Correlation of Different NADPH Oxidase Homologues with Late Endothelial Progenitor Cell Senescence Induced by Angiotensin II: Effect of Telmisartan

Hong Li¹, Qiang Liu², Ningfu Wang¹ and Jian Xu¹

Abstract

Objective  Involvement of different NADPH oxidase (NOX) homologues in late endothelial progenitor cell (EPC) senescence induced by angiotensin II (Ang II) remains rarely studied systemically. The goal of our study was to determine NOX homologues which are correlated with late EPCs senescence induced by Ang II. The inhibitory effect of telmisartan was also studied.

Methods and Materials  Late EPCs were obtained from mononuclear cells isolated from peripheral venous blood. Stimulated by Ang II with telmisartan (Tel) or VAS2870 pretreatment or siRNA prior silencing, NOX was detected by RT-PCR and Western blot. Cell senescence was measured by the acidic β-galactosidase activity assay and cell cycle analysis. Intracellular reactive oxygen species (ROS) were analyzed by flow cytometer based on DCFH-DA.

Results  A bi-phasic change existed in NOX level after Ang II stimulation. Translocated NOX5 was correlated with early and rapid ROS production, but it contributed little to EPCs senescence. NOX2 and NOX4 were correlated with the late and slow phase and contributed greatly to EPCs senescence. There were no significant changes in NOX1 or NOX3. Telmisartan effectively depressed NOX change and delayed late EPCs senescence.

Conclusion  Ang II accelerates late EPCs senescence mainly via increased ROS originating from NOX2 and NOX4 up-regulation or translocated NOX5. Telmisartan effectively inhibited that cascade reaction and delayed EPCs senescence.

Key words: Angiotensin II, late endothelial progenitor cells, NADPH oxidase senescence, telmisartan

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Introduction

The existence of stem cells originating in the bone marrow that can give rise to endothelial cells both in culture and in animal models of ischemic diseases, termed endothelial progenitor cells (EPCs), has come to the limelight with the seminal report by Asahara (1); the number of EPCs is closely correlated to the maintenance of endothelial layer and promotion of neovascularization.

The EPC cell population is heterogeneous in morphology and function including early EPCs and late EPCs which incorporate more readily into the human umbilical vein endothelial cell monolayer and form capillary tube better than early EPCs. Evidence has suggested that early EPCs contribute to neovasculogenesis mainly by secreting angiogenic cytokines, whereas late EPCs enhance neovasculogenesis by providing a sufficient number of endothelial cells (2). Therefore, EPCs directly contribute to vascular healing processes and the pathogenesis of cardiovascular diseases, which is negatively affected by numerous cardiovascular risk factors. Previous research has identified risk factors for atherosclerosis those act on a variety of mechanisms that ultimately culminate in reduced levels or function of circulating EPCs (3).
The mechanism determining low EPC counts in subjects with cardiovascular risk factors has been emphasized by some studies and these studies appear to implicate, at least in part, an accelerated senescence induced by angiotensin II (Ang II) (4). But upon close examination, EPCs studied in those reports were primarily early EPCs and rarely did a study focus on the contribution of late EPCs in Ang II-induced senescence.

Accumulated evidence has shown that Ang II is implicated in a wide variety of pathologies of cardiovascular diseases (5-7). Prominent evidence among those featuring pathologies mediated by Ang II is the excessive generation of reactive oxygen species (ROS) (8-10).

As for the sources of ROS, vascular NADPH oxidase (NOX) has been emphasized. Recent evidence suggests that ROS derived from NOX based on gp91phox (also termed NOX2) importantly contributes to early EPC senescence induced by Ang II (11). Whereas several other homologues to NOX2 including NOX1, NOX3, NOX4 and NOX5 have been identified to be expressed in endothelial cells (12-15), the contribution of these NOX homologues to late EPC senescence induced by Ang II remains poorly understood.

Furthermore, studies in the past decade have shown that Ang II acts via the type 1 receptor (AT1) to induce vascular EPCs senescence through increased ROS production. A case in point was the result from Kobayashi et al where Ang II induced EPCs senescence was mainly responsible to ROS derived from NOX2 and was significantly inhibited by pretreatment of valsartan (16). Indeed, recent clinical studies have revealed that interruption of the renin-angiotensin system (RAS) with angiotensin-converting enzyme (ACE) inhibitors or Ang II type 1 receptor blockers (ARBs) slows cardiovascular disease progression.

Telmisartan (Tel), one of the lipophilic and highly selective ARBs, is receiving more and more attention. According to recent research, Tel may attenuate endothelial inflammation and oxidative cell damage induced by Ang II-dependent stimuli (17-19). But whether telmisartan is able to alter expression of NOX and consequently antagonize Ang II-induced late EPC senescence remains unclear.

Thus, in this study, using siRNA and NOX inhibitor VAS2870, we examined the correlation of different NOX with late EPC senescence induced by Ang II. We further studied whether or not telmisartan, a commercially available ARB, could have an effect on late EPC senescence.

Materials and Methods

Isolation of mononuclear cells and cell culture

To obtain late EPCs, a similar procedure was used based on what described by Ingram (20). Briefly, mononuclear cells (MNC) were isolated from peripheral venous blood from healthy volunteers by Ficoll density-gradient centrifugation (Sigma-Aldrich, St. Louis, MO, USA), washed twice with phosphate-buffered saline and pre-plated onto 10 cm fibronectin-coated plates (Becton, Dickinson and Company, Rutherford, NJ, USA) in complete EGM-2 medium (Lonza Bioscience, Basel, Switzerland). After 24 hours adherent cells were collected and 2 million cells were re-plated onto fibronectin-coated six-well plates in complete EGM-2. Then cells were cultured with changing medium every day up to 21 days. Confirmation of late endothelial progenitor cells was performed by immunofluorescence and flow cytometry. The Institutional Review Board at the Zhejiang University School of Medicine approved all protocols, and informed consent was obtained from all adult donors.

To determine the stimulatory effect of Ang II and the inhibitory effect of Tel on NOX expression, EPCs at day 21 were treated with Ang II at 50, 100 or 200 nmol/L for 24 h with or without pretreatment of 2 kinds of concentration Tel (10⁻⁵ or 10⁻⁶ mol/L) for 0.5 h before Ang II stimulation. VAS2870 inhibitory effect also was analyzed by pretreatment EPCs with 50 μmol/L NOXs inhibitor 3-benzyl-7-(2-benzoazolyl) thio-1,2,3-triazolo[4,5-d] pyrimidine (VAS2870) 30 minutes prior to Ang II. Tel was kindly donated by Novartis Pharma AG (Basel, Switzerland). VAS2870 was provided by vasopharm BIOTECH GmbH, Würzburg, Germany.

RNA interference

To determine the effect of NOX silencing by siRNA on Ang II stimulation, EPCs were also transected with special siRNA with optimal concentration and time duration gained before Ang II stimulation.

We used commercially generated double-strand RNA pair oligonucleotides, duplexed Stealth™ RNAi (Invitrogen, Carlsbad, CA, USA), for RNA interference. NOX2 specific siRNA oligos against the human sequence were as follows: 5'-AUUGGAUAGUGGGGUUCCCAUGUU-3' and 5'-CAUGGG ACCCACAUCCAUUU-3'. NOX4 specific siRNA oligos against the human sequence were as follows: 5'-AUGGAUAGUGGGGUUCCCAUGUU-3' and 5'-CAUGGG ACCCACAUCCAUUU-3'. NOX5 specific siRNA oligos against the human sequence were as follows: 5'-CUUCGACUUGGACGUU CAUCUU-3' and 5'-GAUGAAGCGUGAGUAGAUAGUU-3'. Stealth™ RNAi Negative Control Low GC Duplex (Invitrogen) was used as the negative control. siRNA was transfected to EPCs with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

To determine the optimal concentration and time point of siRNA efficacy, EPCs were grown to subconfluency and transfected with 10, 30, or 100 nmol/L of each of the siRNAs in the presence of 8 nmol/L lipofectin and subjected to analysis 24, 36, 48 and 72 hours after transfection. Transfected cells were used for further experiments.

RNA isolation and RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and cDNA was generated using a commercial kit (Invitrogen) according to manufacturer’s instructions. The specific primers used to amplify NOX cDNA are listed in Table 1. PCR reactions were subjected to the following thermal pro-
Table 1. Primer Used in Current Study and Size of PCR Products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequence 5’-3’</th>
<th>Size of products expected (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOX1</td>
<td>TGACTGCTATAGCAGAAGCGACAG</td>
<td>458</td>
</tr>
<tr>
<td>NOX1</td>
<td>ATGCAAGGATCCTAATGCA</td>
<td>484</td>
</tr>
<tr>
<td>NOX2</td>
<td>TCTTTGGCAAGTTGGAATATCG</td>
<td>472</td>
</tr>
<tr>
<td>NOX3</td>
<td>CTAACGACCACATCCAGACAG</td>
<td>472</td>
</tr>
<tr>
<td>NOX4</td>
<td>CTGTCTACGTCTCAGAAATG</td>
<td>475</td>
</tr>
<tr>
<td>NOX5</td>
<td>CTGAAAGCTTAGAGGCCCATGAG</td>
<td>475</td>
</tr>
</tbody>
</table>

tocol: (94°C×20 s; 60°C×20 s; 72°C×30 s)×35 cycles. PCR products were visualized in 1.5% ethidium bromide-stained agarose gels. β-actin was amplified as a reference.

Western blotting

Cells were washed with cold PBS, harvested by scraping and further lysed by sonication in buffer (mainly including 50 mmol/L Tris-HCl, 1 mmol/L PMSF (α-toluenesulfonyl fluoride), 4 mmol/L NaF). The lysates were centrifuged (800 g, 4°C for 90 min). The supernatant was discarded and deposit of lysates was lysed once more by sonication in buffer (mainly including 50 mmol/L Tris-HCl, 1 mmol/L PMSF (α-toluenesulfonyl fluoride), 4 mmol/L NaF, 1% Triton X-100). The protein concentrations of the supernatant were determined by the Bradford Assay (Sigma-Aldrich). Proteins in cells were separated by 15% SDS-PAGE before transfer to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk. Rabbit polyclonal antibody against human NOX 1 to 5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1 : 100. To normalize for protein loading, a rabbit polyclonal anti β-actin antibody was used (1 : 1,000; Sigma-Aldrich). Anti-rabbit HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were diluted 1 : 1,000 and visualized using chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Identification of late EPCs

To confirm the late EPC phenotype, adherent cells at day 21 were incubated with DIL-labeled acLDL (Molecular Probes, Eugene, OR, USA) for 12 hours and after fixation they were incubated with fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin (Vector Laboratories, Burlingame, CA, USA) for a further 12 hours. Cells were then visualized with an inverted fluorescent microscope.

To determine surface marker difference between 7 and 21 days cultured EPCs, 10⁶ cells were incubated in the dark with FITC-CD45 and PE-31. The number of positive cells was compared with that of IgG isotype controls. Data acquisition was performed on a flow cytometer (BD Biosciences, Palo Alto, CA, USA) including 100,000 cytometric events.

Detection of ROS

To measure reactive oxygen, cells were washed with PBS and incubated with 10 mmol/L 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37°C. The intensity of fluorescence was analyzed by flow cytometry. Results were expressed as the average intensity fluorescence (AIF) in the analyzed cells.

Measurement of cell senescence

Senescence was measured by the acidic β-galactosidase activity assay (Invitrogen). Cells were washed in phosphate-buffered saline, fixed for 3 minutes at room temperature in 2% paraformaldehyde, washed, and incubated for 18h at 37°C (no CO2) with fresh SA-β-gal stain solution. Cells were counterstained with 4’, 6-diamino-phenylindole (DAPI) for 10 min to count the total cell number. The percentage of positive blue cells was determined by counting five random fields (average of 200 cells).

To analyze senescent cell cycle, cells were washed twice with PBS without calcium or magnesium. After fixation in 4°C ethanol overnight, cells were incubated with 10 mg/mL RNase (Sigma-Aldrich) and 500 μg/mL PI at 37°C for 30 min. Cellular DNA content was measured by flow cytometry for cell cycle analysis.

Statistical analysis

Data were expressed as mean ± standard deviation based on at least three independent experiments. The difference
Results

Characterization of peripheral blood-derived late EPCs

Culturing mononuclear cells for 21 days according to the conditions described above yielded a population of slabstone-like acLDL+ and ulex-lectin+ cells matching the previously described late EPCs phenotype. Furthermore, immunophenotype analysis by flow-cytometry demonstrated the altered expression of both hematopoietic and endothelial surface antigens including CD45 and CD31 along with culture time. After 7 or 21 days culture, the mean percentage of CD31+ cells was (6.5 ± 1.5) % vs (67.6 ± 4.5) % (n=3, p<0.05) and CD45+ cells was (79.5 ± 3.5) % vs (5.6 ± 1.4) % (n=3, p<0.05). These orphological, functional and changed surface marker characteristics permitted us to label these cells as late EPCs, Fig. 1.

NOX differential expression stimulated by Ang II in late EPCs

During treatment with different concentrations of Ang II for 24 h, the NOX mRNA level in EPCs at 3h, 6h, 12h and...
24h time point was analyzed by RT-PCR.

Results in late EPCs treated with Ang II, revealed that differential expression of NOX mRNA existed. Within 5 kinds of NOX mRNA, NOX2, NOX4 and NOX5 mRNA levels were significantly increased, whereas there was no significant change in NOX1 and NOX3. Furthermore, in both a dose- and time-dependent manner, Ang II stimulated NOX2, NOX4 and NOX5 mRNA expression. As shown in Fig. 2, NOX5 mRNA reached peak with 200 nmol/L Ang II at 3 h, whereas NOX2 or NOX4 reached a peak at 12 h with the same concentration Ang II (200 nmol/L).

As shown in Fig. 3, Tel also in a dose dependent manner inhibited Ang II (200 nmol/L) induced an NOX mRNA increase. Tel in 10⁻⁵ mol/L reached the peak inhibition effect and attenuated increased NOX mRNA nearly to the control level.

Similarly, in Western blot, Fig. 4, Ang II (200nmol/L) resulted in maximum increase in NOX5 protein level at 3h versus NOX2 and NOX4 at 12h. Pretreatment with Tel (10⁻⁵ mol/L) achieved consistent results on RT-PCR analysis.

Based on the above results, 200 nmol/L or 10⁻⁵ mol/L was the optimal concentration for Ang II or Tel in following siRNA silencing, ROS production and cell senescence study.

**Silencing effect of siRNA on NOXs expression**

After silencing effects analysis, 100 nmol/L was found to be the optimal concentration for all 3 kinds of special siRNAs; 24 hours was the optimal time point for NOX5 targeting siRNA and 48 hours was optimal for NOX2 or NOX4 targeting siRNAs. Under such an optimal condition, siRNAs achieved the maximum silencing effect.

After transfection with 3 kinds of special siRNAs at the
optimal condition described above, EPCs were stimulated by 200 nmol/L Ang II for 3 hours for NOX5, and 12 hours for NOX2 or NOX4 expression assay. As shown in Fig. 5, within 3 kinds of NOXs, NOX2 and NOX4 did not increase significantly in both mRNA and protein level after Ang II stimuli. Different from the expression pattern of NOX2 or NOX4, NOX5 showed unrelated mRNA and protein level change after stimuli; there was almost no change in mRNA, while a significant increase in protein was seen.

**Effects of siRNA, VAS2870 or Tel on Ang II-induced ROS formation in late EPCs**

Figure 6 showed a time-dependent significant increment in ROS level in late EPCs treated with Ang II (200 nmol/L). The level of ROS in cells began to be significantly different from the control group only after 3 h stimulation by Ang II (200 nmol/L). And the maximum effect was reached at 12 h. After pretreatment with 10^{-5} mol/L Tel or 50 μmol/L VAS2870 for 30 minutes, the production of ROS was lowered to control level. As for NOXs silencing, siRNA targeting NOX5 had no effect on ROS increase pattern after Ang II stimuli. siRNA targeting NOX2 or NOX4 attenuated ROS production of time point after 3 h significantly.

**Effects of siRNA, VAS2870 or Tel on Ang II-induced EPC senescence**

Acidic-galactosidase was detected as a biochemical marker for the onset of cellular senescence. The growth of senescent EPCs was measured by cell cycle analysis. After coincubation with Ang II (200 nmol/L) for 24 h, the number of SA-β-Gal-positive or G0/G1 resting cells increased significantly. Pretreatment with Tel (10^{-5} mol/L) or 50 μmol/L VAS2870 for 30 minutes significantly inhibited 200 nmol/L Ang II-induced increase in SA-β-Gal positive or G0/G1 resting cells (Fig. 7, 8). siRNA targeting NOX5 showed no attenuation of the number of SA-β-Gal positive or G0/G1 resting cells. However, siRNA targeting NOX2 or NOX4 yielded a significant decrease in SA-β-Gal positive or G0/G1 resting cells, Fig. 7, 8.
Senescence-induced numerical and functional impairment of circulating endothelial progenitor cells (EPCs) has been identified to contribute to vascular aging and associated increase in cardiovascular risk.

Typically, two types of endothelial progenitor cell colonies can be obtained depending on the timing of their emergence in culture. Early and late colonies have been thus distinguished for having different proliferation potential. Compared with early EPCs, late EPCs may bring a sufficient number of endothelial cells, incorporate more readily into endothelial cell monolayer to form capillary tube and therefore contribute more to neovascularogenesis (21). In this point, anti-late EPC senescence may be a good way to lower cardiovascular risk effectively.

Different from late EPCs, early EPCs might more preferentially originate from monocytes, thus the pan leucocyte CD45 marker is an important marker to distinguish between endothelial and hemopoietic lineage cells (22). Based on our results, mononuclear cells isolated from peripheral venous blood, after 21 days culture, were slabstone-like with positive 5 kinases. As demonstrated in a previous study, Ang II accelerates the onset of EPCs senescence via induction of gp91phox expression and related oxidative stress. In that accelerated EPC senescence process, gp91phox is the main function subunit of NAD(P)H oxidases (11). Similar to phagocyte NADPH oxidase, NAD(P)H oxidases are the major enzymatic sources of ROS in the cardiovascular system and the only enzyme discussed so far whose primary function appears to be ROS production. Interestingly, gp91phox have several homologues, namely NOX1, NOX3, NOX4, and NOX5, all of which are part of NOX family (23). Several recent re-
Figure 5. Silencing effect of siRNA on NOXs expression. The band gray scale ratios of NOX/β-actin represent the normalized NOX mRNA and protein level by β-actin. There were three replicate wells per treatment and the experiment was carried out three times. The left: 3 representative RT-PCR and western blot results after treatment with 200 nmol/L Ang II and different special siRNAs. The upper band and blot graph showed almost no change in mRNA, while significant increase in protein after siRNA targeting NOX5 silencing plus Ang II stimuli from 0 h to 3 h. The middle and lower band and blot graph showed no change in both mRNA and protein after NOX2 or NOX4 silencing plus Ang II stimuli from 0 h to 12 h. The right: upper curve graph showed normalized mRNA level. Lower curve graph showed normalized protein level. *p<0.05 vs 0 h time point.

ports have demonstrated that in addition to gp91phox, other numbers of NOX family also are present in endothelial cells and attribute to various physiopathological processes. For example, NOX1 exists in endothelial cells and is involved in cell growth and angiogenesis (24). NOX5 variants, including a variant lacking calcium binding domains, are expressed in micro vascular endothelial cells and contribute to endothelial ROS production, proliferation, and formation of capillary-like structures, as well as to endothelial response to thrombin (13). NOX4 is also detected in endothelial cells and contributes to ROS generation and proliferation. Although previous studies have correlated NOX family to endothelial ROS production and proliferation; to date the contribution of NOX family, especially members other than NOX2, to Ang II-induced EPC senescence lacks systemic research and remains unclear.

That unclearness promotes us to systemically investigate the role of NOX family including NOX2 and other homologues in Ang II induced late EPCs senescence. Intriguingly, we found, induced by Ang II, 5 kinds of NOX members take on a time-dependent differential expression, in which NOX5 reached expression peak in both mRNA and protein level almost 9 hours sooner than NOX2 and NOX4. Subsequent ROS and EPC senescence measurement indicated, with NOX gene differential expression, ROS production and senescent late EPC counts increased at the same pace, or in other words, showing difference from control and reaching peak in the same time point. Moreover, not only a time-dependent manner, Ang II inducing NOX gene differential expression was also dose-dependent as previously described. Taken together, behind the significant increase in ROS production and senescent EPCs, a bi-phasic change existed in
NOX after Ang II stimulation. NOX5 may be responsible for early and rapid ROS production and EPC senescence, whereas NOX2 and NOX4 are responsible for the late and slow phase. Certainly, NOX1 and NOX3 indeed are expressed in late EPCs, but they show no correlation with Ang II-induced late EPC senescence. Further, siRNA indicated silencing NOX2 or NOX4 achieved a significant attenuation of ROS production and EPC senescence; these are results which support that NOX2 and NOX4 are important for late and slow phase effect of Ang II stimulation.

Current results seem slightly contradict what was found by Petry and colleagues, according to whose data endothelial cells simultaneously express NOX2, NOX4, and NOX1. NOX2 and NOX4, but not NOX1, equally contributed to ROS generation and proliferation under basal conditions (25). In the present study, NOX1 contributed less and NOX2 with the same pace as NOX4 contributed to ROS generation in Ang II-induced late EPC senescence, which was consistent with the results of Petry et al. The difference between their study and the current work is that NOX5, NOX2 and NOX4 took on a bi-phasic expression, NOX5 being for early phasic ROS generation and NOX2 and NOX4 for the late one. Two possible explanations may account for the difference: (a) different cells were used in experiment, we used late EPCs, however Petry and colleagues used human microvascular endothelial cells; and (b) different intervention conditions, the cells in their study being under basal conditions, whereas those of the present study were stimulated by Ang II.

But there remain a puzzle that typically an obvious increase in protein synthesis inside cells needs 6 to 8 hours, while just after 3 hours stimuli, NOX5 took on a significant increase in protein. Considering during the cell lysis we used a protocol of acquiring member protein by re-lysing cell with buffer containing Triton X-100, perhaps the rapidly increased protein came from another part such as cytoplasm.
That is, there may be a translocation mechanism which exists in EPCs stimulated by Ang II. Further, siRNA silencing results showed a significant up regulation of NOX5 protein without the corresponding increase in mRNA, which confirmed the hypothesis of NOX5 translocation.

To our knowledge, the present study provides the first evidence for the bi-phasic expression pattern of different NOX in Ang II-induced EPC senescence. One highlight of that kind of bi-phasic expression exists in NOX5 being responsible for early phase ROS stress. In contrast to other NOX proteins, NOX5 has been described to contain an amino-terminal calmodulin-like domain with four binding sites for calcium (EF hands) (26). It has been suggested that calcium binding induces a conformational change leading to intramolecular interactions between the N-terminus and the C-terminal catalytic domain which may result in enhanced ROS production upon addition of free calcium or ionomycin. But in the current study, silencing NOX5 yielded little effect on EPC senescence. Taken together these findings imply Ang II-induced early ROS stress in EPCs via NOX5 may be mediated by calcium and the calcium channel blockers (CCB) also may attenuate this stress. But regarding the limits of experimental design, calcium channel blocker intervention was not involved in the current research and further studies are needed to investigate the potential effect of CCB on EPC senescence. The other highlight is that, after stimuli, NOX2 and NOX4 had a slow but continuous increase by nearly 10 to 20 times versus base level and with correspondent enhancement of ROS and accelerated EPCs senescence, which means NOX2 and NOX4 may be the main source of ROS for Ang II stimuli and play a key role in Ang II-induced EPC senescence. In this point, several others have shown the same results. For example, Imanishi et al (11) attributed the Ang II-induced ROS stress and following senescence in EPCs mainly to NOX2. Lyle et al also suggested that NOX4 may mediate steady production of low amounts of ROS that are important in the metabolic and differentiation functions of the cell (27).

In addition, there are also observations of a decrease of Ang II stimuli effects by pretreatment of telmisartan or
VAS2870, such as ROS production and senescence of late EPCs. Between two major angiotensin receptor subtypes, most of the actions of angiotensin are mediated by the AT1 receptor. Telmisartan is a selective Ang II type 1 receptor (AT1R) blocker and its preventive roles against ischemic heart diseases (accumulating evidence suggesting that low EPCs count is closely correlated with those kind of diseases) have been demonstrated by several in vivo clinical trials (28). In our in vitro study, high dose telmisartan had potency to attenuate NOX express, ROS production and subsequent late EPCs senescence, which results characterize the AT1R being responsible for AngII-induced late EPC senescence. But whether or not the ROS mainly came from the NOX still remained indirectly identified. Consequently, VAS2870 was used. As a nonselective NOX inhibitor, VAS2870 attenuated ROS production and cells senescence induced by Ang II obviously, which result identified that the increased ROS was mainly from NOX after Ang II stimuli.

Certainly, several studies recently suggest that the effects of telmisartan are mediated via not only blockade of AT1R but also activation of peroxisome proliferators-activated receptor (PPAR)-γ (29). For the reason of experimental design, (PPAR)-γ was not involved in current study, and further research is needed to investigate latent role of telmisartan on regulating NOX gene expression via (PPAR)-γ.