Exendin-4 alleviates angiotensin II-induced senescence in vascular smooth muscle cells by inhibiting Rac1 activation via a cAMP/PKA-dependent pathway

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Zhao L, Li AQ, Zhou TF, Zhang MQ, Qin XM. Exendin-4 alleviates angiotensin II-induced senescence in vascular smooth muscle cells by inhibiting Rac1 activation via a cAMP/PKA-dependent pathway. Am J Physiol Cell Physiol 2014;307:C1130–C1141. First published November 5, 2014; doi:10.1152/ajpcell.00151.2014.—Vascular aging has been implicated in the progression of diabetes and age-related cardiovascular disorders. Glucagon-like peptide-1 (GLP-1) is an incretin hormone capable of cytoprotective actions in addition to its glucose-lowering effect. The present study was undertaken to examine whether Exendin-4, a specific ligand for the GLP-1 receptor, could prevent angiotensin (ANG) II-induced premature senescence in vascular smooth muscle cells (VSMCs) and to determine the underlying mechanism involved. Senescence-associated β-galactosidase (SA β-gal) assay showed that ANG II induced premature senescence of VSMCs. Pretreatment with Exendin-4 significantly attenuated ANG II-induced generation of H2O2 and the subsequent VSMC senescence. These effects were, however, reversed in the presence of exendin fragment 9–39, a GLP-1 receptor antagonist, or PKI14-22. Moreover, a marked increase in the levels of p53 and p21 induced by ANG II was blunted by the treatment with Exendin-4. Nevertheless, Exendin-4 failed to decrease ANG II-induced expression of NAD(P)H oxidase 1 (Nox1), NAD(P)H oxidase 4 (Nox4), p22phox, or p47phox in VSMCs. Mechanistically, Exendin-4 blocked ANG II-induced Rac1 activation through the cAMP/PKA signaling cascade. Specifically, NSC23766, a Rac1 inhibitor, abrogated the suppressive effects of Exendin-4 on ANG II-induced premature senescence and H2O2 generation, respectively. Thus Exendin-4 confers resistance to ANG II-induced superoxide anion generation from NAD(P)H oxidase and the resultant VSMC senescence by inhibiting Rac1 activation via a cAMP/PKA-dependent pathway. These findings demonstrate that GLP-1 as well as its analogs (GLP-1-related reagents) may hold therapeutic potential in the treatment of diabetes with cardiovascular disease.

Exendin-4; angiotensin II; senescence; NAD(P)H oxidase

VASCULAR AGING IS A MAJOR risk factor for many chronic conditions including diabetes and cardiovascular diseases (27, 39). Cellular senescence is a permanent nonreplicating state characterized by growth arrest, increased oxidative stress, telomere and mitochondrial dysfunction (26), and increased expression of senescence-associated β-galactosidase (SA β-gal), p53, p21, and p16 (12). In cultured cells, critical DNA damage triggered by a variety of chemical agents (26) and stresses including oxidative stress and activation of oncogenes (8, 48) induces rapid “stress-induced premature senescence” (SIPS) (52). In addition to SIPS, vascular cells may also undergo replicative senescence via accelerated attrition of telomeres (19, 36, 40). Senescent vascular cells are not benign and may undergo a phenotypic shift to a more inflammatory and atherogenic phenotype. Increased expression of proinflammatory and prothrombogenic molecules has been observed in vascular cells of aged arteries (26, 41). With aging, vasodilation is impaired owing to decreased endothelial production of vasodilators such as nitric oxide (NO) and prostacyclin and to reduced responsiveness of vascular smooth muscle cells (VSMCs) to these vasodilators (6). VSMC senescence in atherosclerotic plaques is a characteristic feature of atherosclerosis and is associated with increased levels of reactive oxygen species (ROS) (26). Prevention of vascular aging may be important for effective therapy for age-related vascular changes in atherosclerotic degeneration.

ROS increases intracellular (DNA) damage and ultimately can elicit the onset of apoptosis or the induction of cellular senescence. The arterial wall is remodeled by the joint effects of numerous protein alterations, in particular, ANG II signaling molecules, with advancing age. ANG II has been demonstrated to stimulate the intracellular accumulation of ROS via NAD(P)H oxidases (Noxs) and to promote VSMC senescence (19, 26); therefore, vascular Noxs may participate in cellular senescence. Nox is a multicomponent enzyme that comprises membranebound subunits, p22phox and gp91phox (Nox2 or its homologues Nox1, 3, 4, and 5), and cytosolic subunits, p47phox, p67phox, and the small guanosine triphosphate (GTP)-binding protein Rac1, which play a role in activating Nox (48). Nox isoforms are differentially regulated by ANG II. In VSMCs, ANG II activates Nox1 and Nox 4, whereas in endothelial cells and cardiomyocytes, Nox2 appears to be the predominant isofrom that responds to ANG II (42, 48).

Glucagon-like peptide-1 (GLP-1) is a hormone secreted from L cells of the small intestine and stimulates glucose-dependent insulin response (34). GLP-1 acts through the GLP-1 receptor (GLP-1R), a G-coupled protein receptor, which is abundantly present in the islet cells, kidney, nervous system, heart, vascular smooth muscle cells, and endothelial cells (4). Activation of the GLP-1R can mainly trigger the generation of the second messenger cAMP (cAMP) followed by activation of protein kinase A (PKA). However, native GLP-1 has a short half-life of minutes before getting degraded rapidly by dipeptidyl peptidase-4 (DPP-4) (13). Therefore, more stable GLP-1 analogs, such as exenatide, and DPP-4 inhibitors, such as vildagliptin, have been developed and have become part of the management of patients with type 2 diabetes mellitus (10). Exendin-4, a GLP-1R agonist that was first isolated from salivary secretions of a South American lizard, is
resistant to DPP-4 cleavage and therefore has long-acting efficacy suitable for the treatment of type 2 diabetes (59).

Considerable evidence demonstrates that GLP-1R agonists could potentially provide cardiovascprotective benefits and enhance cardiovascular function, encouraging further scientific and clinical exploration, such as the modulation of blood pressure (24), myocardial infarction (32, 47, 57), and myocardial ischemia/reperfusion injury (5). Exendin-4 suppresses high glucose-induced cardiomyocyte apoptosis via inhibition of endoplasmic reticulum stress and activation of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase a (SERCA2a) (58). Moreover, a study of diabetic patients found that infusion of GLP-1 was associated with improved endothelial function (43). Recent publications describe that Exendin-4 attenuates neointimal hyperplasia after vascular injury (16, 20). Another GLP-1 analog liraglutide is capable of suppressing macrophage foam cell formation and atherosclerosis (51). Vascular cell senescence may play a pivotal role in the pathogenesis of atherosclerosis (39). Accordingly, a question was raised as to whether sustained GLP-1R activation may also prove beneficial in the process of VSMC senescence. Here, our present study revealed for the first time that Exendin-4 could attenuate ANG II-stimulated NAD(P)H oxidase-derived O\(_2^-\) generation and resultant in premature senescence in VSMCs via the cAMP/PKA-dependent pathway. Importantly, Exendin-4 inhibition of Rac1 activation, which is in turn alleviated ANG II-induced oxidative stress, may contribute to the Exendin-4 suppression of VSMC senescence.

**MATERIALS AND METHODS**

*Chemicals, reagents, and antibodies.* Exendin-4, exendin fragment (9–39), angiotensin II, dibutyryl cAMP, forskolin, N-acetyl-l-cysteine (NAC), and the SA β-gal staining kit were all obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against p53, p21, Nox1, Nox4, and Rac1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing p22phox, p47phox, and Rac1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p53, p21, Nox1, Nox2, Nox4, and Rac1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies recognizing p22phox, p47phox, and Rac1 were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell culture.** Rat aortic VSMCs were obtained by terminal harvest of aortas from male Sprague-Dawley rats (150–200 g) using anesthesia under a protocol approved by the Peking University Institutional Animal Care and Use Committee. A 2-cm segment of artery cleaned of fat and adventitia was harvested in 1 mg/ml collagenase for 3 h at room temperature. The artery was then cut into small sections and fixed to a culture flask for explantation in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 95% air-5% CO\(_2\). Medium was changed every 2–3 days, and VSMCs at passages 3–8 were used for all experiments. At subconfluence, the culture medium was replaced with DMEM containing 0.5% FBS for 12 h to render the cells quiescent. ANG II (100 nmol/l) with or without Exendin-4 was administered to VSMCs for 1 or 3 days by changing the medium every day to new medium containing freshly prepared ANG II and Exendin-4.

SA β-gal assay. Senescent cells were detected using a SA β-gal staining kit according to the manufacturer’s instructions (Sigma-Aldrich). In brief, cells seeded in 12-well plates were preincubated for 30 min with indicated reagents or vehicle before addition of Exendin-4 for 12 h, followed by treatment with ANG II for 3 days by changing the medium every day to new medium containing freshly prepared ANG II and Exendin-4. After being washed twice with ice-cold PBS, the cells were incubated with staining solution (4.2 mM citric acid, 12.5 mM sodium phosphate, 158 mM sodium chloride, 0.21 mM magnesium chloride, 2.21 mg/ml potassium ferrocyanide, and 1 mg/ml X-Gal, pH 6.0) for 6 h at 37°C. Stained cells were visualized using an Olympus CK40-SL light microscope (Tokyo, Japan). The person who was quantifying the SA β-gal-positive cells was blinded to the experimental conditions.

**Cell cycle analysis.** The cell cycle analysis was performed as described previously (45). At 80% confluence, VSMCs were synchronized by serum starvation in 1% FBS-containing DMEM for 12 h and treated with 10 nM Exendin-4 for 12 h followed by stimulation with ANG II (100 nM) for 1 or 3 days. Adherent cells were harvested and fixed in 70% ethanol. Staining for DNA content was performed with 50 μg/ml propidium iodide (Sigma) and 100 μg/ml RNase A for 30 min. The cell cycle distribution was analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

**Amplex red assay for ROS production.** Extracellular hydrogen peroxide (H\(_2\)O\(_2\)) was measured using the Amplex Red Hydrogen Peroxide Assay Kit according to the manufacturer’s protocol (Molecular Probes, Invitrogen). Twelve-well plates were used to cultivate and treat cells. After being washed with freshly prepared warm Krebs-Ringer-phosphate-glucose buffer (KRP: 145 mM NaCl, 5.7 mM Na\(_2\)PO\(_4\), 4.86 mM KCl, 0.54 mM CaCl\(_2\)-H\(_2\)O, 1.22 mM MgSO\(_4\)-7H\(_2\)O, and 5.5 mM glucose, pH 7.35), cells were incubated with 300 μM KRGP containing horseradish peroxide (2 U/ml) and Amplex red (50 μM; Invitrogen) and incubated for 30 min at 37°C in the CO\(_2\) incubator. The supernatant was transferred to 96-well plates, and fluorescence was then measured with a fluorescence microplate reader ( Molecular Devices) using excitation at 530 nm and fluorescence detection at 590 nm. The obtained fluorescence intensities of the sample were normalized to control. Background fluorescence, determined for a no-H\(_2\)O\(_2\) control reaction, has been subtracted from each value.

**Measurement of cAMP level.** To delineate signaling molecules that might contribute to the antisenescent effects of Exendin-4 in VSMCs, intracellular cyclic AMP (cAMP) concentration ([cAMP]) was measured using a competitive ELISA, according to the manufacturer’s instructions (Enzo Life Sciences). VSMCs were plated in 24-well plates at a density of 1 x 10⁴ cells per well and cultured overnight. After 12 h of serum deprivation with 0.5% FBS, VSMCs were incubated with 10 nM Exendin-4 or 30 μM forskolin for 10 min. Cells were lysed in 0.1 M HCl, 0.1% Triton X-100, and results were normalized to protein content. Absorbance was read at 405 nm.

**Preparation of membrane extracts.** Membrane proteins were extracted by using the Nucl-Cyto-Mem Preparation Kit according to the manufacturer’s protocol (Applygen Technologies). All steps were performed at 4°C. Briefly, VSMCs treated with ANG II for various time points were harvested and washed twice with PBS by centrifugation at 800 g for 5 min. The cell pellets were resuspended in 500 μl of cytosol extraction reagent (CER) containing a protease inhibitor cocktail (Roche) and homogenized with 20 passes in a 2-ml dounce homogenizer. The homogenate was centrifuged at 1,000 g for 5 min at 4°C to pellet nuclei and cellular debris. The resulting supernatants were then mixed with 50 μl of membrane extraction reagent (MER) for 5 min at 4°C and subjected to centrifugation at 16,000 g for 45 min at 4°C. The resulting pellet and supernatant fractions were designated membrane and cytosol, respectively. The pellet containing the enriched membrane preparation was then resuspended at 50–100 μl of suspension buffer (Applygen).
Western blot analysis. Protein was extracted from the cells with lysis buffer supplemented with the protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland) as described previously (45). Protein concentration was determined using the BCA Protein Assay (Pierce, Rockford, IL). For Western blot, an aliquot of the cell lysate was subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore). Membranes blocked with 5% nonfat milk in TBS containing 0.1% Tween-20 for 1 h were incubated with the specific antibodies against Nox1, Nox4, p22phox, p47phox, p53, p21, Rac1, or β-actin in TBS containing 1% BSA and 0.05% Tween-20 overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. After being extensively washed in TBS containing 0.1% BSA and 0.1% Tween-20, the immunoblots were visualized with an ECL chemiluminescence system (Cell Signaling Technology) and detected by using the ChemiDoc XRS system (Bio-Rad).

Rac1 activity assay. GTP-bound Rac1 was detected by using Rac1 Activation Assay Kit (Millipore), according to the manufacturer’s instructions. Briefly, VSMCs starved in DMEM containing 0.5% FBS overnight were stimulated with the indicated agents for 30 min in 100-mm culture dishes and then were treated with Exendin-4 for 12 h. Upon ANG II stimulation for 60 min, the cells were lysed on ice with cell lysis buffer supplemented with protease inhibitor cocktail. p21-Activated kinase-conjugated protein beads were incubated with the cell lysates at 4°C for 1 h before being washed three times and boiled in SDS-PAGE sample buffer. Eluted proteins were subjected to SDS-PAGE, followed by immunoblotting with anti-Rac1 antibody. The levels of activated Rac1 (Rac1-GTP) were normalized to total Rac1 levels.

Data analysis. Quantitative data are expressed as means ± SE. Differences were analyzed via one-way ANOVA followed by Student’s-Newman-Keuls test. P < 0.05 was considered significant. Nonquantitative results are representative of at least three independent experiments. The statistical analysis was carried out by Graphpad Prism software.

RESULTS

Exendin-4 alleviates ANG II-induced premature senescence in VSMCs. To investigate the effect of Exendin-4 on ANG II-induced senescence in VSMCs, a SA-β-gal assay was performed. VSMC grown (2 × 10^5/well) on six-well plates were cultured in complete growth media (DMEM medium containing 10% FBS). Then, VSMCs were serum starved for 12 h and preincubated for 12 h with Exendin-4 or vehicle (0.1% BSA) before stimulation with 100 nM ANG II for 3 days. As shown in Fig. 1, A and B, ANG II induced a significant increase in SA-β-gal-positive cells. The Exendin-4-pretreated group showed a decrease in SA-β-gal-positive cells in a dose-dependent manner, with a maximum of an ∼54% reduction of senescent-positive cells at a concentration of 10 nM Exendin-4 (Fig. 1, A and B). A single treatment of Exendin-4 has no meaningful effect on the VSMC senescence. These results demonstrate that Exendin-4 exerts suppressive effect on ANG II-induced premature senescence in VSMCs.

Growing lines of evidence have confirmed that ROS play a major role in the development of cellular senescence (46). Next, we evaluated the effects of NAC, a thiol antioxidant, on the senescence induced by ANG II. VSMCs were cultured as described above, serum starved for 12 h, and preincubated for 30 min with 30 μM NAC, followed by stimulation with 100 nM ANG II for 3 days. As shown in Fig. 1C, NAC completely prevented an ANG II-induced increase in SA-β-gal-positive cells, suggesting the involvement of ROS in the VSMC premature senescence induced by ANG II.

Upregulation of p53 mediates ANG II-induced senescence of VSMCs (26). Next, we examined the effect of Exendin-4 on p53 expression and its target gene p21, key proteins in the senescence pathway. Quiescent VSMCs were preincubated for 12 h with 10 nM Exendin-4, followed by stimulation with 100 nM ANG II for 1 or 3 days. As shown in Fig. 1D, ANG II stimulation for 1 day did not affect the expression of p53 or p21, and Exendin-4 did not show any effect either. However, a marked increase in the levels of p53 and p21 was observed in VSMCs exposed to ANG II for 3 days, and pretreatment with Exendin-4 significantly blunted the effects of ANG II on these proteins (Fig. 1E), indicating that downregulations of p53 and p21 may be responsible for Exendin-4 attenuation of VSMC senescence. Therefore, Exendin-4 resulted in downregulations of both p53 and p21 (Fig. 1, D and E) at day 3 of culture, rather than at day 1.

The ability of Exendin-4 to downregulate p53 and p21 protein levels predicted that Exendin-4 would affect VSMC proliferation. To test this, the effect of Exendin-4 on cell cycle progression of VSMC was examined. Here we showed that treatment with ANG II for 1 day promoted VSMC cell cycle progression modestly, as evidenced by the decreased proportion of cells in the G0/G1 phase (Fig. 1F). Exendin-4 had little effect on the proliferative effect of ANG II (Fig. 1F). Nonetheless, the cells exposed to ANG II for 3 days showed cell cycle arrest at the G0/G1 phase with increased G0/G1-phase cell percentage (Fig. 1G). Pretreatment with Exendin-4 restored the proliferatory capacity in prematurely senescent VSMCs, wherein larger cell populations were found to progress to the S phase in the presence of Exendin-4 (Fig. 1G). Our present results indicate that Exendin-4 improves the proliferatory capacity of prematurely senescent VSMCs, downregulates p53 and p21, and counteracts ANG II-stimulated VSMC senescence.

Exendin-4 attenuated ANG II-induced oxidative stress in VSMCs. Among the enzymes implicated in ANG II-induced ROS formation in vascular cells, NADPH oxidase is the main source of ROS generated in VSMCs. ANG II stimulates NAD(P)H oxidase to generate O2·, which is promptly converted by SOD to a more stable molecule, H2O2 (5). H2O2 is a major determinant of the intracellular redox state. To determine whether Exendin-4 attenuated ANG II-induced superoxide generation in VSMCs, H2O2 generated from VSMCs was determined by the Amplex Red assay, as described in MATERIALS AND METHODS. VSMCs were cultured as described above, serum starved for 12 h, and preincubated for 12 h with 10 nM Exendin-4, followed by stimulation with 100 nM ANG II for 12 h. Amplex red detection (specific for extracellularly released H2O2) showed that H2O2 production was increased in the ANG II group, whereas it was markedly decreased in the Exendin-4-pretreated group (Fig. 2A), suggesting that Exendin-4 has vasoprotective effects against pro-oxidant stimuli. Treatment with Exendin-4 alone did not show any effect on basal H2O2 generation.

Additionally, although ANG II caused a significant elevation of H2O2, pretreatment of VSMCs with 30 μM H2O2-scavenging NAC significantly decreased the fluorescence intensity (Fig. 2B). The combined treatment of VSMCs with Exendin-4 and NAC failed to produce any additive effects on ANG II-induced superoxide production. These findings demonstrate that the anti-senescent effects of Exendin-4 in VSMCs may be mediated through the inhibition of ROS production.
Exendin-4 inhibited ANG II-induced VSMC senescence through a GLP-1R-dependent pathway. GLP-1R was found to be expressed abundantly in VSMCs (4). To further study whether the effects of Exendin-4 noted above were conveyed via a canonical GLP-1R-dependent pathway, the GLP-1R antagonist exendin (9–39) was employed. Quiescent cells were pretreated for 30 min with 100 nM exendin (9–39) before addition of 10 nM Exendin-4 for 12 h, followed by treatment with ANG II for 3 days. As previously shown, pretreatment of VSMCs with Exendin-4 significantly blocked the senescence induced by ANG II. In the presence of exendin (9–39), the inhibitory effect of Exendin-4 on ANG II-induced senescence was abolished, whereas exendin (9–39) alone had no effect on the actions of ANG II (Fig. 3A). To further support the involvement of GLP-1R in the vasculoprotective action of Exendin-4, H$_2$O$_2$ production was measured. As presented in Fig. 3B, pretreatment of VSMCs with exendin (9–39) completely reversed the inhibitory effect of Exendin-4 on H$_2$O$_2$ generation, suggesting that Exendin-4 exerts antioxidative effect via GLP-1R. Altogether, these results support the notion that Exendin-4 attenuates ANG II-elicited senescence and H$_2$O$_2$ generation via GLP-1R.

Exendin-4 suppressed ANG II-induced VSMC senescence through the cAMP/PKA pathway. The GLP-1R is a $G_s$ protein-coupled receptor and activates adenylate cyclase, resulting in cAMP production and downstream PKA activation (34). The
It was of great interest to examine if the enhanced cAMP level in VSMCs produced the same effects as Exendin-4. To test this, we used dibutyl cAMP (db-cAMP) and an adenylyl cyclase activator forskolin to examine whether cAMP can reproduce the downregulations of VSMC senescence and H$_2$O$_2$ production. Treatment with db-cAMP (1 mM) decreased the amounts of senescent VSMCs induced by ANG II (Fig. 4D). Results shown in Fig. 4E indicated that the enhanced production of H$_2$O$_2$ induced by ANG II was restored to control levels by db-cAMP. In addition, forskolin also attenuated the enhanced senescence and superoxidant generation in VSMCs exposed to ANG II (Fig. 4, D and E). We further confirmed this pathway by using the PKA inhibitor PKI14-22. Our results showed that PKI14-22 reversed the suppressive effect of db-cAMP or forskolin on VSMC senescence and H$_2$O$_2$ production induced by ANG II (Fig. 4, D and E).

Fig. 2. Exendin-4 inhibits ANG II-induced superoxide anion generation in VSMCs. A: quantitative analysis of superoxide anion production was determined by Amplex Red Hydrogen Peroxide Assay. Quiescent cells were incubated with Exendin-4 for 12 h before stimulation with 100 nM ANG II for 12 h, and hydrogen peroxide (H$_2$O$_2$) levels were measured by the Amplex red assay. Results are means ± SE of 7 independent experiments. *P < 0.05 vs. control; #P < 0.05 vs. ANG II. B: quiescent VSMCs were preincubated with 30 μM NAC for 30 min, followed by stimulation with 100 nM ANG II for 12 h. Pretreatment of VSMCs with NAC significantly decreased the fluorescence intensity. The combined treatment of VSMCs with Exendin-4 and NAC failed to produce any additive effects on ANG II-induced H$_2$O$_2$ production. Results are means ± SE of 5 independent experiments. *P < 0.05 vs. untreated; #P < 0.05 vs. ANG II-treated group.

Fig. 3. Glucagon-like peptide-1 receptor (GLP-1R) mediates the VSMC protective effects of Exendin-4. A: effect of the GLP-1R antagonist exendin (9–39) on VSMC senescence. Quiescent cells were pretreated for 30 min with 100 nM exendin (9–39) before stimulation with 10 nM Exendin-4 for 12 h, followed by treatment with ANG II for 3 days. Cells were stained for SA β-gal. In the presence of exendin (9–39), the inhibitory effect of Exendin-4 on ANG II-induced senescence was abolished. Data are means ± SE of 5 independent experiments. *P < 0.05 vs. untreated; #P < 0.05 vs. ANG II. B: effect of exendin (9–39) on superoxide anion production in VSMCs. Quiescent VSMCs were treated as in A, but ANG II treatment for 12 h, and H$_2$O$_2$ levels were measured by the Amplex red assay. Data are expressed as means ± SE of 6 independent experiments. *P < 0.05 vs. untreated; #P < 0.05 vs. ANG II.
**Exendin-4 failed to decrease Nox1, Nox4, p47phox, and p22phox expression in VSMCs exposed to ANG II.** Nox is a multicomponent enzyme that comprises membrane-bound subunits, p22phox and gp91phox (Nox2 or its homologues Nox1, 3, 4, and 5), and cytosolic subunits, p47phox and p67phox, and the small G protein Rac1, which play a role in activating NAD(P)H oxidase (7, 48). In rodents, the predominant expression pattern is that VSMCs express Nox1 and Nox4 whereas cardiomyocytes and endothelial cells express Nox2 and Nox4 (48). Although Nox1 mRNA is less abundant than Nox4, Nox1 is inducible and upregulated by ANG II (11, 42). Experiments in knockout mice revealed that Nox1 is involved in the ANG II-induced increase in blood pressure and ROS production in VSMCs (15, 35). Because Exendin-4 protected against ANG II-induced oxidative stress, we next tested the possibility that Exendin-4 modulates the expressions of the Nox1, Nox4, p47phox, and p22phox subunits of Nox. As shown in Fig. 5, A–D, the protein levels of Nox1, Nox4, p47phox, and p22phox were significantly increased in ANG II-induced VSMCs compared with control. Nonetheless, in the presence of Exendin-4, the increased expression of Nox1, Nox4, p47phox, and p22phox in VSMCs exposed to ANG II was not significantly altered compared with ANG II-treated cells.

**Exendin-4 counteracted ANG II-induced senescence and superoxide generation by inhibiting Rac1 activation.** Rac1 translocation is essential to the activation of NAD(P)H oxidase and the production of ROS (48). In ANG II-induced diabetic mouse aorta, Rac1 membrane translocation was increased (54). This translocation favors the assembly of the several subunits of the NAD(P)H oxidase. The function of Nox1-based superoxide by inhibiting Rac1 activation. Rac1 binding site is conserved in Nox1 but was not found in Nox4, indicating a Rac1-independent activity of the latter (23). Treatment with ANG II rapidly elicited the translocation of Rac1 from cytosol to membrane fraction at short stimulation times up to 30 min (Fig. 6A). To examine the effect of Exendin-4 on ANG II-induced Rac1 activation, we next proceeded to test the activation state of endogenous Rac1. Rac1 activity was determined by using the effector domain pull down assay. As demonstrated in Fig. 6B, pretreatment with Exendin-4 signifi-
sults, which significantly suppressed the activation of Rac1 induced by ANG II, suggesting that Exendin-4 suppresses ANG II-induced superoxide generation by modulating Rac1 activation.

To verify whether activation of GLP-1R with Exendin-4 could account for the blockade of Rac1 activation elicited by ANG II, we applied exendin (9–39). As shown in Fig. 6C, exendin (9–39) abrogated the inhibitory effect of Exendin-4 on Rac1 activation. Furthermore, preincubation of VSMCs with PKI14-22 also obliterated the suppressive effect of Exendin-4 on Rac1 activation (Fig. 6D).

To further determine the mechanism of action behind the beneficial effect of Exendin-4 as well as the participation of Rac1, the selective Rac1 inhibitor NSC23766 was used. As previously shown, the elevation of H$_2$O$_2$ by ANG II treatment vanished upon pretreatment with Exendin-4 (Fig. 6E). Pretreatment with NSC23766 decreased the ANG II-induced H$_2$O$_2$ generation, indicating the dominant role of Rac1 in ANG II-induced H$_2$O$_2$ generation (Fig. 6E). However, the combination of Exendin-4 and NSC23766 failed to further attenuate H$_2$O$_2$ production (Fig. 6E). Importantly, NSC23766 showed a tendency to inhibit the senescence of VSMCs in response to ANG II compared with control (Fig. 6F). Nevertheless, the combined treatment with Exendin-4 and NSC23766 failed to produce any additive effects on ANG II-induced VSMC senescence (Fig. 6F). Thus Exendin-4 confers resistance to ANG II-induced superoxide generation and premature senescence in VSMCs by upregulation of cAMP/PKA signaling and ensuing inhibition of Rac1 activity to reduce H$_2$O$_2$ generation.

**DISCUSSION**

GLP-1 analogs and DPP-4 inhibitors have emerged as novel therapeutic agents for the treatment of diabetes mellitus (10). Evidence is accumulating that GLP-1 and its analogs can also favorably affect cardiovascular function (33, 44, 55, 57). In this study, we have provided the first evidence that the GLP-1R agonist Exendin-4 counteracts ANG II-induced VSMC senescence by reducing intracellular ROS generation through a GLP-1R/cAMP/PKA-dependent pathway. Specifically, the phenomenon that Exendin-4 suppresses the activation of Rac1, an important event in the initiation of ROS production by Nox1, seems to play a major role in the alleviation of ANG II-induced VSMC senescence.

Novel GLP-1R agonists offer good glycemic control, weight loss benefits, and low risk of hypoglycemia in patients with type 2 diabetes (34). The therapeutic use of GLP-1 is severely compromised by its rapid degradation by DPP-4 (13). Exendin-4 is a peptide incretin mimetic that shares many biological functions with GLP-1. In particular, acute administration of GLP-1 improves endothelial dysfunction in type 2 diabetes...
Fig. 6. Exendin-4 prevents ANG II-induced senescence and superoxide generation through the inhibition of Rac1 activity. A: effect of ANG II on the translocation of Rac1. VSMCs were treated with ANG II for the time indicated. Treatment with ANG II rapidly caused the translocation of Rac1 from cytosol to membrane fraction at short stimulation times up to 60 min. Western blot analysis was performed using anti-Rac1 antibody. Representative blots from 3 independent experiments are shown. B: Exendin-4 suppressed ANG II-induced activation of Rac1. Quiescent VSMCs were pretreated with 10 nM Exendin-4 or vehicle (0.1% BSA) for 30 min were incubated with Exendin-4 for 12 h and exposed to ANG II for 60 min. Data are expressed as means ± SE of 3 independent experiments. *P < 0.05 vs. untreated; #P < 0.05 vs. ANG II. C: VSMCs pretreated for 30 min with exendin (9–39) were incubated with Exendin-4 for 12 h and exposed to ANG II for 60 min. Rac1 activity was determined by a pull-down assay. Representative blots from 3 independent experiments are shown. D: Exendin-4 decreased ANG II-induced superoxide anion production in VSMCs through a cAMP/PKA-dependent pathway. VSMCs pretreated with PKI14-22 for 30 min were incubated with Exendin-4 for 12 h and exposed to ANG II for 60 min. Data are expressed as means ± SE of 3 independent experiments. *P < 0.05 vs. untreated. E: Exendin-4 decreased ANG II-induced superoxide anion production through the inhibition of Rac1-dependent NADPH oxidase activity. Quiescent cells pretreated with 300 μM NSC23766 for 30 min were incubated with 10 nM Exendin-4 for 12 h, followed by treatment with ANG II for 12 h, and H2O2 levels were measured by the Amplex red assay. Data are expressed as means ± SE of 5 independent experiments performed. *P < 0.05 vs. untreated; #P < 0.05 vs. ANG II. F: Exendin-4 counteracted ANG II-induced senescence by inhibiting Rac1-dependent NADPH oxidase. Quiescent VSMCs were treated as in E, but ANG II treatment for 3 days, and VSMC senescence was analyzed as described in MATERIALS AND METHODS. Data are expressed as means ± SE of 6 independent experiments. *P < 0.05 vs. untreated; #P < 0.05 vs. ANG II.

Patients with coronary heart disease (43). Exendin-4 is able to induce endothelial cell proliferation and neovascularization (14, 22). Moreover, Exendin-4 inhibits the monocyte adhesion to endothelial cells and retards the formation of atherosclerotic lesion (3). Although there is much evidence indicating a beneficial function for Exendin-4, there is still a paucity of information on whether Exendin-4 exerts effects on VSMC senescence. Here, our results exhibited that Exendin-4 signif-
EXENDIN-4 ATTENUATES ANG II-INDUCED VSMC SENESCENCE

C1138

p53, the guardian of the genome that is important for cellular responses to oxidative stresses, might be a key coordinator of oxidative stress and aging. Persistent activation of p53 in response to high levels of oxidative stresses can result in cell senescence (31). At day 1 of ANG II stimulation, there was no SA β-gal expression in VSMCs. By day 3 of ANG II stimulation, VSMCs had entered senescence as detected by SA β-gal expression (25, 26, 38). This was supported by the very high level of expression of p53 and p21 at day 3 of culture compared with day 1. Because Exendin-4 also has an antioxidant effect in our study, it is possible that reduction of oxidative stress is responsible for the p53/p21 downregulation by Exendin-4. The upstream signaling of Exendin-4 that inhibits ANG II-induced p53/p21 expression is not clear at this point. It is reported that the signaling mechanisms involve activation of the Ras/Raf-1/ERK 1/2/NF-κB, AP-1/p53/p21 cascade (31, 38). There is increasing evidence that ANG II promotes vascular senescence via the ANG II type-1 (AT1) receptor (19, 38). In the present study, we did not investigate if Exendin-4 affects the expression of AT1R in VSMCs. Also, we did not examine if Exendin-4 affects the binding of ANG II to AT1 in VSMCs. Therefore, we cannot exclude the possibility that AT1R downregulation may contribute to the attenuating effect of Exendin-4 on ANG II-induced VSMC senescence. The signaling mechanisms of AT1 receptor-mediated vascular senescence-promoting effects involve at least two discrete cell signaling axes where one, represented by ERK1/2 and its downstream targets, is redox-independent, and the other involves activation of redox-dependent pathways including activation of Rac, c-Src, or Akt. Activation of these redox-dependent pathways involves stimulation of NADPH oxidases that produce superoxide (O2−) and hydrogen peroxide (H2O2) (38). Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway leads to translocation of Rac to elicit prolonged oxidase-dependent ROS generation (49). The PI3K/Akt pathway plays a dominant role in ANG II-induced VSMC senescence and ROS generation (25). To characterize the signal(s) induced by ANG II that is (are) altered by Exendin-4, we investigated the effect of Exendin-4 on ANG II-induced signaling pathway such as phosphorylation of ERK1/2 and Akt. Treatment with ANG II leads to phosphorylations of Akt and ERK1/2 in a time-dependent manner (data not shown). However, Exendin-4 did not alter the activation of these proteins in rat VSMCs (data not shown). Future research, beyond the scope of the current study, should focus on identifying and characterizing these signaling proteins to advance our understanding of the mechanisms underlying the antisenescent and antioxidant effects of Exendin-4.

The type of senescence in VSMC exposure to ANG II seems to be dependent on the period of treatment with ANG II. Min et al. (38) have reported the time course of ANG II stimulation in inducing senescence of VSMCs. With ANG II treatment, the total cell number increased time dependently and reached a plateau at ~5 days, whereas SA β-gal-positive cells were enhanced in a time-dependent manner starting at 5 days after the start of ANG II stimulation (38). These effects suggest that ANG II initially stimulates a mitogenic response of VSMCs and then sustained ANG II in turn promotes VSMC senescence. Consistent with our study, Kim et al. (25) and Kunieda et al. (26) also treated human VSMC with ANG II for 3 days
and examined markers of senescence. They showed that ANG II treatment provoked stress-induced premature senescence and cell cycle arrest in VSMCs within 3 days, which was supported by the very high expression levels of p53 and p21 at day 3 of culture compared with day 1 (25, 26). In the present study, we demonstrated that Exendin-4 recovered the proliferative capacity of prematurely senescent VSMCs, downregulated p53 and p21, and counteracted ANG II-stimulated VSMC senescence on day 3. As reported earlier, Ang II induces premature senescence in VSMCs through an increased superoxide anion production by NADPH oxidase activity (19, 26). Oxidative stress produces DNA damage, inducing cell cycle inhibitors that make cells exit the cell cycle. The lower proliferative capacity and the appearance of premature senescence in ANG II-treated cells could result from the higher expression of the cell cycle inhibitors p53 and p21, implicated in the senescent phenotype (26). In our current study, it is possible that Exendin-4 recovered the proliferative rates of senescent VSMCs as a result of the blockade of ANG II/NADPH oxidase-mediated ROS generation. The above data set establishes an important role for Exendin-4 in regulating the commitment of a VSMC population between proliferation and senescence. Each of these processes has been shown to be important in VSMC biology, and we propose that by shifting the balance between them, Exendin-4 has a beneficial effect on the overall VSMC population and biological function.

The GLP-1R is a G protein-coupled receptor and activates adenylyl cyclase, resulting in cAMP production and downstream PKA activation (34). It was recently revealed that activation of the cAMP/PKA pathway may be involved in GLP-1-offered protective effects. In pancreatic β-cells, GLP-1 enhanced insulin secretion via cAMP/PKA signaling pathway-mediated Nox 2 suppression (29). Exendin-4 attenuated neointimal hyperplasia after vascular injury through the cAMP/PKA pathway in C57BL/6 mice (20). Furthermore, it is reported that db-cAMP via the PKA pathway attenuates the hyperproliferation of VSMCs, the increased production of O$_2^-$, and the overexpression of Nox1/Nox2/Nox4 and p47$^{phox}$ proteins (18). Here, we showed that Exendin-4 suppressed ANG II-induced VSMC senescence and superoxidant production through the cAMP/PKA pathway. In addition, db-cAMP or adenylyl cyclase activator mimicked the role of Exendin-4 in attenuating VSMC senescence and H$_2$O$_2$ production. Several previous studies have demonstrated that sustained stimulation of ANG II increased senescence and oxidative stress in VSMCs, which is attributed to the decreased levels of intracellular cAMP (18, 56). Similarly, at the physiological level, β-adrenergic receptor-stimulated cAMP production declines with advancing age (17). In the present study, we report that the enhanced senescence and superoxidant production induced by ANG II stimulation may also be attributed to the decreased levels of intracellular cAMP, because augmenting the intracellular levels of cAMP by db-cAMP or forskolin, a cAMP-elevating agent, attenuated ANG II-elicted VSMC senescence and superoxidant generation. Furthermore, our results showing that inhibition of PKA by PKI14-22 reversed the attenuating effect of Exendin-4 or db-cAMP on the premature senescence and superoxidant production suggest the implication of the cAMP-PKA pathway in Exendin-4 or db-cAMP-mediated antisenescent effects. In this regard, the role of cAMP/PKA pathway in attenuating oxidative stress and senescence induced by different interventions has been well documented. Klotho, a recently discovered antiaging gene, has been shown to attenuate the superoxidant production and Nox2 expression stimulated by ANG II through the cAMP-PKA-dependent pathway in rat VSMCs (56). Adenosine receptor A$_{2A}$ activation reduces senescence by decreasing p53 and Rb protein levels through the cAMP-PKA/Rac1 pathway in hepatic stellate cells (1). It is well known that cAMP/PKA was an essential negative regulator of Rho A phosphorylation (9). Phosphorylation of Rho A activates Rac1 by the enhanced binding of phospho-RhoA to Rho GDI, leading to the release of Rac1 from the Rac1-RhoGDI complex and Rac1 translocation to the membrane, where Rac1 binds with p22$^{phox}$ that activates NAD(P)H oxidase and produces superoxide (30). Our data revealed that cAMP/PKA may contribute to Exendin-4-mediated suppression of ANG II-evoked Rac1 activation. This conclusion was supported by the fact that the Exendin-4 inhibition of Rac1 activation was abrogated when the cAMP/PKA pathway was blocked.

Our results indicate that pharmacological intervention targeting on GLP-1 may represent a promising therapeutic strategy to maintain VSMC function in patients with diabetes and cardiovascular disease. So far, this is the first study that links activation of the GLP-1R to a protective effect on VSMC senescence. Nonetheless, it is noteworthy that our findings were mainly based on in vitro experiments. Therefore, caution must be taken in evaluating the effects of GLP-1 on VSMCs and in patients with diabetes.

In conclusion, Exendin-4 confers resistance to ANG II-induced superoxide generation and the resultant senescence, en route to improved vasculature function. Notably, the protective effects of Exendin-4 are dependent on downstream inhibition of Rac1 activation through a GLP-1R/cAMP/PKA-dependent manner, resulting in a subsequent decrease in the ROS level generated from the Nox1. These findings should provide important implications for diabetes with cardiovascular injury, for which Exendin-4 may hold promise for prevention and treatment.

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

AUTHOR CONTRIBUTIONS

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EXENDIN-4 ATTENUATES ANG II-INDUCED VSMC SENESCENCE


