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

Liang Zhao,1,2 Ai Q. Li,1,2 Teng F. Zhou,1,2 Meng Q. Zhang,1,2 and Xiao M. Qin1,2

1Institute of Cardiovascular Science, Peking University Health Science Center, Beijing, China; and 2Key Laboratory of Molecular Cardiovascular Science of Ministry of Education, Peking University Health Science Center, Beijing, China

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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) (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 (Nos) and to promote VSMC senescence (19, 26); therefore, vascular Nos 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).

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 cardiovascularprotective 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\textsuperscript{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 thickening 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\textsuperscript{-} generation and result in premature senescence in VSMCs via the cAMP/PKA-dependent pathway. Importantly, Exendin-4 inhibition of Rac1 activation, which 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 p2\textsuperscript{g}bhol, p4\textsuperscript{g}bhol, and Rac1 were from Cell Signaling Technology (Beverly, MA). The myristoylated PKA inhibitor amide14-22 (PKI14-22) and Rac1 inhibitor NSC23766 were from Cell Signaling Technology (Santa Cruz, CA). The Cyclic AMP Assay Kit was from Enzo Life Sciences (Farmingdale, NY). The Rac1 Activation Assay Kit was from Cell Signaling Technology (Beverly, MA). The Neurotrophic Membrane Preparation Kit was from Invitrogen (Carlsbad, CA). Exendin-4 was prepared in PBS containing 0.1% BSA to prevent the peptide from adhering to plastic surfaces.

**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 incubated 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% FBS, 100 mM Penicillin, and 100 µg/ml Streptomycin. Cells were incubated in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 95% air-5% CO\textsubscript{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.
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 proliferative 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 proliferative capacity of prematurely senescent VSMCs, downregulates p53 and p21, and counteracts ANG II-stimulated VSMC senescence.

Exendin-4 attenuates 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 O_2^- , which is promptly converted by SOD to a more stable molecule, H_2O_2 (5). H_2O_2 is a major determinant of the intracellular redox state. To determine whether Exendin-4 attenuated ANG II-induced superoxide generation in VSMCs, H_2O_2 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 H_2O_2) showed that H_2O_2 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 vasculoprotective effects against pro-oxidant stimuli. Treatment with Exendin-4 alone did not show any effect on basal H_2O_2 generation.

Additionally, although ANG II caused a significant elevation of H_2O_2, pretreatment of VSMCs with 30 μM H_2O_2-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 antisenescent 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 $\mathrm{G}_s$ 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 dibutylryl cAMP (db-cAMP) and an adenylyl cyclase activator forskolin to examine whether cAMP can reproduce the downregulations of VSMC senescence and H2O2 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 H2O2 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 H2O2 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 (H2O2) 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 H2O2 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 6 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 H2O2 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 NAD(P)H oxidase requires Rac1 activation. A putative 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-
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 and reduces the risk of cardiovascular events (36). 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.

**Fig. 5.** Exendin-4 has no effects on the expression of Nox1, Nox4, p22phox, or p47phox in VSMCs exposed to ANG II. Quiescent VSMCs were preincubated with 10 nM Exendin-4 for 12 h, followed by stimulation with 100 nM ANG II for 24 h. Western blot analyses for Nox1 (A), Nox4 (B), p47phox (C), or p22phox (D) (20 μg of total protein) were performed with primary antibodies against Nox1, Nox4, p22phox, or p47phox. Quantitative analysis of protein expression was presented as the ratio of Nox1, Nox4, p22phox, or p47phox bands relative to β-actin and expressed as fold of control. Statistical analysis of the densitometric evaluation of the 3 independent experiments are shown. Data are means ± SE. *P < 0.05 vs. control.
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 preincubated with 10 nM Exendin-4 or vehicle (0.1% BSA) for 12 h, followed by stimulation with 100 nM ANG II for 60 min. Cells were lysed and activated Rac1 was determined by pull-down assay. GTP-bound Rac1 levels were determined by immunoblotting. Top: representative blots for GTP-Rac1 and total Rac1 from 3 experiments. Bottom: quantification of Rac1 activity. Results are presented as intensities of Rac1-GTP relative to total Rac1 and expressed as fold of control. 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. Data are expressed as means ± SE of 5 independent experiments performed. *P < 0.05 vs. untreated; #P < 0.05 vs. ANG II. D: Exendin–4 decreased ANG II-induced Rac1 activation 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. Rac1 activity was determined by a pull-down assay. Representative blots from 3 independent experiments are shown. 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 6 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 signifi-
ificantly ameliorated ANG II-induced VSMC senescence by reducing H$_2$O$_2$ generation, consistent with a recent report in which the DPP-4 inhibitor vildagliptin was found to protect against endothelial cell senescence in Zucker diabetic fatty rats (44). Since VSMCs with a senescence phenotype have been identified at sites prone to atherosclerosis, it may be possible to control the development of age-associated cardiovascular disease, such as atherosclerosis, through application of GLP-1-related reagents.

Involvement of ROS has been documented in ANG II-induced cellular senescence (19). Nox is a main source of ROS generated in VSMCs. The present findings demonstrated for the first time that Exendin-4 inhibits Nox-derived superoxide generation stimulated by ANG II in VSMCs. A recent study showed that GLP-1 inhibits Nox activity and decreases superoxide (O$_2^-$) generation in rat myocardial tissues after ischemia and reperfusion injury (55). Therefore, the O$_2^-$ generated from Nox may be one of the major molecules targeted by GLP-1 in exerting its cardiovascular protective effects. ROS derived from different Nox isoforms may play distinct roles in modulating intracellular ROS-mediated signaling. For example, Nox4 appears to be important in maintaining basal O$_2^-$ and hydrogen peroxide (H$_2$O$_2$) production, the activation of which does not require p67$^{phox}$, p47$^{phox}$, or Rac1. However, Nox1 might be responsible for O$_2^-$ production and redox signaling after stimulation and in pathological conditions, such as atherosclerosis, diabetes, and hypertension (2, 11, 42). Nox1 appears to be important in ANG II-dependent hypertension and vascular remodeling (39). Insulin-like growth factor-I induces ROS production and VSMC migration through Nox4 and Rac1 (37). Nox1 is activated mainly through Rac1 membrane translocation in VSMCs, which favors the assembly of active Nox1, leading to superoxide generation (23). ANG II has been considered to be responsible for the activation of Rac1-dependent Nox1 in VSMCs (28). Moreover, oxidative stress and Rac1 activation are enhanced in the heart of C57BL/6 mice treated with ANG II infusion (53). In the present study, the blockades of superoxide production and senescence by Rac1 inhibitor NSC23766 indicate that Nox1-based NAD(P)H oxidase is essential to ANG II-induced VSMC senescence. However, the precise molecular targets that could inhibit these cellular events have not been identified to date. No direct evidence is available on Exendin-4 and Rac1 activation in VSMCs. In this study, we have established that Exendin-4 prevented ANG II-induced H$_2$O$_2$ production, possibly through inhibition of Rac1 activation. Notably, Rac1 activation induced by ANG II was suppressed by Exendin-4 via GLP-1R. These findings demonstrate that the protective effects of Exendin-4 may be mediated through Rac1 inhibition, which in turn alleviated ANG II-induced oxidative stress in VSMCs. This conclusion is further substantiated using the Rac1 inhibitor NSC23766. In particular, joint therapy with Exendin-4 and NSC23766 failed to further suppress senescence and H$_2$O$_2$ production. Therefore, it is possible that the antioxidative effect of Exendin-4 is partially achieved by the modulation of this key regulatory factor, thereby suppressing excessive ROS generation in the vasculature. To our knowledge, this is the first report in which activation of GLP-1R is linked to decreased Rac1 activation and NAD(P)H oxidase-derived O$_2^-$ generation, initiating antisenescent property in the ANG II environment.

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 ß-gal expression in VSMCs. By day 3 of ANG II stimulation, VSMCs had entered senescence as detected by SA ß-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. Consistent with these results, Exendin-4 resulted in downregulations of both p53 and p21 at day 3 of culture, rather than at 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 (AT$_1$) receptor (19, 38). In the present study, we did not investigate if Exendin-4 affects the expression of AT$_1$R in VSMC. Also, we did not examine if Exendin-4 affects the binding of ANG II to AT$_1$ in VSMCs. Therefore, we cannot exclude the possibility that AT$_1$R downregulation may contribute to the attenuating effect of Exendin-4 on ANG II-induced VSMC senescence. The signaling mechanisms of AT$_1$ 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 (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) (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 ß-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 proliferatory 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 proliferatory 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 proliferatory 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 Nox2 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 hyperpolarization of VSMCs, the increased production of $\mathrm{O}_2^-$, and the overexpression of Nox1/Nox2/Nox4 and p47phox 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 $\mathrm{H}_2\mathrm{O}_2$ production. Several previous studies have demonstrated that sustained stimulation with 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 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 anti-senescent 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 A2A 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 p22phox 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
Author contributions: L.Z. and X.M.Q. conception and design of research; L.Z., A.Q.L., T.F.Z., M.Q.Z., and X.M.Q. performed experiments; L.Z., A.Q.L., T.F.Z., M.Q.Z., and X.M.Q. analyzed data; L.Z. and X.M.Q. prepared figures; L.Z. and X.M.Q. drafted manuscript; L.Z. and X.M.Q. edited and revised manuscript; L.Z., A.Q.L., and X.M.Q. interpreted results; L.Z., T.F.Z., M.Q.Z., and X.M.Q. approved final version of manuscript.

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