Exendin-4 suppresses VSMC senescence via Nrf2 activation

Activation of Nrf2 contributes to the protective effect of Exendin-4 against angiotensin II-induced vascular smooth muscle cell senescence

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Abstract

Oxidative stress and impaired antioxidant defense are believed to be contributors to the cardiovascular aging process. The transcription factor NF-E2 related factor 2 (Nrf2) plays a key role in orchestrating cellular antioxidant defenses and maintaining redox homeostasis. Our previous study showed that Exendin-4, a GLP-1 analogue, alleviates angiotensin II (Ang II)-induced VSMC senescence by inhibiting Rac1 activation via cAMP/PKA. The objective of this study is to investigate if Nrf2 mediates the anti-senescent effect of Exendin-4 in Ang II-induced VSMCs. Here we report that Exendin-4 triggered Nrf2 nuclear translocation, a downstream target of cAMP responsive element-binding protein (CREB) and expressions of antioxidant genes heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase-1 (NQO-1) in a dose- and time-dependent manner. In addition, knock-down of Nrf2 attenuated the inhibitory effects of Exendin-4 on Ang II-induced superoxidant generation and VSMC senescence. PKA/CREB pathway participated in the upregulations of HO-1 and NQO-1 induced by Exendin-4. Notably, our study revealed that Exendin-4 dose-dependently increased the acetylation of Nrf2 and the recruitment of transcriptional coactivator CREB binding protein (CBP) to Nrf2. The Exendin-4-induced Nrf2 transactivation was diminished in the presence of CBP siRNA. Microscope imaging of Nrf2 as well as immunoblotting for Nrf2 showed that the Exendin-4-evoked Nrf2 acetylation favored its nuclear retention. Importantly, CBP silencing attenuated the suppressing effects of Exendin-4 on Ang II-induced VSMC senescence and superoxidant production. In conclusion, these results provide a
mechanistic insight into how Nrf2 signaling mediates the anti-senescent and anti-oxidative effects induced by Exendin-4 in VSMCs.

**Keywords:** vascular smooth muscle cells; Glucagon-like peptide-1; cellular senescence; NF-E2 related factor 2
INTRODUCTION

Cellular senescence is an important risk factor for cardiovascular diseases, such as hypertension, atherosclerosis, heart failure and stroke (30). Unlike replicative senescence, which is characterized by shortened telomere length and eventually induces incomplete chromosomal replication (42), stress-induced premature senescence (SIPS) is triggered by a variety of stresses including chemical or oxidative stresses and oncogene activation. Enhanced production of reactive oxygen species (ROS) and insufficient removal by scavenging systems are hallmarks of vascular aging (2). Increased levels of cellular senescence have been observed in the vasculature of patients with coronary artery disease (30). Aged vascular smooth muscle cells (VSMCs) become synthetic, exhibiting enhancement in the capability of migration/invasion, proliferation, secretion and inflammation (25, 29). Prevention of vascular aging may be important for effective therapy to age-related vascular changes in atherosclerotic degeneration.

A key modulator of redox balance and signaling is the transcription factor, nuclear-factor-E2-related factor 2 (Nrf2), which drives the expression of a battery of genes that protect against electrophilic and oxidative stress (33). Under basal conditions, Nrf2 is sequestered in the cytoplasm by its interaction with Kelch-like ECH-associated protein 1 (Keap1), which promotes ubiquitin-mediated degradation of Nrf2 (32). Increased oxidant load in cells results in the oxidation of cysteine residues in Keap1 (24), phosphorylation of Nrf2 at Ser⁴⁰ and the release of Nrf2 from
Keap1, thereby allowing Nrf2 translocation into the nucleus and binding to the antioxidant response elements (AREs) in the promoters of its target genes, including NAD(P)H quinone oxidoreductase-1 (NQO-1) and heme oxygenase-1 (HO-1) (18). Another level of control is modulation of the availability of transcription coactivators, such as cAMP responsive element-binding protein (CREB) binding protein (CBP), which has been shown to interact with and activate Nrf2 (47). CBP is a histone acetyltransferase (HAT) involved in chromatin opening, and plays a key role as a nuclear coactivator for a wide variety of transcription factors (26). CBP is normally recruited to Nrf2 and directly modulates Nrf2 association with ARE sequences by acetylation Nrf2 (47). Kawai et al (20) showed that acetylation/deacetylation plays a crucial role in the nucleocytoplasmic shuttling of Nrf2. Nrf2 acetylation can increase Nrf2 sequence-specific DNA binding capacity and facilitate transcription of its downstream target genes (20).

Glucagon-like peptide-1 (GLP-1) is an endogenous incretin hormone released from the intestinal L cells in response to nutrient ingestion and stimulates insulin secretion in a glucose-dependent manner (21). GLP-1-related therapies are currently approved for the treatment of type 2 diabetes (21). In addition, GLP-1 has beneficial effects in the heart (27, 52, 56) and the vasculature (1, 35, 50). We showed recently that Exendin-4, a GLP-1 analogue, alleviates angiotensin II (Ang II)-induced VSMC senescence by inhibiting Rac1 activation via cAMP/PKA (54). However, the contribution of Exendin-4-operated antioxidant expression in attenuating Ang
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II-induced VSMC senescence remains unclear. Here, we demonstrate that Exendin-4 suppresses Ang II-induced premature senescence of VSMCs by inhibiting superoxidant production through the activation of Nrf2 and up-regulation of Nrf2-targeted antioxidant genes. Notably, Exendin-4 greatly enhances the acetylation of Nrf2 by recruiting CBP to Nrf2 via PKA in Ang II-treated VSMCs. Therefore, increasing acetylation of Nrf2 through CBP may constitute a novel regulatory mechanism for the protective effects of Exendin-4.
MATERIALS AND METHODS

Chemicals, reagents, and antibodies. Exendin-4, Exendin fragment (9-39), angiotensin II, and SA β-galactosidase staining kit were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against Nrf2 (sc-722), HO-1 (sc-7695), NQO-1 (sc-16464), phospho-CREB (sc-7978), CREB (sc-186), p300 (sc-584), CBP (sc-25748), PCAF (sc-8999) were from Santa Cruz. Anti-acetyl-lysine antibody was from Cell Signaling Technology (Danvers, MA). Nrf2 promoter-driven reporter plasmids was purchased from Beyotime (Beyotime, Beijing). Myristoylated PKA inhibitor amide14-22 (PKI14-22) were from Calbiochem (La Jolla, CA).

Cell culture. Rat aortic VSMCs were obtained by terminal harvesting of aortas from male Sprague-Dawley rats, as described previously (54). Cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS at 37°C in a humidified atmosphere of 95% air-5% CO₂. VSMCs at passages 3 to 8 were used for all experiments.

Senescence-associated β-galactosidase (SA β-gal) assay. Senescent cells were detected using an SA β-galactosidase staining kit, as described previously (54). In brief, cells seeded in 6-well plates were preincubated for 30 min with indicated reagents before addition of Exendin-4 for 12 h, followed by treatment with Ang II for 3 days. After washing twice with phosphate-buffered saline (PBS), the cells were incubated with staining-solution for 6 h. Stained cells were visualized using an
Olympus CK40-SL light microscope (Tokyo, Japan).

Amplex red assay for ROS production. H$_2$O$_2$ level was measured using the Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes, Invitrogen) as previously described (54). After being washed with freshly prepared Krebs-Ringer-phosphate-glucose buffer, cells were incubated with 300 μl KRPG containing horse-radish peroxide and Amplex red (50 μM) for 30 min at 37 °C. The supernatant was transferred to 96 well plates and fluorescence was then measured with a fluorescence microplate reader (Molecular Devices). The obtained fluorescence intensities were normalized to control.

Transfection and reporter gene assay. Human VSMC aorta cell line (T/G HA-VSMC, CRL-1999, ATCC) at ~80% confluence in 24-well plates were transiently cotransfected using Lipofectamine 2000 (Invitrogen) with 1 μg of ARE4-luciferase reporter construct containing four tandem copies of an ARE sequence in the pGL3 basic vector (Beyotime, Beijing) and 5 ng of pSV-β-galactosidase luciferase construct as an internal control, as described previously (40). After transfection for 24 h, cells were incubated with Ang II, Exendin-4 or Ang II in combination with Exendin-4. Luciferase activity was measured using the Reporter Luciferase assay kit (Promega) and β-galactosidase activity was measured using O-nitrophenyl β-galactopyranoside. Variations in transfection efficiency were normalized using β-galactosidase activity.
Immunofluorescence staining. Confluent VSMCs grown on cover slips were fixed with 4% paraformaldehyde for 15 min, permeabilized in 0.2% Triton X-100 for 10 min, and blocked for 1 h with 1% BSA. Thereafter, cells were incubated with Nrf2 antibody (1:200) at 4°C overnight followed by a FITC-labeled goat anti-rabbit IgG.

Nuclei were stained with DAPI (Molecular Probes). Images were captured using Leica TCS SP2 laser scanning confocal microscopy (Leica Microsystems, Wetzlar, Germany).

Nuclear and cytosolic protein extraction. Nuclear and cytosolic proteins were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) following the manufacturer’s instructions (40). Briefly, the cells were harvested and then resuspended in 200 μl of Cytoplasmic Extraction Reagent I (CER I) and then vortexed for 15 s. Then, cells were added to 11 μl of Cytoplasmic Extraction Reagent II (CER II) and vortexed for 5 s. Following centrifugation at 16000 g for 5 min at 4°C, the supernatants (cytoplasmic fraction) were collected. Nuclear Extraction Reagent (NER, 100 μl) was then added to the pellet and the samples were vortexed for 15 s. Following centrifugation at 16000 g for 10 min, the supernatants (nuclear fraction) were collected.

Western blotting. Western blotting of whole cell lysates or subcellular fractions was performed as previously described (54). Equal amounts of protein homogenate were resolved on 12% SDS-PAGE and transferred onto PVDF membranes. Membranes
were blocked in TBST buffer containing 5% nonfat milk and probed with primary antibodies against HO-1, NQO-1, phospho-CREB, CREB or Nrf2 overnight at 4°C. Blots were then incubated with respective horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. Blots were analyzed with an enhanced chemiluminescence detection system.

Co-immunoprecipitation and detection of acetylated Nrf2 in cells. The cells were harvested and then lysed in ice-cold lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, 0.05% SDS, 1 mM phenylmethylsulfonyl fluoride, and a 1% protease inhibitor mixture). The lysates were centrifuged at 13,000 rpm for 10 min at 4°C. Lysates (100 μg total protein) were incubated with antibody against CBP, p300, PCAF or Nrf2 for 3 h at 4°C. Protein A/G plus agarose (Invitrogen) was added to the samples and mixed by rolling for 1 h. After washing the beads three times, the pellets were dissolved into 2× SDS-PAGE sample buffer, and subjected to western blotting using anti-Nrf2 or anti-acetyl-lysine antibody.

Silencing with siRNA. Transfection of cells with siRNA was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Commercially available Nrf2, CREB and CBP specific siRNAs (Santa Cruz Biotechnology) and negative control siRNAs were used for transfection. Gene silencing was measured after 48 h by western blot.
Statistical analysis. Quantitative data are expressed as means ± SE. Differences were analyzed via one-way analysis of variance (ANOVA) followed by a Newman-Keuls test. A $P$ value of < 0.05 was considered statistically significant. Nonquantitative results were representative of at least 3 independent experiments.
RESULTS

*Exendin-4 promotes Nrf2 nuclear translocation, thereby inducing Nrf2 target genes in VSMCs.* In agreement with our previous study (54), treatment of VSMCs with Exendin-4 reduced the number of senescent cells induced by 3 days of Ang II treatment, as determined by SA β-galactosidase staining (Fig. 1A). Because Ang II-induced oxidative stress causes DNA damage, we examined the effects of Ang II on DNA stability, using Western blot with an antibody against phospho-γ-H2AX, a marker for double-stranded DNA breakage. We studied the phosphorylation of γ-H2AX using time points from 0 to 24 h (Fig. 1B). Western blot analysis on nuclear proteins showed that phosphorylation of γ-H2AX was induced by Ang II at 1 h, which even increased over 24 h (Fig. 1B). In parallel to the SA β-galactosidase staining, Exendin-4 significantly attenuated Ang II-induced superoxidant production using the Amplex Red assay (Fig. 1C).

Nrf2-modulated signaling is vital for the antioxidative response. We therefore examined whether Exendin-4 promotes Nrf2 nuclear translocation, thereby inducing antioxidant gene expression. As shown in Fig. 2A, Ang II caused an increase of Nrf2 nuclei content in VSMCs at 1 h and 3 h, evidence for the presence of oxidative stress in these cells (Fig. 2A). But at 12 h and 24 h, Ang II showed on significant effects on the translocation of Nrf2 into the nucleus (Fig. 2A). In Exendin-4 alone-treated VSMCs, Exendin-4 stimulated Nrf2 translocation at 1 h, 3 h, 6 h (Fig. 2A). The combination of Exendin-4 and Ang II promoted Nrf2 translocation from the cytoplasm to the nucleus time-dependently, reaching a peak at 1 h and declining.
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thereafter until 6 h. Accordingly, levels of Nrf2 in cytoplasmic fraction were
gradually decreased by Exendin-4. When we treated cells with various concentrations
of Exendin-4 for 1 h, Nrf2 translocation to the nucleus increased in a dose-dependent
manner and a maximum translocation was detected at 10 nM (Fig. 2C).

Exendin-4-induced rapid nuclear translocation of Nrf2 in Ang II-treated cells
was further confirmed by double fluorescence staining with anti-Nrf2 and DAPI. Nrf2
was localized in cytoplasm and nucleus in Ang II-treated cells (Fig. 2D). Nrf2
translocations from cytosol to nucleus were observed within 30 min after Exendin-4
treatment (10 nM) and Nrf2 was remained in the nucleus at 6 h post-treatment (Fig.
2D), with its effects peaking at 1 h (Fig. 2D).

The phosphorylation of Nrf2 was also investigated, as it has been reported to
play a critical role in the dissociation of Nrf2 from Keap1 and nuclear distribution of
Nrf2. As shown in Fig. 2E, compared to slightly increased Nrf2 phosphorylation in
Exendin-4 treated cells, a significant level of Nrf2 phosphorylation occurred in the
cells treated with Exendin-4 together with Ang II. Next, we transfected VSMCs with
ARE4-Luc plasmid DNAs, which contain the luciferase gene driven by the Nrf2
responsive element. ARE activity at 6 h was increased by Ang II (Fig. 2F). However,
the increase in mean ARE activation was not significant. At 12 h and 24 h, ARE
activity were almostly not affected by Ang II (Fig. 2F), but at the same time,
Exendin-4 alone treatment increased the ARE activity compared with control (Fig.
2F). The combination of Exendin-4 and Ang II further increased the activity of ARE
(Fig. 2F), showing maximal efficiency in promoting Nrf2 activity.
Moreover, HO-1 protein level was moderately elevated by Ang II alone at 6 h (Fig. 2G). After 12 to 24 h, HO-1 and NQO-1 expressions in the Ang II group were not affected (Fig. 2G). Exendin-4 may markedly increase the protein levels of HO-1 and NQO-1 in the presence or absence of Ang II (Fig. 2G). The combination of Exendin-4 and Ang II increased the protein levels of NQO-1 and HO-1 dose- and time-dependently (Fig. 2H).

**Effects of silencing Nrf2 on Exendin-4-induced anti-senescent effect and antioxidant gene expression.** In order to provide direct evidence of the involvement of Nrf2 pathway in Exendin-4-mediated anti-senescent effect, we transiently transfected VSMCs with either the scrambled or Nrf2 siRNA. As shown in Fig. 3A, transfection with Nrf2 siRNA significantly inhibited Nrf2 expression. The Exendin-4-induced upregulations of HO-1 and NQO1 were reduced by Nrf2 siRNA (Fig. 3B).

Next, we examined the effect of Nrf2 siRNA on the suppressing effects of Exendin-4 on Ang II-induced VSMC senescence and H₂O₂ production. In VSMCs transfected with the scrambled siRNA, Exendin-4 inhibited Ang II-induced VSMC senescence (Fig. 3C). However, Exendin-4 was less effective in attenuating the Ang II-induced premature senescence in VSMCs transfected with the Nrf2 siRNA (Fig. 3C). Moreover, knockdown of Nrf2 expression by siRNA significantly reversed the attenuating effect of Exendin-4 on Ang II-induced H₂O₂ production (Fig. 3D).
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**PKA-CREB signaling contributes to Exendin-4-induced Nrf2/ARE activation during Ang II treatment.** An earlier study reported that GLP-1 receptor agonists activate cAMP/PKA/CREB, thereby promoting β-cell growth and survival (21).

Expression of CREB in VSMCs is regulated in response to vascular injury. It is reported that CREB can be activated by TNFα, Ang II and thrombin (32). Stimulation of VSMCs with Ang II for various intervals resulted in transient CREB phosphorylation (Fig. 4A). CREB phosphorylation was detectable within 2 min after treatment of VSMCs with Ang II, and thereafter returned to basal levels by 15 min (Fig. 4A). Our results were consistent with those of Molnar et al (31). Here, we reported that Exendin-4 increased the phosphorylation of PKA (Fig. 4B). Moreover, Exendin-4 significantly induced the phosphorylation of CREB on Ser133 (Fig. 4B), which was blunted by PKA inhibitor PKI14-22 (Fig. 4C).

To identify a connection between cAMP/PKA and Exendin-induced Nrf2/ARE activation, induction of ARE-driven luciferase reporter was analyzed in VSMCs pretreated with PKI14-22. As shown in Fig. 4D, addition of PKI14-22 decreased the Exendin-4-induced ARE4-luc activation in VSMCs exposed to Ang II.

In addition, the upregulations of HO-1 and NQO-1 induced by Exendin-4 were also reduced by either PKI14-22 or CREB siRNA (Fig. 4, E and F). These results support a role for cAMP/PKA/CREB cascade as an upstream regulator of Exendin-4-mediated Nrf2/ARE activation.

Exendin-4 increases the acetylation of Nrf2 and modulates CBP interaction with
Nrf2 in VSMCs. Since acetylation is essential for binding of Nrf2 to specific ARE-containing promoters (47), we next investigated whether Nrf2 was acetylated under the treatment of Exendin-4. Co-immunoprecipitation (Co-IP) assays showed that Nrf2 acetylation was not significantly observed in Ang II-treated cells (Fig. 5A), whereas Exendin-4 increased the acetylation of Nrf2 dose-dependently (Fig. 5B). The whole cell content of Nrf2 was not changed by Exendin-4. These results provide strong support for our hypothesis that Exendin-4 could induce changes in the acetylation status of Nrf2, not changes in the whole cell content of Nrf2.

The key cofactor related to cAMP-mediated signaling that has been associated with Nrf2 nuclear translocation and acetylation is histone acetyltransferase (HAT) p300/CBP (20). CBP was identified by its association with the transcription factor CREB (19). It has been shown that Nrf2 was acetylated only by p300 and CBP, but not PCAF (10). To further investigate which enzymes are responsible for the Exendin-4-induced lysine acetylations of Nrf2, Co-IP assays were performed to test the interaction of co-activators p300, CBP and PCAF with Nrf2. As shown in Fig. 5C, Ang II had no effect on the recruitment of p300, CBP or PCAF to Nrf2. In the Exendin-4 treatment group, increased Nrf2 was specifically detected with anti-Nrf2 antibody in the immunoprecipitate made by anti-CBP antibody, but not in the p300, PCAF, or control IgG immunoprecipitates (Fig. 5D). CBP expression remained unchanged after Exendin-4 treatment. Therefore, CBP was the most potent in acetylating Nrf2 in response to Exendin-4 and was chosen for the subsequent studies. In addition, the Exendin-4-stimulated acetylation of Nrf2 was reduced by the addition
of PKA inhibitor PKI14-22 (Fig. 5E). Pretreatment with GLP-1R antagonist Exendin
(9-39) or PKI14-22 decreased the binding of Nrf2 and CBP (Fig. 5F).

**CBP is essential for Exendin-4-mediated Nrf2 acetylation and nuclear translocation.**

To establish a functional role for CBP in Exendin-4-mediated acetylation of Nrf2, RNAi was employed. As shown in Fig. 6A, CBP knockdown greatly decreased nuclear CBP expression in VSMCs. Importantly, the Exendin-4-induced acetylation of Nrf2 was reduced in the presence of CBP siRNA (Fig. 6B).

To assess the nucleocytoplasmic redistribution of Nrf2 under conditions of Exendin-4 with or without CBP siRNA, we used western blotting to determine the relative levels of Nrf2 in cytoplasmic and nuclear fractions. Pretreatment of CBP siRNA significantly reversed the increasing effect of Exendin-4 on nuclear translocation of Nrf2 in Ang II-treated cells, resulting in decreased content of Nrf2 in the nuclear fraction (Fig. 6C). The decreased nuclear content of Nrf2 in Ang II-treated cells incubated with Exendin-4 plus CBP siRNA was accompanied by an increased content of Nrf2 in the cytoplasmic fraction (Fig. 6C), indicating that the Exendin-4-induced acetylation of Nrf2 favors its nuclear retention, whereas deacetylation caused by CBP knockdown results in relocalization of Nrf2 to the cytoplasmic compartment. Taken together, these results demonstrate that CBP is required for the nuclear retention of Nrf2 induced by Exendin-4.

**CBP-mediated Nrf2 acetylation contributes to Exendin-4 suppressions of Ang**
II-induced superoxidant production and VSMC senescence. Functionally, the
Exendin-4-inhibited VSMC senescence and superoxidant generation were observed
only in scrambled siRNA- transfected cells, but not in CBP siRNA-transfected cells
(Fig. 7, A and B), indicating that the Exendin-4-elicited protective effects may result
from the Nrf2-associated antioxidant gene expressions which are dependent on Nrf2
posttranslational modifications including acetylation.
DISCUSSION

In addition to excessive production of ROS, inadequate antioxidant responses in the vasculatures are thought to contribute to the pathogenesis and progression of vascular aging (2). Aging is associated with Nrf2 dysfunction in the vasculature (48). Here we show that Exendin-4 protects against Ang II-induced VSMC senescence in association with upregulations of HO-1 and NQO-1 dependent of Nrf2 signaling. Exendin-4 promotes Nrf2-CBP interaction, which results in CBP accumulation in the Nrf2 complex assembled on the target genes under oxidative stress and in turn increases Nrf2 acetylation and transcriptional activity. Most notably, acetylated Nrf2 plays an important role in Exendin-4-inhibited VSMC senescence and superoxidant production. To our knowledge, the present study is the first to provide evidence that Exendin-4 activates the Nrf2 signaling pathway in VSMCs, resulting in upregulation of antioxidant genes and eventually decreases VSMC senescence.

Nrf2 transcriptionally governs the cellular response to harmful electrophiles, xenobiotics, and ROS. Its nuclear levels decline with age (46, 48). Puddu et al (38) reported that GLP-1 abrogates AGE-induced intracellular ROS production in pancreatic β cells through upregulating Nrf2. There is evidence that Exendin-4 protects kidney from ischemia-reperfusion injury through induction of NQO-1 and HO-1 (7). GLP-1R agonist liraglutide improves outcomes after experimental myocardial infarction via Nrf2/ARE pathway (34). GLP-1 improves hepatic insulin sensitivity and antioxidant activity through upregulating Nrf2 in type 1 diabetic mice.
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(37). GLP-1 induces the expression of Nrf2-detoxifying enzyme targets to attenuate
the high glucose-induced apoptotic phenotype in endothelial cells (39). Therefore, the
increase in Nrf2 activity may represent an important mechanism for the protective
effects of GLP-1-related agents. However, little work has yet characterized the role of
Nrf2 in modulating the Exendin-4-mediated anti-senescent effect in VSMCs. In the
present study, Ang II evoked a rapid and transient increase in Nrf2 activation. The
detected increase of nuclei translocation of Nrf2 at 1 h to 3 h in Ang II-treated
VSMCs underlines the treatment-mediated formation of oxidative stress. However, at
12 h and 24 h, the Nrf2 response was impaired. No significant increases in Nrf2
translocation and ARE activation were detected in Ang II-treated cells at 12 h and 24
h. In vitro, a suppression of Nrf2 signaling by Ang II was also found after 24 hours
(18). In the spontaneously hypertensive rat, the Nrf2 response was impaired (53). In
aldosterone-mediated hypertension, the Nrf2-activation was not sufficient to protect
the organs from oxidative DNA damage (41). Similar results were reported previously
in Ang-II-treated kidney (5, 22). A possible explanation for Nrf2 suppression by Ang
II might be that Nrf2 was first upregulated, and during chronic oxidative stress,
downregulated again. Acute and transient increase in H$_2$O$_2$ production in Ang-treated
VSMCs may be beneficial during Ang II short-term stimulation, since intracellular
H$_2$O$_2$ can activate the Nrf2-mediated antioxidant response. However, chronic and
long-term stimulation with Ang II may result in overproduction of H$_2$O$_2$ in VSMCs,
which is associated with antioxidant capacity impairment and may contribute to
VSMC senescence, and cardiovascular disease. Therefore, the fact that Ang II
increased transient Nrf2 activation may indicate incomplete VSMC protection in this condition. The observed relationship between Ang II treatment, Nrf2 and antioxidant enzyme activities suggests that Ang II long-term treatment diminishes the redox-mediated, antioxidant scavenging capacity in VSMCs. Our results are consistent with those of Brand et al (5). Importantly, we demonstrate that Exendin-4 significantly promoted the nuclear translocation of Nrf2 in Ang II-treated VSMCs in a time- and dose-dependent manner. We observed that Exendin-4 triggered Nrf2 nuclear translocation with the activation of ARE-driven transcriptional activity. Therefore, these results demonstrate a posttranscriptional mechanism of Nrf2 regulation by Exendin-4. Furthermore, the Exendin-4-mediated inhibitions of VSMC senescence, superoxidant production and upregulations of HO-1 and NQO-1 were attenuated by Nrf2 siRNA. These findings support the notion that the Exendin-4-induced Nrf2 activation contributes to the suppressing effects of Exendin-4 on Ang II-induced VSMC senescence and superoxidant generation.

Our results here are particularly novel because we reveal for the first time that Exendin-4 suppressed Ang II-induced VSMC senescence by modulating Nrf2 acetylation. Phosphorylation at serine 40 allows Nrf2 accumulation in the nucleus, where it is acetylated by p300/CBP (20, 47). p300/CBP are believed to serve as transcription coactivators by acetylating core histones to facilitate chromatin decondensation (4). Histone acetyltransferase CBP is normally recruited to Nrf2 and directly modulates Nrf2 association with ARE sequences through acetylation of Nrf2.
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(47). Dl-3-n-butylphthalide, a small molecule compound has been shown to ameliorate oxidative stress by increasing the recruitment of CBP to Nrf2 in the Alzheimer's disease (49). In airway epithelial cells, Rp-cAMPS, a cAMP competitor, significantly shifts CBP association away from NF-κB and toward Nrf2, which could have potential therapeutic efficacy for reducing inflammation in patients with cystic fibrosis (55). In the present study, the induction of Nrf2 acetylation and the recruitment of CBP to Nrf2 were not observed in Ang II-treated cells. However, a significant dose-dependent increase in Nrf2 acetylation was observed in Exendin-4-treated VSMCs. To further investigate the reason for Exendin-4-induced Nrf2 acetylation, a Co-IP assay was performed to test the interaction of coactivators with Nrf2. Our results showed that Exendin-4 induced a recruitment of CBP to Nrf2; however, Exendin-4 could not enhance interaction of p300, or PCAF with Nrf2. Moreover, the Exendin-4-increased acetylation of Nrf2 was inhibited in the presence of CBP siRNA, indicating that CBP plays a central role in Exendin-4-mediated acetylation of Nrf2. It is of particular interest that Exendin-4 suppressions of Ang II-stimulated superoxidant generation and VSMC senescence were not observed in CBP siRNA-transfected VSMCs. These results clearly suggest that the Nrf2 acetylation induced by Exendin-4 is indispensable for Exendin-4-induced anti-oxidant and anti-senescent effects in VSMCs. With aging, the activity of Nrf2 is diminished (46, 48). There is decreased nuclear Nrf2 activity and decreased expression of Nrf2 target genes in aging vessels (48). Interestingly and in relation to the present study, levels of CBP decline with age in the liver, kidney, and cerebral cortex of rats (3, 8).
Thus, CBP directly binds and acetylates Nrf2 in response to Exendin-4 may constitute a novel regulatory mechanism for Exendin-4-mediated anti-senescent effect in VSMCs.

Acetylation/deacetylation plays a crucial role in the nucleocytoplasmic shuttling of Nrf2 (20, 47). We also show that Exendin-4-induced acetylation condition is crucial for the nuclear retention of Nrf2. In our study, use of CBP siRNA to knockdown CBP resulted in redistribution of Nrf2 in favor of relocalization to the cytoplasmic compartment in response to Exendin-4. It seems reasonable to conclude that the inhibitory effect of CBP siRNA on Exendin-4-induced Nrf2 nuclear translocation resulted from its inhibition of the acetyltransferase activity of CBP.

An additional aspect of the current study was to identify the signaling pathway, which mediates the activation of Nrf2/ARE pathway induced by Exendin-4. Recent evidence suggests that the cAMP/PKA cascade activates CREB, which is also known to interact with Nrf2, and synergistically increase Nrf2 target gene transcription (36). GLP-1 increased the translocation of Nrf2 to improves β-cell antioxidant capacity via PKA/ERK pathway (12). We have previously reported that phosphorylation of PI3K, p38 MAPK, JNK1/2 or ERK1/2 was not affected by Exendin-4 in Ang II-induced VSMCs (54). Here, we investigated whether Exendin-4 could activate Nrf2/ARE in Ang II-stimulated VSMCs through cAMP/PKA/CREB pathway. Our results showed that preincubation with PKI14-22 decreased Exendin-4-induced Nrf2 nuclear
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translocation and HO-1 and NQO1 upregulations. Furthermore, knock-down of CREB significantly attenuated the increasing effects of Exendin-4 on HO-1 and NQO-1 upregulations. Importantly, the Exendin-4-increased association of CBP with Nrf2 and acetylation of Nrf2 were also abrogated by PKI14-22. The involvement of cAMP/PKA/CREB in Exendin-4-induced Nrf2 activation is consistent with at least two previous reports. It is reported that GLP-1 stimulates CBP nucleo/cytoplasmic translocation via PKA (23). In addition, GLP-1 induces HO-1 and NQO-1 expressions in endothelial cells via PKA-mediated phosphorylation of CREB (3).

Activation of CREB in VSMCs by Ang II was previously reported (14, 31, 43). It is demonstrated that Ang-II-induced superoxide stimulates CREB activation in a p38MAPK and ERK1/2-dependent manner (45). In our study, a time course of Ang II treatment established an early transient induction of CREB phosphorylation in VSMCs. CREB phosphorylation was detectable within 2 min after treatment of VSMCs with 100 nM Ang II, and returned to basal levels after 15 min (Fig. 4A). Our data were consistent with the results of Molnar et al (31). Our RNA silencing experiments indicate that CREB/CBP is important for the Exendin-4-evoked Nrf2 acetylation, whereas the CREB/CBP-mediated Nrf2 acetylation is not required for Ang II induction of Nrf2. The differences may be agonist specific. The cellular and physiological consequences of CREB activation in VSMCs remain highly contested. There are some convincing reports showing that CREB is down-regulated in response to signals of proliferation and migration, and vascular or metabolic disease (17),
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implying that CREB activity is important for normal vascular physiology. In contrast, there is much evidence that CREB is activated in vascular cells by agents associated with the development of vascular disease (14). Our observations indicate that Ang II is responsible for short-term CREB phosphorylation in quiescent VSMCs, supporting the hypothesis that rapid CREB activation likely promotes defense effects by activating Nrf2. Acute, transient activation (phosphorylation from 0 to 10 min) could be a factor relating to disease progression, where transient activation may modulate VSMC function, predisposing the cells to a different response in the case of subsequent chronic stimulation. Ang II elevation of CREB phosphorylation in VSMC represents a potentially important negative feedback mechanism to attenuate Ang II signaling and maintain VSMC homeostasis.

It has been hypothesised that, vascular smooth muscle cell senescence promotes the thinning of fibrous caps and the instability of atherosclerotic plaques (16). Telomere dysfunction is involved in replicative and stress-induced senescence (13). Telomere shortening might be an independent risk factor for atherosclerosis through various biological ageing pathways, such as cellular senescence (6, 15, 51). There is conflicting evidence in the literature on the association between telomere length and cardiovascular disease. It is reported that there is no causal role of short telomere length for the development of cardiovascular diseases (9, 11, 44). Therefore, defining with certainty the direct role of telomeres in and the effect of telomere length on the atherosclerotic process is still difficult.
In conclusion, our present study demonstrates a novel mechanism for the anti-senescent and anti-oxidant effects of Exendin-4 in VSMCs, namely that increased cAMP/PKA signaling in response to Exendin-4 enhances CBP interaction with Nrf2, thereby significantly increasing the acetylation and accumulation of Nrf2 in the nucleus and promoting its transcriptional activity. Thus, Nrf2 posttranslational modifications including phosphorylation at Ser40 and acetylation by CBP as a novel regulatory mechanism will shed light on how Exendin-4 inhibits Ang II-induced VSMC senescence and superoxidant production. GLP-1-based therapies are currently in use for glycemic control in type 2 diabetes. Based on our findings, GLP-1 as a direct antioxidant may be an effective treatment to preserve normal vascular function under conditions of oxidative stress, and thus improves outcomes in this significant patient population.
This study was supported by National Natural Science Foundation of China Grants 30700292, 30971160 and 31271493 (to X. M. Qin).
DISCLOSURES

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

Author contributions: T.F.Z., and X.M.Q. conception and design of research; T.F.Z., L.Z., A.Q.L. and M.Q.Z. performed experiments; T.F.Z. and X.M.Q. analyzed data; T.F.Z. and X.M.Q. interpreted results of experiments; T.F.Z. and X.M.Q. drafted manuscript; T.F.Z. and X.M.Q. edited manuscript; T.F.Z., M.Q.Z., L.Z., A.Q.L. and X.M.Q. approved final version of manuscript.
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47. **Sun Z, Chin YE, Zhang DD.** Acetylation of Nrf2 by p300/CBP augments promoter-specific DNA binding of Nrf2 during the antioxidant response. *Mol Cell*
Exendin-4 suppresses VSMC senescence via Nrf2 activation


FIGURE LEGENDS

Fig. 1. Exendin-4 treatment protects VSMCs from Ang II-induced VSMC senescence and superoxidant production. A: Representative images of VSMCs stained with SA β-galactosidase. Serum starved VSMCs were preincubated with 10 nM Exendin-4 for 12 h before stimulation with 100 nM Ang II for 3 days. Scale bar, 500 μm. The lower bars show the mean ± S.E. percentage of SA β-gal-positive cells of eight independent experiments.* P < 0.05 vs. untreated; # P <0.05 vs. Ang II. B: Phosphorylation of γ-H2AX at various time points from 0 to 24 h. VSMCs were preincubated with 10 nM Exendin-4 for 12 h before stimulation with 100 nM Ang II for 1, 3, 6, 12 and 24 h. C: Quantitative analysis of superoxide anion production. 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. Results are means ±S.E. of six independent experiments. *P < 0.05 vs. untreated; #P<0.05 vs. Ang II.

Fig. 2. Exendin-4 increases Nrf2 nuclear localization, antioxidant response element (ARE) activation, and HO-1 and NQO-1 expression in Ang II-stimulated VSMCs. A: Effects of Ang II or Exendin-4 alone on the translocation of Nrf2 into the nucleus. VSMCs were incubated with 100 nM Ang II or 10 nM Exendin-4 for the indicated periods of time. Nuclear proteins were prepared as described in MATERIALS AND METHODS and subjected to western blotting. Anti-histone H3 was used as loading controls for nuclear proteins. B: Representative blots of nuclear and cytosolic Nrf2 expression in VSMCs. VSMCs were incubated with 10 nM Exendin-4 for the indicated periods of time before stimulation with 100 nM Ang II for 12 h. Cytoplasmic and nuclear proteins were prepared and subjected to western
Exendin-4 suppresses VSMC senescence via Nrf2 activation

blotting. Anti-histone H3 and β-actin were used as loading controls for nuclear and cytosolic proteins, respectively. n=4. C: VSMCs were incubated with various concentrations of Exendin-4 for 1 h before stimulation with 100 nM Ang II for 12 h. Cytoplasmic and nuclear fractions of VSMCs were extracted and Nrf2 protein was examined as in B. D: Exendin-4 enhanced Nrf2 nuclear localization. VSMCs were treated with 10 nM Exendin-4 for the indicated periods of time before stimulation with 100 nM Ang II for 12 h. Subcellular localization of Nrf2 was monitored under a confocal microscope. Scale bar, 50 μm. n=9. E: Representative western blots of phosphorylated Nrf2 at Ser40 (phospho-Nrf2) and total Nrf2 in the whole cell lysates of VSMCs. Quiescent VSMCs were incubated with Exendin-4 for 1 h before stimulation with 100 nM Ang II for 12 h. n=3. F: Relative ARE-driven luciferase activity in human VSMC cell line. Human VSMC cell line were transfected with the ARE4-luciferase constructs for 24 h before incubation with 10 nM Exendin-4 for 12 h, followed by treatment with 100 nM Ang II for 0, 1, 3, 6 or 12 h. Results are means ±S.E. of five independent experiments. *P < 0.05 vs. untreated. G: Effects of Ang II alone or Exendin-4 alone on the expression of HO-1 and NQO-1. H: Representative western blotting results showing that Exendin-4 upregulates HO-1 and NQO-1 time- and dose-dependently in Ang II-treated cells. Cells were cultured with 10 nM Exendin-4 for 3 h, 6 h, 12 h and 24 h, or various concentrations of Exendin-4 for 12 h before stimulation with 100 nM Ang II for 12 h, after which HO-1 and NQO-1 expression were determined by western blotting.
Fig. 3. Blockage of Nrf2 signaling attenuated the suppressive effects of Exendin-4 on VSMC senescence and superoxidant generation. A: VSMCs were transfected with Nrf2 siRNA or scramble siRNA (100 nM) for 48 h, and western blot analyses were performed using Nrf2 antibody. B: VSMCs were transfected with Nrf2 siRNA or scramble siRNA for 48 h before stimulation with 10 nM Exendin-4 (1 h for Nrf2, 12 h for HO-1 and NQO-1). β-Actin was used as a loading control. Representative images from three independent experiments were shown. C: Nrf2 knockdown decreased the attenuating effect of Exendin-4 on Ang II-induced VSMC senescence. VSMCs were transfected with Nrf2 siRNAs or scramble siRNA for 48 h before incubated with Exendin-4 (10 nM) for 12 h, followed by Ang II treatment for 3 days. Cells were stained for SA β-gal. n=7. * P < 0.05 vs. untreated; # P<0.05 vs. Ang II. D: VSMCs were treated as in C, but Ang II treatment for 12 h, and H2O2 levels were measured by the Amplex red assay. n=6. * P < 0.05 vs. untreated; # P<0.05 vs. Ang II.

Fig. 4. PKA-CREB pathway is required for the Exendin-4-activated Nrf2/ARE signaling. A: VSMCs were incubated with Ang II for indicated periods of time. phospho-CREB and CREB expressions were determined by western blot. n=3. B: VSMCs were incubated with Exendin-4 for indicated periods of time. phospho-PKA, PKA, phospho-CREB and CREB expression were determined by western blot. n=3. C: Exendin-4 activated CREB via PKA. VSMCs were preincubated PKA inhibitor PKI14-22 for 30 min before stimulation with 10 nM Exendin-4 for 30 min. The cell lysates were immunoblotted for phosphorylated CREB at Ser 133 and reprobed for
Exendin-4 suppresses VSMC senescence via Nrf2 activation

D: Effect of PKA inhibition on Exendin-4-mediated increase in Nrf2 activity. Human VSMC cell line were transfected with the ARE4-luciferase constructs for 24 h before incubation with PKI14-22 for 30 min. The cells were then stimulated with 10 nM Exendin-4 for 12 h, followed by treatment with Ang II for 12 h. Relative ARE4-driven luciferase activity was measured. n=6. Open bar represents the empty vector group. *P < 0.05 vs. untreated; #P <0.05 vs. Ang II+Exendin-4.

E: Representative western blots of HO-1 and NQO-1 expression. VSMCs were preincubated with PKI14-22 for 30 min before stimulation with 10 nM Exendin-4 for 12 h, followed by treatment with 100 nM Ang II for 12 h. Whole cell lysates were subjected to western blot analysis with antibodies against HO-1 and NQO-1. n=3.

F: Representative western blots of NQO-1 and HO-1 in VSMCs transfected with CREB siRNA or scramble siRNA for 48 h before incubation with 10 nM Exendin-4 for 12 h, followed by treatment with 100 nM Ang II for 12 h.

Fig. 5. Exendin-4 enhanced the acetylation of Nrf2 and the association of CBP with Nrf2 via GLP-1R/PKA pathway.

A: Ang II had no effect on Nrf2 acetylation in VSMCs. VSMCs were treated with 100 nM Ang II for different periods of time. Acetylation was measured in whole cell lysates by a co-immunoprecipitation (Co-IP) assay. The whole cell lysates (100 μg of protein) were first subjected to immunoprecipitation (IP) with anti-Nrf2 antibody, followed by immunoblotting (IB) of the IP samples, using anti-acetyl-lysine antibody. β-actin was used as loading control. n=3.

B: Representative blots indicated the changes in Nrf2 acetylation in
VSMCs treated with Exendin-4 at 0.1-100 nM for 1 h. Acetylation was measured as described in A. C: Ang II had no effect on the binding of CBP and Nrf2. VSMCs were treated with 100 nM Ang II for 1 h, and whole cell protein was extracted for performing Co-IP with anti-p300, anti-CBP, anti-PCAF and IgG (as negative control), followed by western blotting with anti-Nrf2. The lower panels show Co-IP efficiency by reprobing with anti-p300, anti-CBP and anti-PCAF. D: Exendin-4 increased the recruitment of CBP to Nrf2. VSMCs were treated with 10 nM Exendin-4 for 1 h, and whole cell protein was extracted for performing Co-IP as described in C. E: VSMCs were pretreated with PKA inhibitor PKI14-22 for 30 min before incubation with Exendin-4 (10 nM) for 1 h. Nrf2 acetylation levels were measured. β-actin was used as loading control. F: VSMCs were pretreated with GLP-1R antagonist exendin (9-39) or PKI14-22 for 30 min before incubation with Exendin-4 (10 nM) for 1 h. The cell lysates were immunoprecipitated with anti-CBP antibody, followed by western blotting with anti-Nrf2 antibody. The lower panels show Co-IP efficiency by reprobing with anti-CBP. β-actin were used as loading controls.

Fig. 6. CBP is responsible for Exendin-4-mediated acetylation of Nrf2 and nuclear translocation. A: VSMCs were transfected with CBP siRNA or scramble siRNA (80 nM) for 48 h, and western blotting analyses were performed by using antibodies against CBP and histone H3. B: Effects of CBP siRNA on the Exendin-4-evoked acetylation of Nrf2. VSMCs were transfected with CBP siRNA or scramble siRNA for 48 h before incubated with 10 nM Exendin-4 for 1 h. Cytoplasmic extracts were
isolated and subjected to IP with Nrf2 antibody followed by IB for acetyl-lysine. β-actin were used as loading controls. C: CBP contributes to the Exendin-4-induced Nrf2 nuclear translocation. VSMCs were transfected with CBP or scramble siRNA for 48 h before incubated with 10 nM Exendin-4 for 1 h. Cytoplasmic and nuclear fractions of VSMCs were extracted and endogenous Nrf2 protein was probed with anti-Nrf2 antibody. Anti-histone H3 and β-actin were used as loading controls for nuclear and cytosolic proteins, respectively.

Fig. 7. The CBP-mediated Nrf2 acetylation contributes to the attenuating effects of Exendin-4 on premature senescence and superoxidant production in VSMCs. A: VSMCs were transfected with CBP siRNA or scramble siRNA for 48 h before incubated with 10 nM Exendin-4 for 12 h, followed by Ang II treatment for 3 days. SA β-gal was examined. n=7. \( *P < 0.05 \) vs. untreated; \( #P < 0.05 \) vs. Ang II. B: VSMCs were treated as in A, but Ang II treatment for 12 h, and \( \text{H}_2\text{O}_2 \) levels were measured. \( n=6. \) \( *P < 0.05 \) vs. untreated; \( #P < 0.05 \) vs. Ang II.
Fig. 2

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Luciferase activity (fold of control)

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control  EX-4  ANG II+EX-4

* * *
Fig. 3

A

Scramble  si Nrf2

57 kDa

Nrf2

42 kDa

β-actin

B

EX-4  -  +  +  +  +  +

ANG II  -  +  +  +  +  +

scramble  -  -  +  +  +  +
si Nrf2  -  -  -  +  +  +

HO-1

NQO-1

β-actin

C

SA β-gal positive cells (%)

ANG II  -  +  +  +  +  +

EX-4  -  +  +  +  +  +

scramble siRNA  -  -  +  +  +  +
si Nrf2  -  -  -  +  +  +

D

H₂O₂ production (% of control)

ANG II  -  +  +  +  +  +

EX-4  -  +  +  +  +  +

scramble siRNA  -  -  +  +  +  +
si Nrf2  -  -  -  +  +  +
Fig. 7

A

SA β-gal cells (%)

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B

H$_2$O$_2$ production (% of control)

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