Research paper

CO-releasing molecules CORM2 attenuates angiotensin II-induced human aortic smooth muscle cell migration through inhibition of ROS/IL-6 generation and matrix metalloproteinases-9 expression

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ABSTRACT

Ang II has been involved in the pathogenesis of cardiovascular diseases, and matrix metalloproteinase-9 (MMP-9) induced migration of human aortic smooth muscle cells (HASMCs) is the most common and basic pathological feature. Carbon monoxide (CO), a byproduct of heme breakdown by heme oxygenase, exerts anti-inflammatory effects in various tissues and organ systems. In the present study, we aimed to investigate the effects and underlying mechanisms of carbon monoxide releasing molecule-2 (CORM-2) on Ang II-induced MMP-9 expression and cell migration of HASMCs. Ang II significantly up-regulated MMP-9 expression and cell migration of HASMCs, which was inhibited by transfection with siRNA of p47 phox, Nox2, Nox4, and pretreatment with the inhibitors of NADPH oxidase, ROS, and NF-κB. In addition, Ang II also induced NADPH oxidase/ROS generation and p47phox translocation from the cytosol to the membrane. Moreover, Ang II-induced oxidative stress and MMP-9-dependent cell migration were inhibited by pretreatment with CORM-2. Finally, we observed that Ang II induced IL-6 release in HASMCs via AT1R, but not AT2R, which could further cause MMP-9 secretion and cell migration. Pretreatment with CORM-2 reduced Ang II-induced IL-6 release. In conclusion, CORM-2 inhibits Ang II-induced HASMCs migration through inactivation of suppression of NADPH oxidase/ROS generation, NF-κB inactivation and IL-6/MMP-9 expression. Thus, application of CO, especially CORM-2, is a potential countermeasure to reverse the pathological changes of various cardiovascular diseases. Further effects aimed at identifying novel antioxidant and anti-inflammatory substances protective for heart and blood vessels that targeting CO and establishment of well-designed in vivo models properly evaluating the efficacy of these agents are needed.
1. Introduction

Angiotensin II (Ang II) plays an important role in the pathogenesis of several cardiovascular diseases including cardiomyopathy, coronary artery disease, atherosclerosis and vascular injury [1]. Ang II is the key effector peptide of the rennin-angiotensin system (RAS) and exerts the vasculature remodeling through mechanisms of promoting cell proliferation, fibrosis, oxidative stress and inflammation [2,3]. All these processes lead to initiation of vascular damage. There have been several studies that demonstrated the beneficial effect of inhibition of the RAS system, or directly inhibition of Ang II, on the prevention or treatment of cardiovascular disease [4–6].

After vascular damage, the pathogenesis of these cardiovascular diseases includes the degradation of the extracellular matrix, through various inflammatory reactions, generation of reactive oxygen species (ROS) and matrix metalloproteinase (MMPs) [7,8]. The relationship of cardiovascular disease includes the degradation of the extracellular matrix, through Ang II is the key of several cardiovascular diseases including cardiomyopathy, coronary artery disease, atherosclerosis and vascular injury [1]. Ang II activates signaling response through the Ang II type 1 receptor (AT1R) and NADPH oxidase, which lead to ROS generation in vascular smooth muscle cell (VSMC), and subsequently, the proliferative, migratory and inflammatory responses of VSMC [9,12]. Carbon monoxide (CO) is endogenously produced in cells and tissues as the byproduct of heme oxygenase (HO) enzymes after heme catalytic activity [13,14]. Although CO is toxic to human bodies at high concentration, low-dose exogenous CO (approximately 250–500 ppm) has been demonstrated to have protective functions, anti-inflammatory and antiapoptotic effects in various human diseases [15–18]. Furthermore, accumulating studies over the past decade have shown that low concentration of CO, or CO-releasing molecules (CORMs), can confer several molecular mechanisms, including eliminating microbes, regulating cell death and organ dysfunction, and promote immune response through resolving mediator-heme oxygenase-1 circuits [19], activation of p38 MAPK, enhanced PPAR-γ singaling, and suppression of TLRs translocation [13,16,20]. Among them, the most characterized CO-RMs is the lipid-soluble tricarbonyl-dichlororuthenium (II) dimmer (CORM-2) [21–23]. We hypothesized that CORM-2 might be potentially as an anti-inflammatory modulator and therapeutic agent of vascular inflammation.

CORM-2 has been proven to inhibit MMP-1 and MMP-2 expression in human lung epithelial cell [24]; however, the most important MMPs involved in cardiovascular diseases is MMP-9 [25]. MMP-9 plays an important role of vascular remodeling and inflammation through directly cleaving the extracellular matrix and activation of various chemokines and cytokines [25], MMP-9 inhibition has been proven significantly beneficial in multiple animal models of cardiovascular disease [26–28]. In this present study, we aimed to investigate the therapeutic potential of CORM2 on inhibition of VSMC migration, a hallmark of cardiovascular disease, and the underlying mechanisms of CORM2 on MMP-9 inhibition.

2. Results

2.1. Ang II induces MMP-9-dependent cell migration

Here, we examined the effect of Ang II on the cell viability of human aortic smooth muscle cells (HASMCs). As shown in Fig. 1A, Ang II (1, 5, and 10 μM) had no effects on the cell viability of HASMCs. However, treatment of 100 μM Ang II for 24 h significantly increased the cell viability of HASMCs. Ang II acts via binding to receptor types AT1R and AT2R [12]. In our study, we found that 10 μM Ang II markedly induced AT1R and AT2R mRNA levels in HASMCs (Fig. 1B). We further determined the effect of Ang II treatment on MMP-9 expression in HASMCs. As shown in Fig. 1C, cells were treated with Ang II for 2, 4, 6, 16, or 24 h, and then the protein expression of MMP-9 was determined by Western blot analysis. We showed that Ang II induced MMP-9 protein expression in a time-dependent manner. Ang II-induced MMP-9 expression was significantly increased within 6 h and continued to increase over 24 h. In addition, Ang II also induced MMP-9 mRNA levels and promoter activity in these cells (Fig. 1D and E). The biological importance of MMPs has been described in multiple cellular processes including proliferation, angiogenesis, migration, host defense, cancer invasion, and metastasis [29]. Indeed, we showed that Ang II induced migration of HASMCs, which was reduced by transfection with MMP-9 siRNA. Taken together, these data suggest that Ang II induces MMP-9-dependent migration of HASMCs.

2.2. CORM-2 inhibits Ang II-induced MMP-9 expression and cell migration

Rodriguez et al. found that induction of HO-1 or CO, but not biliverdin or bilirubin, inhibited VSMC migration [30]. Here, we examined the effect of CORM-2 on the cell viability of HASMCs. As shown in Fig. 2A, CORM-2 (1, 10, 50, and 100 μM) had no effects on the cell viability of HASMCs. To further examine the mechanisms which end metabolites of heme catalysis by HO-1, including CO, contribute to the attenuation of Ang II-mediated responses in HASMCs, cells were incubated with the CO donor, CORM-2 before the addition of Ang II. The results showed that pretreatment with CORM-2 caused a significant decrease in Ang II-induced MMP-9 concentration, pro-MMP-9 expression, cell migration, MMP-9 mRNA levels, and MMP-9 promoter activity (Fig. 2B–E). However, as shown in Fig. 2B, D and E, the addition of CO scavenger, hemoglobin, significantly reversed the attenuating effects of CORM-2 on MMP-9 concentration, cell migration, MMP-9 mRNA levels, and MMP-9 promoter activity induced by Ang II, indicated that CORM-2 contributes to protection of cells from Ang II insult through at least partly a CO-dependent attenuation of MMP-9 expression and migration of HASMCs.

2.3. Ang II induces MMP-9 expression via NADPH oxidase/ROS in HASMCs

Sun et al. found that salusin-β promotes vascular smooth muscle cell migration and intimal hyperplasia after vascular injury via ROS/NF-κB/MMP-9 pathway [31]. In addition, ROS is a family of non-phagocytic NADPH oxidases, including the prototypic Nox2 homologous-based NADPH oxidase, as well as other NADPH oxidases, such as Nox1 and Nox4. Thus we investigated whether NADPH oxidase was involved in Ang II-induced MMP-9 expression in HASMCs. As shown in Fig. 3A, transfection with Nox2 or Nox4 siRNA markedly inhibited Ang II-induced pro-MMP-9 expression. Activated NADPH oxidase is a multi-meric protein complex consisting of at least three cytosolic subunits of p47phox, p67phox, and p40phox. Phosphorylation of p47phox leads to a conformational change allowing its interaction with p22phox [32]. Moreover, we showed that transfection with siRNA of p47phox, Nox2, or Nox4 markedly inhibited Ang II-induced MMP-9 concentration in HASMCs (Fig. 3B). Ang II-enhanced migration of HASMCs was also reduced by transfection with these three siRNAs (Fig. 3C). Finally, we demonstrated that pretreatment with the inhibitor of ROS (NAC) or NADPH oxidase ( DPI or APO) inhibited Ang II-induced MMP-9 mRNA levels and promoter activity in these cells (Fig. 3D). Taken together, these results suggest that Ang II induces MMP-9 expression via NADPH oxidase and ROS in HASMCs.

2.4. CORM-2 inhibits Ang II-induced NADPH oxidase activation and ROS generation

Cepinskas et al. showed that CORM-2-released CO attenuated LPS-induced production of ROS and NO [33]. Here we also investigated whether CORM-2 could inhibit Ang II-induced ROS generation and...
NADPH oxidase activation in HASMCs. As shown in Fig. 4A, treatment of Ang II markedly enhanced intracellular ROS production in a time-dependent manner. Moreover, transfection with siRNA of Nox2, Nox4, or p47phox inhibited Ang II-induced ROS generation in HASMCs (Fig. 4B). On the other hand, Ang II time-dependently induced NADPH oxidase activity in these cells (Fig. 4C). We further found that...
Ang II induced p47phox translocation from the cytosol to the membrane, which was inhibited by pretreatment with CORM-2 (Fig. 4D). Finally, we demonstrated that CORM-2 could reduce Ang II-induced ROS generation and NADPH oxidase activity in HASMCs (Fig. 4E). Thus these data suggest that CORM-2 can reduce MMP-9 expression via inhibition of the ROS generation and NADPH oxidase activation in HASMCs.

2.5. CORM-2 reduces Ang II-induced NF-κB activation in HASMCs

NF-κB was shown to be involved in MMP-9 regulation in response to various stimuliators [34,35]. ROS have been shown to regulate NF-κB activation [36]. Moreover, we showed that transfection with p65 siRNA markedly reduced Ang II-induced MMP-9 concentration and migration of HASMCs (Fig. 5A and B). In addition, we also found that pretreatment with the inhibitor of NF-κB (HLN or Bay11-7082) inhibited Ang II-induced MMP-9 mRNA levels and promoter activity in these cells (Fig. 5C). On the other hand, Ang II time-dependently induced NF-κB promoter activity (Fig. 5D), which was reduced by pretreatment with CORM-2, NAC, APO, DPI, and HLN (Fig. 5E). Finally, we showed that Ang II markedly induced NF-κB p65 activation, which was reduced by CORM-2, NAC, or HLN (Fig. 5F). Therefore we suggest that CORM-2 also can inhibit MMP-9-dependent migration via inhibition of the NF-κB activation in HASMCs.

2.6. CORM-2 reduces Ang II-induced MMP-9 expression via inhibition of the AT1R/IL-6 pathway in HASMCs

IL-6 has been shown to regulate cell migration in various cell types [37,38]. Ang II has also been shown to induce interleukin-6 (IL-6) expression in astrocytes [39]. Here we investigated whether Ang II could induce IL-6-dependent MMP-9 expression in HASMCs. As shown in Fig. 6A, Ang II induced IL-6 release in a time-dependent manner. Moreover, we found that Ang II-induced MMP-9 concentra-
tion and migration of HASMCs were reduced by transfection with IL-6 siRNA (Fig. 6B and C). On the other hand, we showed that pretreatment with HLN, NAC, APO, or CORM-2 could inhibit Ang II-induced IL-6 release (Fig. 6D). Ang II acts via binding to receptor types AT1R and AT2R [12]. Finally, we demonstrated that Ang II-induced IL-6 and MMP-9 production was reduced by transfection with AT1R siRNA, but not AT2R siRNA in HASMCs (Fig. 6E and F). Taken together, we suggest that CORM-2 inhibits Ang II-induced MMP-9 expression via inhibition of the AT1R-dependent IL-6 release in HASMCs.

3. Discussion

In this study, we demonstrated that Ang II induced migration of HASMCs is through AT1R and activation of NADPH oxidase/ROS/NF-κB/IL-6-dependent MMP-9 expression. We also demonstrated CORM-2 inhibited Ang II-induced NADPH oxidase/ROS generation, NF-κB activation, IL-6 release, and MMP-9-dependent HASMCs migration. We found the benefits of CORM-2 in reducing the HASMCs, a hallmark of vascular remodeling, so administration of exogenous CO may serve as a potential therapeutic strategy for many cardiovascular diseases. Therefore, further efforts aimed at identifying novel antioxidant and anti-inflammatory substances protective for heart and blood vessels that targeting CO and establishment of well-designed in vivo models properly evaluating the efficacy of these agents are needed.

Ang II is previously identified as a potent vasoconstrictor but now it has been recognized as the key vasoactive peptide that plays an important role in the pathogenesis of vascular disease, including cell growth, inflammation, migration, and apoptosis of VSMCs [40,41]. It is well known that Ang II contributes to hypertension and atherosclerosis. Recently, there have been more studies investigating the molecular mechanisms and effects of Ang II on the blood vessels, including induction of Egr-1 expression [42], AT1R and p38MAPK pathway interactions [43], sphingosine-1-phosphate induced signaling axis [44], and Toll-like receptor 4 (TLR4) signaling pathway [45]. Although it has been demonstrated that Ang II can induce MMP-9 expression in VSMCs via a NF-κB dependent pathway [46], we found the NF-κB binding activity is through AT1R, but not AT2R. Furthermore, the effect of Ang II to induce IL-6 and ROS generation on the HASMCs was
also found in the central nervous system [47]. Therefore, more detailed elucidation of the mechanisms involved in Ang II pro-inflammatory effects in the cardiovascular system can contribute to the development of novel therapeutic strategies, which can also treat hypertension through central nervous system [48].

Unbalanced production of ROS has been reported in various pathological conditions of cardiovascular systems [49–51]. NADPH oxidase in the plasma membrane and mitochondria are the major sources of ROS. In this report we found Ang II triggers the formation of ROS via AT1R and NADPH oxidase in the plasma membrane, with a subsequent increase in oxidative damage. In this study, we also found that Ang II induced MMP-9 expression and cell migration is related to NADPH oxidase activation and intracellular ROS production. In HASMCs challenged with CORM-2, Ang II-enhanced ROS generation and NADPH oxidase activity in HASMCs were attenuated. Thus CORM-2 may inhibit MMP-9 expression through this antioxidant property.

Heme oxygenase-1 (HO-1) and its enzymatic product, carbon monoxide (CO), has been demonstrated using transgenic animal models to show its protective effects against the inflammatory response and oxidative stress in rats since more than decades ago [52–54]. CORMs have been proven as a potential therapeutic regimen in several...
cardiovascular diseases, especially those involved vascular inflammation [54–56]. Several pathways were demonstrated to be involved in the anti-inflammatory effect of CORMs on VSMCs in various models of systemic or pulmonary vascular remodeling [54–58], which confirmed the protective role of CORMs through relaxation of vascular tone and reduction of VSMCs migration. In this paper we have described for the first time to our knowledge that CORM-2 can be used to reverse the pathological features of common cardiovascular disease. We found Ang II induced over-production of ROS was paralleled to NF-κB activation and the pro-inflammatory cytokine IL-6, which result in the activation of MMP-9 and migration of HASMCs. CORM-2 can exert anti-inflammatory effects through inhibition of NADPH oxidase [59,60].
Therefore, the blockade of ROS generation by CORM-2 can be a preventive strategy of cardiovascular diseases. NF-κB is the transcriptional factor that involves both pro- and anti-inflammatory roles [61]. Because NF-κB is extensively involved in the signaling pathways of various human diseases, there have been numerous studies toward development of inhibitors in this mediator [61,62]. In this report, we found NADPH oxidase/ROS have a significant regulatory role in NF-κB activation, which has been demonstrated in various cell types [63,64]. In addition, activation of NF-κB activity has been shown to induce MMP-9 expression in multiple types of human diseases and human cancer cells [63,65,66]. Hence, we examined whether NF-κB is involved in Ang II-induced upregulation of MMP-9 in HASMCs. Here we showed that Ang II induced NF-κB activation and enhanced NF-κB promoter activity in HASMCs, which could regulate MMP-9 expression and cell migration. Moreover, CORM-2 could inhibit Ang II-induced MMP-9 expression via inhibition of the activation of NF-κB in HASMCs.

Although NF-κB has been shown to induce expression of MMP-9, and then promotes cell migration, metastasis, and invasion [65–67], rare studies have investigated whether NF-κB directly binds to MMP-9 and controls the transcription of MMP-9 in these cells. Here we showed that the interaction between NF-κB and MMP-9 in HASMCs is mediated through IL-6 activation via IL-6 receptors. Increased IL-6 expression has been found to be associated with a variety of cardiovascular diseases, including atherosclerosis, obesity, myocardial infarction and type II diabetes [68,69]. IL-6 release can be stimulated by chronic inflammations, acute infections, physiological stress and sometimes obesity [69]. Furthermore, IL-6 is one of the most highly

Fig. 6. CORM-2 reduces Ang II-induced MMP-9 expression via inhibition of the AT1R/IL-6 pathway in HASMCs. (A) Cells were treated with 10 μM Ang II for the indicated times, and then the production of IL-6 was measured. Cells were transfected with siRNA of scrambled or IL-6, and then treated with Ang II for 24 h. The concentration of MMP-9 was determined (B). The cell migration was determined by migration assay (C). (D) Cells were pretreated with HLN, NAC, APO, or CORM-2, and then treated with Ang II for 24 h. The production of IL-6 was measured. (E, F) Cells were transfected with siRNA of scrambled, AT1R, or AT2R, and then treated with Ang II for 24 h. The production of IL-6 and MMP-9 was measured. Data are expressed as mean ± S.E.M. of three independent experiments. *P < 0.05; #P < 0.01, as compared with the basal level (A). *P < 0.01, as compared with the cells exposed to Ang II + scrambled siRNA (B, C, E, F).
cytokines induced by NF-κB [70]. Although IL-6-induced expression of MMP-9 in macrophage has been demonstrated in the pathogenesis of chronic inflammation and cancer [71,72], the role of IL-6 linked to MMP-9 in cardiovascular diseases was rarely investigated. Indeed we found that Ang II markedly induced IL-6 release, which further caused MMP-9 induction and HASMCs migration. Moreover these responses induced by Ang II were mediated via AT1R, but not AT2R. CORM-2 can reduce MMP-9 expression via inhibition of IL-6 release in HASMCs.

Indeed, we also observed that Ang II induced MMP-2 concentration and the inhibition of MMP-2 also reduced Ang II-induced cell migration (Supplementary Fig. 1). Finally, we found that CORM-2 pretreatment could decrease Ang II-induced MMP-2 concentration. Thus, we suggest that MMP-2 also possibly plays a role in regulating Ang II-induced cell migration. However, in this study, we focused on the signaling pathways involved in Ang II-induced COX-2/PGE2/IL-6/MMP-9 expression and migration of HASMCs. Moreover, pretreatment with CORM-2 inhibited Ang II-induced NADPH oxidase/ROS generation, NF-κB activation, IL-6 release, and MMP-9-dependent cell migration. Thus, as induction of exogenous CO may serve as a potential therapeutic target, continuous efforts toward identifying novel heart and blood vessels protective antioxidants/anti-inflammatory substances that targeting CO and establishment of well-designed in vivo models properly evaluating the efficacy of these agents will be warranted.

4. Materials and methods

4.1. Materials

Cell Signaling (Danver, MA) provided us the anti-phospho-p65 antibody. Helenalin (HLN), Bay11-7082 (Bay), diphenyleneiodonium (DPI), N-acetyl-l-cysteine (NAC), and apocynin (APO) were purchased from Biomol (Plymouth Meetings, PA). Anti-GAPDH, anti-GoS, and anti-MMP-9 antibodies were purchased from Santa Cruz (Santa Cruz, CA). We bought the Bicinchoninic acid (BCA) protein assay from Pierce (Rockford, IL). Tricarboxydichlororuthenium (II) dimer (CORM-2), hemoglobin (Hb), enzymes, Ang II, and other chemicals were obtained from Sigma (St. Louis, MO).

4.2. Cell culture

HASMCs were obtained as cryopreserved tertiary cultures from Cascade Biologies (OR) and grown in culture flasks in smooth muscle cells growth medium M231 (Cascade Biologies, Inc.) This medium was supplemented with fetal bovine serum (FBS, 5%), human basic fibroblast growth factor (3 ng/ml), human epidermal growth factor (10 ng/ml), Fungizone (1.25 mg/ml), streptomycin (100 pg/ml), penicillin (100 U/ml), and insulin (10 mg/ml) in a humidified 5% CO2 atmosphere at 37 °C. We changed the growth medium every other day until the cells became confluent, then we passed these cells, and cultured until the cells grew to confluence again. We used cells at between passages 3 and 7 in this study.

4.3. Transient transfection with siRNAs

We purchased the human siRNAs of scrambled, Nox2, MMP-9, p47phox, p65, IL-6, Nox4, AT1R, and AT2R from Sigma (St. Louis, MO). Transient transfections with various siRNAs (100 nM) were performed using Lipo2000 transfection reagent in accordance with the manufacturer’s protocols. The siRNA used in this study were labeled with EGFP. The transfection efficiency was evaluated by transfection with EGFP, and was determined to be approximately 60%.

4.4. Real-Time PCR

Total RNA of HASMCs was extracted using TRIzol reagent and reverse-transcribed into cDNA. We used real-time PCR and SYBR Green PCR reagents (Applied Biosystems, Branchburg, NJ, USA) and primers specific for MMP-9 and GAPDH mRNAs to analyze the reverse-transcribed cDNA from mRNA. We determined the levels of MMP-9 mRNA expression by normalizing to that of GAPDH expression.

4.5. Migration assay

HASMCs were cultured to confluence in 10-cm dishes and starved with serum-free M231 for 24 h. We used a blade to scratch the monolayer cells to create extended and clear scratches in the center of the dishes in order to make a bright and clear field. We washed the cells once with PBS to remove the detached cells. After preincubation with the various inhibitors for 1 h, we added serum-free M231 with or without Ang II to each dish which contained a DNA synthesis inhibitor hydroxyurea (10 μM). We obtained images of migratory cells from the scratched boundary at 0 and 24 h using a digital camera (Canon, Japan) and a light microscope (Olympus, Japan). We counted the migratory cells numbers from the resulting four phase images for each point and then made an average for each experimental condition. This experiment was repeated, and we generated the data from at least four independent assays.

4.6. Isolation of cell fractions

The cytosolic and membrane fractions were isolated according to the standard procedure [32]. Cells were lysed in bysis buffer, and then centrifuged at 16,000g for 20 min at 4 °C. The supernatant which represented the cytosolic fraction was collected. Following centrifugation, the pellet (representing the membrane fraction) was collected.

4.7. Cell viability

The MTT assay was used for evaluation of cell viability. HASMCs were seeded onto 96-well plates overnight. After treatment with different concentrations of Ang II or CORM-2, MTT solution (5 mg/ml) was added. Following incubation for 2 h at 37 °C, the plates were read using a microplate spectrophotometer at a wavelength of 540 nm.
4.8. Measurement of MMP-9 luciferase activity

The human MMP-9 promoter activity was evaluated using the luciferase assay kit (Promega, Madison, WI, USA) as described previously [73]. The cells were transfected with the MMP-9-luc plasmid (~720 to ~11 bp). Following treatment, luciferase assay solution was added, and the luminescence was determined using a luminometer. The promoter luciferase activities were standardized to β-galactosidase.

4.9. Gelatin zymography

Gelatin zymography is used to detect gelatinase activity, specially MMP-2 and -9. In this study, we seeded HASMCs onto 6-well culture plates and made them quiescent at confluence after incubation in serum-free M231 for 24 h. We used growth-arrested cells, which were incubated with Ang II for the indicated time intervals at 37 °C. We collected the culture medium and then the medium was centrifuged at 10,000×g for 5 min at 4 °C to remove cell debris. The MMP-9 expression was analyzed according to standard procedure, which has been described in our previous studies [74].

4.10. Western blot analysis

HASMCs were seeded onto 12-well plates and serum starved for 24 h. Following treatment with Ang II with or without inhibitors or siRNAs for different periods of time, cells were collected and lysed in lysis buffer. The protein concentration was measured by the BCA kit. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer onto nitrocellulose membranes, the proteins were incubated with various antibodies overnight at 4 °C, including anti-MMP-9, anti-p47phox, and anti-phospho-p65. Secondary anti-rabbit or anti-mouse horseradish peroxidase antibodies (1:2000 dilution) were then added for 1 h, and the membranes, the proteins were incubated with various antibodies for 1 h, and the bands were visualized using ECL reagents and developed by Hyperfilm-ECL.

4.11. RT-PCR analysis

Here, we isolated total RNA using TRizol according to the protocol of the manufacturers’ instructions. We obtained the cDNA from 0.5 µg total RNA and used it as a template for PCR amplification, which has been described in our previous study [32]. We used the primers in this study as the following:

- GAPDH:
  5'-ACCACAGTCCATGCAATC-3' (sense).
  5'-TCCACACCTGTTGCATG-3' (anti-sense).
  ATIR:
  5'-CCGATTCACATGACCCATTAGCTATCCATGACCAAC-3' (sense).
  5'-CCGATTCACATGACCCATTAGCTATCCATGACCAAC-3' (anti-sense).
- ATIR:
  5'-CCGATTCACATGACCCATTAGCTATCCATGACCAAC-3' (sense).
  5'-CCGATTCACATGACCCATTAGCTATCCATGACCAAC-3' (anti-sense).

4.12. Measurement of MMP-9 and IL-6 generation

Cells were cultured in 6-well culture plates. After we found HASMCs became confluent, the cells were incubated with Ang II for the indicated time intervals. The media were collected and stored at ~80 °C until next experiments. Moreover, we assayed the levels of MMP-9 and IL-6 using a MMP-9 ELISA kit (Thermo Scientific, Rockford, USA) and an IL-6 ELISA kit (BioSource International, Camarillo, CA), respectively, according to the manufacturer’s instructions.

4.13. Measurement of intracellular ROS accumulation

We used the CellROX Green Reagent (Molecular Probes, Eugene, OR) to measure oxidative stress in the cells. Following treatment, 5 µM CellROX Green Reagent was added to cells for 30 min at 37 °C. The cells were then harvested and the fluorescence was determined using a Fluoroskan Ascent FL pla-rerader with wavelengths of 485 nm (excitation) and 520 nm (emission).

4.14. Determination of NADPH oxidase activity

The NADPH oxidase activity in these cells was evaluated by the lucigenin chemiluminescence assay as described previously [59]. Following treatment, the cell membrane fraction was collected, NADPH (Sigma) (1 µM) and lucigenin (Sigma) (20 µM) were added, and chemiluminescence was determined using a Fluoroskan Ascent FL (Thermo*) in an out-of-coincidence mode.

4.15. Statistical analysis of data

Data were showed as the mean or mean ± SEM of three independent experiments and estimated using GraphPad Prism Program (GraphPad, San Diego, CA, USA). All the data were analyzed by one-way ANOVA followed with Tukey’s post-hoc test. A p value of less than 0.05 was considered significant.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2017.02.019.

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