Role of oxidative stress in angiotensin II-induced enhanced expression of G_\alpha_\delta proteins and adenyl cyclase signaling in A10 vascular smooth muscle cells

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Li Y, Lappas G, Anand-Srivastava MB. Role of oxidative stress in angiotensin II-induced enhanced expression of G_\alpha_\delta proteins and adenyl cyclase signaling in A10 vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 292: H1922–H1930, 2007. First published December 8, 2006; doi:10.1152/ajpheart.01166.2006.—We have previously reported that angiotensin II (ANG II) treatment of A10 vascular smooth muscle cells (VSMCs) increased inhibitory G proteins (G_\alpha_\delta proteins) expression and associated adenyl cyclase signaling which was attributed to the enhanced MAP kinase activity. Since ANG II has been shown to increase oxidative stress, we investigated the role of oxidative stress in ANG II-induced enhanced expression of G_\alpha_\delta proteins and examined the effects of antioxidants on ANG II-induced enhanced expression of G_\alpha_\delta proteins and associated adenyl cyclase signaling in A10 VSMCs. ANG II treatment of A10 VSMCs enhanced the production of O_2^- and the expression of Nox4 and P47H20k, different subunits of NADPH oxidase, which were attenuated toward control levels by diphenyleneiodonium (DPI). In addition, ANG II augmented the expression of G_\alpha_\delta-2 and G_\alpha_\delta-3 proteins in a concentration- and time-dependent manner; the maximal increase in the expression of G_\alpha_\delta was observed at 1 to 2 h and at 0.1–1.0 \mu M. The enhanced expression of G_\alpha_\delta-2 and G_\alpha_\delta-3 proteins was restored to control levels by antioxidants such as N-acetyl-L-cysteine, \alpha-tocopherol, DPI, and apocynin. In addition, ANG II also enhanced the ERK1/2 phosphorylation that was restored to control levels by DPI. Furthermore, the inhibition of forskolin-stimulated adenyl cyclase activity by low concentrations of 5\'-O-(3-triophosphate) (receptor-independent G_\alpha_\delta functions) and ANG II, des(Glu18,Ser19,Glu20,Leu21,Gly22)atrial natriuretic peptide4-23-NH2 (natriuretic peptide receptor-C agonist), and oxotremorine-mediated inhibitions of adenyl cyclase (receptor-dependent functions) that were augmented in ANG II-treated VSMCs was also restored to control levels by antioxidants. In addition, G_\alpha_\delta-mediated diminished stimulation of adenyl cyclase by stimulatory hormones in ANG II-treated cells was also restored to control levels by DPI. These results suggest that ANG II-induced enhanced levels of G_\alpha_\delta proteins and associated functions in VSMCs may be attributed to the ANG II-induced enhanced oxidative stress, which exerts its effects through mitogen-activated protein kinase signaling pathway.

G protein; mitogen-activated protein kinase; adenyl cyclase

ANGIOTENSIN II (ANG II), a vasoactive peptide and key component of the renin-angiotensin system, regulates a variety of physiological functions including blood pressure, cell proliferation, cell differentiation, and apoptosis (27, 31, 33). ANG II elicits its physiological effects by interacting with two distinct receptor subtypes designated as ANG II types 1 and 2 (AT_1 and AT_2, respectively) receptors (35). The presence of AT_1 receptor subtype has been shown in rat vascular tissues; however, a small proportion of AT_2 receptors are also present in rat aorta (9, 39). Most of the physiological effects of ANG II are mediated through the activation of AT_1 receptors that are coupled to several signaling pathways including adenyl cyclase/cAMP inhibition through G_\alpha_\delta proteins (4, 5, 28), mitogen-activated protein kinases (MAPKs) (10, 24, 38), and phosphatidylinositol turnover (15).

The adenyl cyclase/cAMP system is composed of three components: receptor, catalytic subunit, and guanine nucleotide regulatory proteins (G proteins). The G proteins act as transducers and, in the presence of guanine nucleotides, transmit the signal from the hormone-occupied receptor to the catalytic subunit. The agonist-mediated stimulation and inhibition of adenyl cyclase are mediated through stimulatory (G_\alpha_s) and inhibitory (G_\alpha_i) guanine nucleotide protein, respectively (29, 32), resulting in the increased or decreased formation of cAMP, respectively. G proteins are heterotrimeric, consisting of \alpha-, \beta-, and \gamma-subunits. The \alpha-subunits bind and hydrolyze GTP and confer specificity in receptor and effector interactions. Four different isoforms of G_\alpha_s have been identified, which appear to be products of an alternate splicing of a single gene (8, 19), whereas three different isoforms of G_\alpha_i (G_\alpha_i-1, G_\alpha_i-2, and G_\alpha_i-3) have been identified and are shown to be the products of three different genes (17, 18).

Alterations in the levels of G_\alpha_i proteins that result in the impaired cellular functions lead to various pathological states such as hypertension. We have recently shown an increased expression of G_\alpha_i proteins and G_\alpha_i protein mRNA in hearts and aortas from spontaneously hypertensive rats (SHR) and in hearts from experimental hypertensive rats including deoxycorticosterone acetate (DOCA)-salt hypertensive rats and 1 kidney 1 clip (1K1C) rats with established hypertension (3, 6, 12, 13, 34). In addition, we have further shown that vascular smooth muscle cells (VSMCs) from SHR also exhibit enhanced levels of G_\alpha_i protein compared with Wistar-Kyoto rats, which were restored to control levels by antioxidants (21), suggesting a role of oxidative stress in enhanced levels of G_\alpha_i proteins in SHR. The levels of various vasoactive peptides, including ANG II, that have been shown to be enhanced in hypertension may be responsible for the enhanced expression of G_\alpha_i proteins. This was supported by studies showing that treatment of hypertensive rats with captopril, an antihypertensive drug that acts by blocking the ANG II-converting enzyme, and losartan; an AT_1 receptor-antagonist decreased the blood pressure and the enhanced levels of G_\alpha_i proteins (16, 26).
Furthermore, ANG II has been shown to increase the levels of G\(_i\) proteins in A10 smooth muscle cells (25), which was attenuated by losartan and a mitogen-activated protein/extracellular signal-regulated (ERK) kinase inhibitor (1, 11). ANG II also increases oxidative stress by activating NADPH oxidase (14). Taken together, it may be possible that ANG II-induced enhanced expression of G\(_\alpha\) proteins in A10 cells is also attributed to the enhanced oxidative stress. To examine this possibility, the present studies were therefore undertaken to investigate the effect of antioxidant treatment on ANG II-induced enhanced expression of G\(_\alpha\) proteins and adenylyl cyclase signaling in A10 VSMCs.

**MATERIALS AND METHODS**

**Materials.** 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Aldrich Chemical (Milwaukee, WI). N-acetyl-t-cysteine (NAC), diphenyleneiodonium (DPI), apocynin, and a-tocopherol were from British Drug House (Toronto, Ontario, Canada). [\(\alpha\)-32P]ATP was purchased from Amersham (Oakville, Ontario, Canada). Antibodies L-5, C-10, and monoclonal phosphospecific-Tyr204 ERK1/2, p47phox (NADPH oxidase subunit antibody), and ERK1/2 were purchased from Amersham (Oakville, Ontario, Canada). Antibodies L-5, C-10, and monoclonal phosphospecific-tyrosine204 ERK1/2, p47phox antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Myokinase (EC 2.7.4.3) and all other chemicals used in the experiments were purchased from Sigma Chemical (St. Louis, MO).

**Cell culture and incubation.** A-10 cell line from embryonic thoracic aorta of rat was obtained from American Type Culture Collection (Rockville, MD). The cells were plated in 75-cm\(^2\) flasks and incubated at 37°C in a 95% air-5% CO\(_2\)-humidified atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) (with glucose, l-glutamine, and sodium bicarbonate) containing antibiotics and 10% heat-inactivated fetal bovine serum (FBS) as described previously (21, 25, 26). Confluent cell cultures were starved by incubation for 3 h in DMEM without FBS at 37°C. These cells were then incubated with ANG II (10\(^{-7}\) M) for different time periods at 37°C. To examine the effect of antioxidants on ANG II-induced responses, the cells were pretreated with or without antioxidants for 24 h and then challenged with ANG II (10\(^{-7}\) M) for 1 h. After incubation, cells were washed twice with ice-cold homogenization buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA). The cells were scraped into ice-cold homogenization buffer using a rubber policeman and collected by centrifugation at 4°C for 10 min at 600 g. The cells were then homogenized in a Dounce homogenizer (10 strokes), and the homogenate was used for adenylyl cyclase assay and immunoblotting.

**Adenylyl cyclase activity determination.** Adenylyl cyclase activity was determined by measuring [\(\beta\)-32P]cAMP formation from [\(\alpha\)-32P]ATP, as previously described (3, 6). Briefly, the assay medium contained 50 mM glycyglycine (pH 7.5), 0.5 mM Mg\(_2\)ATP, [\(\alpha\)-32P]ATP (1.5 \times 10\(^6\) counts/min), 5 mM MgCl\(_2\) (in excess of the ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 1 mM IBMX, 0.1 mM EGTA, 10 \(\mu\)M guanosine 5’-O-(3-thiotriphosphate) (GTP\(_\gamma\)S), and an ATP-regenerating system (consisting of 2 mM phosphocreatine, 0.1 mg/ml creatine kinase, and 0.1 mg/ml myokinase) in a final volume of 200 \(\mu\)l. Incubations were initiated by the addition of the membrane preparations (20–30 \(\mu\)g) to the reaction mixture, which had been thermally equilibrated for 2 min at 37°C. The reactions were conducted in triplicate for 10 min at 37°C and were terminated by the addition of 0.6 ml of 120 mM zinc acetate. CAMP was purified by coprecipitation of other nucleotides with ZnCO\(_3\), an addition of 0.5 ml of 144 mM Na\(_2\)CO\(_3\), and subsequent chromatography by the double-column system, as described by Salomon et al. (30). Under the assay conditions used, adenylyl cyclase activity was linear with respect to protein concentrations and time of incubation. Protein was determined essentially as described by Lowry et al. (22) with bovine serum albumin as standard.

**Immunoblotting.** Immunoblotting of G proteins, Nox4, p47phox (NADPH oxidase subunits), and ERK1/2 was performed by using specific antibodies as described previously (3, 21). After SDS-PAGE, the separated proteins were electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) with a semidyry transblot apparatus (Bio-Rad, Mississauga, Ontario, Canada) at 15 V for 45 min. After transfer, the membranes were washed twice in phosphate-buffered saline (PBS) and were incubated in PBS containing 5% dehydrated milk at room temperature for 2 h. The blots were then incubated with respective primary antibodies: L-5 for G\(_\alpha\)-2, C-10 for G\(_\alpha\)-3, monoclonal phosphospecific-Tyr204 ERK1/2 antibody for p-ERK1/2, N-15 for Nox4, and C-20 for p47phox antibodies in PBS containing 3% dehydrated milk and 0.1% Tween 20 at room temperature for 2 h. The antibody-antigen complexes were detected by second antibody, and protein bands were visualized by enhanced-chemiluminescence Western-blotting detection reagents from Amer sham as described previously (3, 21). Quantitative analysis of specific bands was performed by densitometric scanning of the autoradiographs with an enhanced laser densitometer (LKB Ultrascan XL, Pharmacia, Dorval, Quebec, Canada) and quantified by using gel-scan XL evaluation software (version 2.1) from Pharmacia.

**Superoxide anion measurements.** Basal superoxide anion production was measured by using the lucigenin-enhanced chemiluminescence with low concentration (5 \(\mu\)M) of lucigenin as described previously (21). The cells after treatment with ANG II (10\(^{-6}\) M) or DPI (10 \(\mu\)M) alone or in combination 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 Sciences, St. Laurent, Quebec, Canada) for 5 min. The average luminescence value was estimated, the background value subtracted, and the result was divided by the total weight of proteins in each sample.

**Statistical analysis.** Results are expressed as means \(\pm\) SE. Comparisons between groups were made with analysis of variance in conjunction with Newman-Keuls tests. Results were considered significant at a value of \(P < 0.05\).

**RESULTS**

**Effect of ANG II on G\(_\alpha\) protein expression in A10 VSMCs.** We have previously reported that exposure of A10 VSMCs to ANG II for 24 h increased the expression of G\(_\alpha\) proteins; however, it was of interest to examine whether the short-term treatment of VSMCs with ANG II also resulted in the enhanced levels of G\(_\alpha\) proteins. Figure 1 shows the temporal relationship between ANG II treatment and the levels of G\(_\alpha\)-2 and G\(_\alpha\)-3 proteins. ANG II treatment (10\(^{-7}\) M) of the cells increased the expression of both G\(_\alpha\)-2 (Fig. 1A) and G\(_\alpha\)-3 (Fig. 1B) proteins in a time-dependent manner. The levels of G\(_\alpha\)-2 and G\(_\alpha\)-3 started increasing as early as 30 min and peaked off at about 125–135% of control at 1 to 2 h and remained elevated up to 24 h as determined by densitometric scanning. On the other hand, the levels of G\(_\alpha\) were not affected by such treatment (data not shown).

Figure 2 shows the effect of various concentrations of ANG II on the expression of G\(_\alpha\)-2 (Fig. 2A) and G\(_\alpha\)-3 (Fig. 2B) proteins in A10 VSMCs. ANG II treatment for 1 h increased the levels of G\(_\alpha\)-2 and G\(_\alpha\)-3 in a concentration-dependent manner. The maximal increase of about 170% in G\(_\alpha\)-2 and G\(_\alpha\)-3 protein was observed at 10\(^{-5}\) and 10\(^{-7}\) M, respectively.

**Effect of ANG II on superoxide anion production in A10 VSMCs.** ANG II has been shown to increase oxidative stress by activating NADPH oxidase (14). A role of ANG II in enhanced production of superoxide anion in VSMCs from SHR has been
reported (21). To investigate whether ANG II treatment of A10 VSMCs also resulted in enhanced production of superoxide anion, the levels of superoxide anion (O$_{2}^-$) were measured by lucigenin in A10 VSMCs. The results shown in Fig. 3 show that treatment of cells with ANG II for 24 h increased the production of O$_{2}^-$ by sixfold, which was significantly restored toward control levels by DPI, an inhibitor of NADPH oxidase.

Effect of ANG II on the expression of NADPH oxidase subunits. To investigate whether ANG II-induced enhanced O$_{2}^-$ production is attributed to its ability to increase the expression of different subunits of NADPH oxidase, we determined the effect of ANG II on the levels of Nox4 and p47phox in A10 VSMCs. The results shown in Fig. 4 indicate that ANG II (10$^{-7}$ M) increased the levels of Nox4 (Fig. 4A) and p47phox (Fig. 4B) by about 75%, which were restored toward control levels by DPI.

Effect of antioxidants on ANG II-induced increased expression of G$\alpha$ proteins. Since ANG II increases reactive oxygen species (ROS) production, it was of interest to investigate whether ANG II-induced increased ROS production in these cells contributes to the increased expression of G$\alpha$ proteins. To test this, we examined the effect of various antioxidants on ANG II-induced increased expression of G$\alpha$ proteins, and the results are shown in Fig. 5. Treatment of cells with ANG II (10$^{-7}$ M) for 1 h increased the levels of G$\alpha$-2 and G$\alpha$-3 by about 50%, which were restored to control levels by apocynin (Fig. 5A); NADPH oxidase inhibitor, NAC (Fig. 5B); scavenger of O$_{2}^-$, $\alpha$-tocopherol (Fig. 5C); and DPI (Fig. 5D), NADPH...
oxidative stress inhibitor, suggesting the implication of ROS in ANG II-induced enhanced expression of Giα proteins in A10 VSMCs.

Implication of ROS in ANG II-induced enhanced ERK1/2 phosphorylation. We have previously shown that ANG II-induced enhanced expression of Giα proteins was inhibited by mitogen extracellular signal-regulated kinase inhibitor PD-98059 in A10 VSMCs (11), suggesting the implication of MAPK signaling in ANG II-induced enhanced expression of Giα proteins. To investigate whether ANG II could also increase the ERK1/2 phosphorylation in these cells and whether it is attributed to the increased oxidative stress, we examined the effect of DPI on ANG II-induced ERK1/2 phosphorylation in A10 cells. The results shown in Fig. 6 indicate that ANG II (10−7 M) increased the ERK1/2 phosphorylation by about 50%, which was completely abolished by DPI treatment.

Effect of antioxidants on receptor-independent function of Giα. Since antioxidants restored the ANG II-induced enhanced levels of Giα-2 and Giα-3 proteins toward control levels, it was of interest to examine whether the restoration of the enhanced levels of Giα proteins by antioxidants is also reflected in the restoration of increased Go functions. To investigate this, the effect of DPI on receptor-independent and -dependent functions was examined in ANG II-treated cells. For the receptor-independent functions of Go, the effect of DPI was investigated on the inhibitory effect of GTPyS (10−12–10−8 M) on forskolin (FSK)-stimulated adenylyl cyclase activity in control and ANG II-treated A10 cells. The results shown in Fig. 7 indicate that the enhanced inhibition of FSK-stimulated adenylyl cyclase activity by different concentrations of GTPyS in ANG II-treated cells which was attributed to the enhanced levels of Giα proteins compared with untreated control cells was reversed to control levels by DPI.

Effect of antioxidants on receptor-dependent functions. To investigate the effect of antioxidant on the receptor-dependent functions of Giα proteins, the effect of DPI was examined on the hormonal inhibitions of adenylyl cyclase activity in control and ANG II-treated A10 cells. For this, the effect of DPI on ANG II, des(Glu18,Ser19,Glu20,Leu21,Gly22)atrial natriuretic peptide4-23-NH2 (C-ANP4-23), and oxotremorine that inhibit adenylyl cyclase through Giα proteins (2, 4, 5, 9, 23) was examined on adenylyl cyclase activity in control and ANG II-treated cells. Figure 8 shows that ANG II-, C-ANP4-23-, and oxotremorine-mediated inhibitions of adenylyl cyclase which were significantly augmented in ANG II-treated cells were restored to control levels by DPI treatment. Figure 9 shows the effect of DPI on concentration-dependent inhibition of adenylyl cyclase by C-ANP4-23. C-ANP4-23 inhibited adenylyl cyclase activity in a concentration-dependent manner, with an apparent Ki of about 0.5 nM. The maximal inhibition observed was about 25%. However, as reported earlier (25), the inhibition was greater in ANG II-treated cells compared with untreated control cells. For example, C-ANP4-23 at 10−6 M inhibited adenylyl cyclase by about 25% in control cells and about 45% in ANG II-treated cells. DPI, on the other hand, completely abolished the C-ANP4-23-mediated enhanced inhibition of adenylyl cyclase activity in ANG II-treated cells.

Effect of antioxidants on Giα-mediated stimulation of adenylyl cyclase activity. The interaction between Giα and Giα has been well established. We have previously shown that treatment of A10 VSMCs with ANG II for 24 h attenuated the Giα-mediated functions (25). Therefore, it was of interest to

Fig. 3. Effect of diphenyleneiodonium (DPI) on superoxide anion (O2•−) production in A10 VSMCs. A10 VSMCs were pretreated without (CTL) or with 10−6 M DPI for 24 h and challenged with 10−6 M ANG II for 1 h, and O2•− production was determined as described in MATERIALS AND METHODS. Values are means ± SE of 3 separate experiments. ***P < 0.001 vs. CTL.

Fig. 4. Effect of DPI on the expression of Nox4 and p47phox protein expression in control and ANG II-treated A10 VSMCs. A10 VSMCs were pretreated without (CTL) or with 10−6 M DPI for 24 h and challenged with 10−6 M ANG II for 1 h. Membrane proteins (30 μg) were separated and transferred to nitrocellulose, which was then immunoblotted using specific antibodies against Nox4 (N-15, A) and p47phox (C-20, B) as described in MATERIALS AND METHODS. The β-actin was used to assess the loading of the protein. Values are means ± SE of 3 separate experiments. ***P < 0.001 vs. CTL; ##P < 0.01 vs. ANG II-treated groups.
examine whether DPI could also reverse the ANG II-mediated $G_\alpha$ effects. For this, the effect DPI on concentration-dependent stimulation of adenylyl cyclase by GTP$_\gamma$S was investigated in ANG II-treated cells, and the results are shown in Fig. 10. GTP$_\gamma$S stimulated adenylyl cyclase activity in a concentration-dependent manner in control and ANG II-treated A10 VSMCs; however, as reported earlier (25), the extent of stimulation was significantly decreased in ANG II-treated VSMCs compared with untreated control cells. For example, at $10^{-4}$ M, GTP$_\gamma$S augmented the adenylyl cyclase activity by fourfold in control cells compared with twofold in ANG II-treated A10 cells, and the treatment of ANG II-treated cells with 10 $\mu$M DPI restored the decreased stimulation of adenylyl cyclase to control levels.

Effect of antioxidants on hormonal stimulation of adenylyl cyclase activity. To investigate whether antioxidant could also modulate ANG II-induced decreased stimulation of adenylyl cyclase by stimulatory hormones (25), the effect of DPI on
isoproterenol and glucagon-stimulated adenylyl cyclase activity was examined in ANG II-treated A10 cells. As shown in Fig. 1, both isoproterenol and glucagon stimulated adenylyl cyclase activity to various degrees in A10 VSMCs; however, as reported earlier (25), the extent of stimulation was significantly decreased by about 30% and 40%, respectively, in ANG II-treated cells compared with control cells, and this decrease was restored to control levels by DPI treatment. In addition, the decreased stimulation of adenylyl cyclase by sodium fluoride (−30%) and FSK (−60%) that stimulate the enzyme activity by a receptor-independent mechanism in ANG II-treated cells was also restored to control levels by DPI treatment.

DISCUSSION

We have previously reported that SHR and 1K1C hypertensive rats, which have high levels of ANG II, exhibited enhanced expression of Giα-2 and Giα-3 protein mRNA in the

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Fig. 6. Effect of DPI on ANG II-induced ERK1/2 phosphorylation (p-ERK1/2) in A10 VSMCs. A10 VSMCs were pretreated without (CTL) or with 10−6 M DPI for 24 h and challenged with 10−7 M ANG II for 1 h. Membrane proteins (30 µg) were separated and transferred to nitrocellulose, which was then immunoblotted with specific antibodies against phospho-specific-Tyr204-ERK1/2 (E-4) or ERK1/2 (C-14) as described in MATERIALS AND METHODS. ERK1/2 (C-14) was used to assess the loading of the protein. Data presented as means ± SE of 3 separated experiment. ***P < 0.001 vs. CTL.

Fig. 7. Effect of DPI on 5′-O-(3-triotriphosphate) (GTPγS)-mediated inhibition of forskolin (FSK)-stimulated adenylyl cyclase activity in control and ANG II-treated A10 VSMCs. A10 VSMCs were pretreated without (CTL) or with 10−6 M DPI for 24 h and challenged with 10−7 M ANG II for 1 h. Membranes were prepared as described in MATERIALS AND METHODS. The adenylyl cyclase activity in these membranes was determined in the presence of various concentrations of GTPγS (10−12–10−7 M). Values are means ± SE of 3 separate experiments. *P < 0.05 and **P < 0.01 vs. CTL; §§P < 0.01 vs. ANG II-treated group.

Fig. 8. Effect of DPI on hormonal inhibition of adenylyl cyclase activity in CTL and ANG II-treated A10 VSMCs. A10 VSMCs were pretreated without (CTL) or with 10−6 M DPI for 24 h and challenged with 10−7 M ANG II for 1 h. Membranes were prepared as described in MATERIALS AND METHODS. The adenylyl cyclase activity in these membranes was determined in the presence 10−5 M ANG II, 10−7 M des(Glu18,Ser19,Glu20,Leu21,Gly22)atrial natriuretic peptide4-23-NH2 (C-ANP4-23), or 5 µM oxotremorine (Oxo). Values are means ± SE of 3 separate experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. CTL.

Fig. 9. Effect of DPI on C-ANP4-23-mediated inhibition of adenylyl cyclase activity in CTL and ANG II-treated A10 VSMCs. A10 VSMCs were pretreated without (CTL) or with 10−6 M DPI for 24 h and challenged with 10−7 M ANG II for 1 h. Membranes were prepared as described in MATERIALS AND METHODS, and adenylyl cyclase activity in these membranes was determined in the presence 10−5 M C-ANP4-23. Values are means ± SE of 3 separate experiments. **P < 0.01 vs. CTL; §§§P < 0.001 vs. ANG II-treated group.
Although a role of oxidative stress in ANG II-mediated cell signaling has been well established (37), evidence for a direct role of oxidative stress in ANG II-mediated increased expression of Gα protein and associated adenylyl cyclase is lacking. Our results showing that antioxidants such as apocynin, α-tocopherol, NAC, and DPI restored ANG II-evoked enhanced expression of Gα2 and Gα3 to control levels suggest the implication of oxidative stress in ANG II-induced enhanced levels of Gα proteins in smooth muscle cells. In support of this are our earlier findings showing that enhanced oxidative stress caused by enhanced levels of ANG II contributes to the enhanced expression of Gα proteins in SHR (21). We have also shown that antioxidants that result in the restoration of ANG II-induced enhanced expression of Gα proteins to control levels also restored to the control levels the enhanced inhibition of adenylyl cyclase by ANG II, C-ANP4-23, and oxotremorine as well as GTPγS-mediated enhanced inhibition of FSK-stimulated adenylyl cyclase activity, the receptor-dependent and -independent functions of Gα proteins, respectively.

In addition, ANG II-induced diminished stimulation of adenylyl cyclase by GTPγS and stimulatory hormones such as isoproterenol and glucagon were also restored to control levels by DPI. This may be attributed to the Gα proteins and not to Gα proteins, because ANG II was unable to alter the levels of Gα proteins in these cells. This is further substantiated by our studies showing that restoration of the enhanced levels of Gα proteins to control levels by antioxidant treatments also restored the GTPγS-mediated diminished stimulation of adenylyl cyclase to control levels. In addition, ANG II-evoked enhanced levels of Gα proteins may also be responsible for the diminished stimulation of adenylyl cyclase by glucagon, isoproterenol, FSK, and sodium fluoride in ANG II-treated cells, because the restoration of the ANG II-induced enhanced levels of Gα proteins to control levels was also able to restore the ability of isoproterenol, glucagon, sodium fluoride, and FSK to stimulate adenylyl cyclase activity toward control levels. Our
results are consistent with our studies performed in SHR, showing that the restoration of enhanced levels of Go proteins by antioxidants also resulted in the restoration of enhanced Gi functions and diminished Gt-mediated functions to control levels (21). In addition, the restoration of enhanced levels of Go proteins toward control levels by captopril (13, 26) or losartan has also been reported to restore the enhanced Go functions to control levels (1, 14, 16).

ANG II has been shown to activate various signaling pathways including MAPK (24, 28, 38). We have shown previously the implication of MAPK signaling in ANG II-induced enhanced expression of Go proteins in A10 VSMCs (11). We also reported that the enhanced expression of ERK1/2 phosphorylation in VSMCs from SHR was attenuated to control levels by antioxidants, suggesting a role of oxidative stress in enhanced ERK1/2 phosphorylation (21). Thus it can be suggested that oxidative stress by increasing MAPK signaling may be responsible for the enhanced expression of Go proteins. This notion is supported by our findings showing that ANG II-induced increased ERK1/2 phosphorylation is also attenuated by DPI in A10 VSMCs. Taken together, it may be suggested that ANG II-induced enhanced oxidative stress caused by O2\textsuperscript{−} production may be responsible for the activation of MAPK signaling which then results in the enhanced expression of Go proteins in VSMCs.

In conclusion, we have provided the first evidence that ANG II-induced increased oxidative stress through increased MAPK activity may be responsible for the enhanced expression of Go proteins in A10 VSMCs. The increased expression of Go proteins that results in decreased levels of cAMP and thereby increased vascular resistance may be one of the contributing factors in the pathogenesis of hypertension. Thus it may be suggested that antioxidant treatment by decreasing oxidative stress, and thereby MAPK activity, results in the attenuation of ANG II-induced enhanced levels of Go proteins and increased levels of cAMP, which by decreasing vascular resistance may attenuate the development of high blood pressure.

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