siRNA was used as a negative control. Cell lysates were prepared and subjected to Western blot analysis or presence or absence of PLC-β1-siRNA for 12 h. A scramble siRNA was used as a negative control. Cell lysates were prepared and subjected to Western blot analysis using a specific antibody against PLC-β1 (B). Protein synthesis was determined by [3H]leucine incorporation (C). Results are expressed as a percentage of control taken as 100%. Values are means ± SE of 4 separate experiments. **P < 0.01, ***P < 0.001 vs. WKY rats; #P < 0.05, ##P < 0.01 vs. SHR. D: effect of PLC inhibitor on protein synthesis in PLC-β1-silenced VSMC from 16 wk-old SHR and age-matched WKY rats. Confluent PLC-β1-silenced VSMC from 16 wk-old SHR and age-matched WKY rats were further incubated in the absence or presence of U73122 (5 μM) for 24 h. Protein synthesis was determined by [3H]leucine incorporation as described in MATERIALS AND METHODS. Results are expressed as percentage of WKY control (CTL; taken as 100%). Values are means ± SE of 3 separate experiments. ***P < 0.001 vs. WKY rats; #P < 0.01 vs. SHR.
significant effect on the enhanced expression of $G_{q,\alpha}$ or PLC-$\beta$1 in VSMC from SHR. Furthermore, these antagonists did not have any significant effect on the levels of $G_{q,\alpha}$/PLC-$\beta$1 proteins in VSMC from WKY rats.

To further investigate if the enhanced protein synthesis in VSMC from SHR is also attributed to the enhanced levels of endogenous ANG II and ET-1, the effects of losartan, BQ123, and BQ788 were examined on protein synthesis in VSMC from 16-wk-old SHR and age-matched WKY. Confluent VSMC from 16-wk-old SHR and age-matched WKY rats were incubated in the absence or presence of BQ123 (1 M), BQ788 (1 M), and losartan (10 M) for 16 h. Cell lysates were prepared and subjected to Western blot analysis using specific antibodies against $G_{q,\alpha}$ (A) and PLC-$\beta$1 (B) and protein synthesis in VSMC (control and treated) was determined by $[^3H]$leucine incorporation (C). Results are expressed as 100% of WKY control. Values are means ± SE of 3 separate experiments. **$P < 0.01$, ***$P < 0.001$ vs. WKY rats; # $P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. SHR.
VSMC from SHR and WKY rats. Results shown in Fig. 5C indicate that the enhanced protein synthesis exhibited by VSMC from SHR was attenuated to control level by losartan and BQ123, whereas BQ788 did not have any significant effect on protein synthesis. On the other hand, these antagonists were ineffective in attenuating the protein synthesis in VSMC from WKY rats.

Role of MAPK on enhanced expression of Gαq/PLC-β1 proteins and enhanced protein synthesis in VSMC from SHR. Since MAPK pathway has been implicated in the regulation of protein synthesis (14), it was desirable to investigate the role of MAPK signaling in the enhanced protein synthesis in VSMC from SHR. To test this, the effect of PD98059 (MAPK/ERK kinase inhibitor) on the protein synthesis was examined in VSMC from SHR. Results shown in Fig. 7A indicate that the treatment of VSMC from SHR with PD98059 at 10 and 20 μM significantly inhibited the enhanced protein synthesis in VSMC from SHR by about 30 and 80%, respectively. On the other hand, PD98059 did not have any significant effect on the protein synthesis in VSMC from WKY rats.

We further examined the role of MAPK signaling in the enhanced expression of Gαq/PLC-β1 proteins in VSMC from SHR. To test this, the effect of PD98059 (MAPK/ERK kinase inhibitor) on the expression of Gqα proteins was examined in VSMC from SHR. Results shown in Fig. 7B indicate that the treatment of VSMC from SHR with PD98059 at 10 and 20 μM significantly inhibited the enhanced expression of Gqα proteins by about 60 and 80%, respectively. In addition, the increased expression of PLC-β1 protein was also significantly inhibited by PD98059 at 10 and 20 μM by ~70% and ~85%, respectively (Fig. 7C). On the other hand, PD98059 did not have any significant effect on the expression of Gqα and PLC-β1 proteins in VSMC from WKY rats.

To further investigate if the ERK1/2 phosphorylation is also augmented in VSMC from SHR and whether this augmented phosphorylation of ERK1/2 is attributed to the enhanced levels of endogenous ANG II and ET-1, the effects of losartan, BQ123, and BQ788 were examined on ERK1/2 phosphorylation in VSMC from SHR and the results are shown in Fig. 7D. The phosphorylation of ERK1/2 was significantly augmented by ~80% in VSMC from SHR compared with WKY and was restored to control levels by losartan and BQ123 but not by BQ788. However, these treatments did not have any significant effect on ERK1/2 phosphorylation in VSMC from WKY rats.

**Fig. 7. Effect of MAPK inhibitor on the enhanced expression of Gqα and PLC-β1 proteins and protein synthesis in VSMC from 16-wk-old SHR and age-matched WKY rats. Confluent VSMC from 16-wk-old SHR and age-matched WKY rats were incubated in the absence or presence of PD98059 (10 μM; 20 μM) for 16 h. Protein synthesis was determined by [3H]leucine incorporation (A). Cell lysates were prepared and subjected to Western blot analysis using specific antibodies against Gqα (B) and PLC-β1 (C). Results are expressed as 100% of WKY control. Values are means ± SE of 3 separate experiments. **P < 0.01, ###P < 0.001 vs. WKY rats; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. SHR. Effect of antagonists of ETa, ETb, and AT1 on ERK1/2 phosphorylation (p) in VSMC from 16-wk-old SHR and age-matched WKY. Confluent VSMC from 16-wk-old SHR and age-matched WKY rats were incubated in the absence or presence of BQ123 (1 μM), BQ788 (1 μM), and losartan (10 μM) for 16 h. Cell lysates were prepared and subjected to Western blot analysis using specific antibodies against p-ERK and total ERK (D) as described in MATERIALS AND METHODS. Results are expressed as a percentage of control taken as 100%. Values are means ± SE of 4 separate experiments. **P < 0.01 vs. WKY rats; ###P < 0.01 vs. SHR.
DISCUSSION

The role of Gqα signaling in ANG II-induced VSMC hypertrophy has been demonstrated (40). We have shown earlier the implication of Gqα and MAPK/phosphatidylinositol 3-kinase signaling in A10 VSMC hypertrophy induced by vasoactive peptides (34). However, in the present study using animal model of pressure overload hypertrophy, we report for the first time that aortic VSMC from 16-wk-old SHR that express cardiac hypertrophy exhibit enhanced expression of Gqα and PLC-β1 proteins. On the other hand, the expression of Gqα/PLC-β1 was not altered in VSMC from 12-wk-old SHR that do not express cardiac hypertrophy. These results suggest that overexpression of vascular Gqα and PLC-β1 proteins may play a role in cardiac hypertrophy in SHR. In this regard, Keys et al. (28) by using transgenic mice with vascular specific Gq inhibitor peptide (Gqβl) have shown the contribution of vascular Gqα-coupled signaling in the development of cardiac hypertrophy in response to chronic administration of GqPCR agonists.

We also show that the enhanced expression of Gqα protein in VSMC from 16-wk-old SHR contributes to increased protein synthesis (vascular hypertrophy) compared with WKY rats because the silencing of Gqα protein by specific siRNA or antisense attenuated the enhanced protein synthesis in VSMC from SHR. However, Gqα is not implicated in hyperproliferation (hyperplasia) of VSMC from SHR because knocking down of Gqα by antisense treatment did not attenuate the hyperproliferation of VSMC (4). Since knocking down of Gqα inhibited the protein synthesis by about 70–80%, it further suggests that other additional mechanisms may also contribute to the enhanced protein synthesis in 16-wk-old SHR. We earlier reported that VSMC from SHR exhibit enhanced expression of Gqα protein, which was implicated in protein synthesis (33). Taken together, it may be possible that the enhanced expression of Gqα protein may be the other mechanism contributing to the enhanced protein synthesis in 16-wk-old SHR. This notion is further supported by our study showing that pertussis toxin treatment that inactivates Gqα proteins attenuated the enhanced protein synthesis in VSMC from 16-wk-old SHR (unpublished data). On the other hand, the increased protein synthesis in VSMC from 12-wk-old SHR may not be attributed to Gqα protein because the expression of Gqα was not altered in VSMC from 12-wk-old SHR and may be due to the enhanced expression of Gqα proteins in 12-wk-old SHR (31).

A role of PLC in norepinephrine-induced cardiomyocyte hypertrophy has been shown (42). In the present study, we also report that U73122, an inhibitor of PLC, significantly attenuated the enhanced protein synthesis in VSMC from 16-wk-old SHR and provide the first evidence that PLC activation may be directly involved in VSMC hypertrophy. Furthermore, we report for the first time that VSMC from 16-wk-old SHR exhibit enhanced expression of PLC-β1 protein and the fact that silencing the PLC-β1 isofrom with specific siRNA resulted in a significant reduction of protein synthesis in VSMC from SHR suggest its implication in protein synthesis. Our results showing that the inhibition of protein synthesis by silencing PLC-β1 by PLC-β1siRNA was not further potentiated by the PLC inhibitor U73122 further suggests that PLC-β1 may be the major isoform implicated in protein synthesis. On the other hand, PLC-β1 does not appear to contribute to enhanced protein synthesis in VSMC from 12-wk-old SHR because the expression of PLC-β1 did not augment in VSMC from 12-wk-old SHR. To our knowledge, a direct role of PLC-β1 in VSMC hypertrophy has not been demonstrated previously. Thus, taken together, it may be suggested that PLC-β1 isofrom is a mediator of VSMC hypertrophy after the establishment of hypertension in 16-wk-old SHR. Our results are in accordance with the other studies showing the upregulation of PLC-β1 in viable and scar heart tissue after myocardial infarction (26) and in hearts from volume overload induced cardiac hypertrophy (11). In addition, Filtz et al. (15) have also reported the role of PLC-β1b in Gqβ-induced cardiomyocyte hypertrophy. The overexpression of Gqα and PLC-β1 proteins in VSMC from 16-wk-old SHR appears to be at the transcriptional level because AD, an inhibitor of RNA synthesis, inhibited the enhanced expression level of Gqα/PLC-β1 proteins in VSMC from SHR. Furthermore, we showed for the first time the implication of transcription factors AP1 and NF-κB in enhanced expression of Gqα and PLC-β1 proteins in SHR because curcumin and helenalin, selective inhibitors of AP1 and NF-κB, respectively, attenuated the enhanced expression of Gqα and PLC-β1 in VSMC from SHR.

We also examined the possible mechanism by which the expression of Gqα and PLC-β1 proteins is upregulated in VSMC from SHR. We showed that endogenous ANG II and ET-1, important factors of vascular hypertrophy (6, 10, 19, 32) and whose levels are augmented in VSMC from SHR (18, 37), contribute to the enhanced expression of Gqα and PLC-β1 proteins and hypertrophy of VSMC from 16-wk-old SHR. This is supported by our results showing that the AT1 receptor antagonist losartan and the ETA receptor antagonist BQ123 attenuated the enhanced expression of Gqα and PLC-β1 proteins and hypertrophy of VSMC from SHR to WKY levels and suggest that these effects may be mediated by an autocrine production of ANG II and ET-1. On the other hand, the ETB receptor may not be implicated in the enhanced expression of Gqα/PLC-β1 proteins and VSMC hypertrophy. The enhanced levels of endogenous ANG II and ET-1 in VSMC from 16-wk-old SHR through the activation of AT1 and ETA receptor, respectively, activate MAPK signaling, which increases the expression of Gqα and PLC-β1 proteins and results in enhanced protein synthesis.

Fig. 8. The possible intracellular signaling mechanisms implicated in the enhanced expression of Gqα/PLC-β1 proteins and VSMC hypertrophy. The enhanced levels of endogenous ANG II and ET-1 in VSMC from 16-wk-old SHR through the activation of AT1 and ETA receptor, respectively, activate MAPK signaling, which increases the expression of Gqα and PLC-β1 proteins and results in enhanced protein synthesis.
Gαq/PLC-β1 proteins and associated cell hypertrophy in VSMC from SHR induced by endogenous ET-1 because the blockade of ETB receptor with BQ788 did not significantly attenuate the enhanced expression of Gαq/PLC-β1 proteins and the enhanced protein synthesis in VSMC from SHR. Thus, taken together, it may be suggested that endogenous ET-1 and ANG II through the activation of ETA and AT1 receptor, respectively, induce the hypertrophic response by increasing the expression of Gαq and PLC-β1 proteins.

The role of MAPK signaling in hypertension and vascular hypertrophy has been well established (29, 44, 45). Furthermore, treatment of various cell types including VSMC with ANG II and ET-1 has been reported to activate ERK1/2 phosphorylation (9, 25, 44). In this study, we also report that the enhanced levels of endogenous ET-1 and ANG II augmented the phosphorylation of ERK1/2 in VSMC from SHR, because AT1 and ETA receptor blockade by losartan and BQ123, respectively, attenuated the enhanced phosphorylation of ERK1/2 in VSMC from SHR but not in VSMC from WKY. Our results are in agreement with other investigators, who have shown that MAPK activity is inhibited by losartan and BQ123 in rat aorta (30). The fact that the inhibitor of MAPK PD98059 attenuated the enhanced expression of Gαq and PLC-β1 proteins and VSMC hypertrophy further suggests a role of MAP kinase in the enhanced expression of Gαq/PLC-β1 proteins in VSMC from SHR.

In conclusion, we provide the first evidence showing that the expression of Gαq and PLC-β1 proteins is enhanced in VSMC from 16-wk-old SHR and not from 12-wk-old SHR and result in VSMC hypertrophy. The enhanced levels of endogenous ANG II and ET-1 in VSMC from SHR through the activation of AT1 and ETA receptor, respectively, and MAPK signaling contribute to the overexpression of Gαq and PLC-β1 proteins and resultant enhanced protein synthesis (Fig. 8). From these studies, it may be suggested that Gαq and PLC-β1 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. performed experiments; M.E.A. and M.B.A.-S. analyzed data; M.E.A. and M.B.A.-S. drafted manuscript; M.E.A. and M.B.A.-S. approved final version of manuscript; M.B.A.-S. conception and design of research; M.E.A. and M.B.A.-S. approved final version of manuscript; M.B.A.-S. interpreted results of experiments; M.B.A.-S. edited and revised manuscript.

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