Biochemical and Biophysical Research Communications 450 (2014) 1261–1265

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Cleaved high molecular weight kininogen stimulates JNK/FOXO4/MnSOD pathway for induction of endothelial progenitor cell senescence

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ARTICLE INFO

*Article history:*
Received 11 June 2014
Available online 28 June 2014

*Keywords:*
Kininogen
Endothelial progenitor cells
Reactive oxygen species
Senescence

ABSTRACT

*Objective:* Recently we have reported that cleaved high molecular weight kininogen (HKa) accelerates the onset of endothelial progenitor cells (EPCs) senescence by induction of reactive oxygen species (ROS). However, the mechanisms by which HKa induces production of ROS remain unknown. In this study, we have shown that HKa induces EPC senescence via stimulation of c-Jun N-terminal kinases (JNK)-related pathway.

*Methods and results:* Treatment of human EPCs with HKa for 72 h stimulated JNK phosphorylation at Thr183/Tyr185, and FOXO4 phosphorylation at Thr451, Concomitantly, upregulated the expression of MnSOD at protein and mRNA levels in a concentration-dependent manner. HKa increased intracellular level of $H_2O_2$, without affecting the expression of catalase. To narrow down the functional domain of HKa, recombinant proteins of human HK heavy chain (HC, 19–380aa) and light chain (LC, 390–644aa) were generated. HC, but not LC, increased senescence of EPCs and intracellular ROS levels, to a similar extent with HKa. Moreover, HC at 50 nM increased FOXO4 phosphorylation at Thr451 and the protein level of MnSOD in EPCs.

*Conclusion:* These results demonstrate that HKa accelerates the onset of EPC senescence by stimulating JNK/FOXO4/MnSOD pathway, its effect is mediated by the HC.



## 1. Introduction

Endothelial progenitor cells (EPCs) are precursors to mature endothelial cells that display distinct characteristics [1]. These cells have the ability to be mobilized to the site of vascular injury or tissue ischemia, and differentiate into mature endothelial cells, promoting re-endothelialization and neovascularization [1]. Thus, EPCs play an important role in vascular repair, thereby maintaining vascular homeostasis [1]. However, in patients with atherosclerosis and cardiovascular diseases, the number and function of EPCs are negatively correlated with the atherosclerotic risk factors, which may contribute to vascular dysfunction [2]. For example, the frequency of circulating EPCs is reduced 50% in patients with coronary artery disease and patient EPCs display an impaired migratory response [3]. Moreover, the clinical administration of EPCs to patients with cardiovascular diseases has had limited efficacy, whereas in animal models EPCs successfully restore endothelial function and enhance angiogenesis after tissue ischemia [4]. Very likely, EPCs are targets of endogenous angiogenic inhibitors elaborated in the setting of atherosclerosis [4]. Therefore, understanding the factors that affect EPC function and number is important, because it could improve the specific therapies to ultimately correct EPC dysfunction and prevent progression of atherosclerosis.

The plasma kallikrein–kinin system (KKS) consists of the proteins factor XII, prekallikrein and high molecular weight kininogen (HK) [5]. This system displays multiple physiologic and pathophysiological activities, such as blood pressure adjustment, modulation of thrombosis, and regulation of endothelial cell function and angiogenesis. Plasma HK is a major component of the KKS and is responsible for the association of this system with cell surface. The membrane surface of endothelial cells is an important site for the assembly and activation of the KKS [5]. Activation of the KKS is triggered *in vivo* by tissue destruction or by thrombus development [6], and results in cleavage of HK by kallikrein into two-chain HK (HKa). We have previously shown that HKa exposes its domain 5 to the surface upon cleavage and thus acquires new activities of inhibition of endothelial cell functions [7]. Recently, we have also shown that HKa inhibits clonogenic and proliferative

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http://dx.doi.org/10.1016/j.bbrc.2014.06.112
0006-291X/

capacities of EPCs and accelerated their senescence by increasing intracellular reactive oxygen species (ROS) generation [8,9]. ROS propagate cellular signaling induced by growth factors and thereby regulate a variety of cellular processes [10]. However, when ROS levels rise above a certain threshold, ROS react with proteins and lipids, then damage the cellular interior. Excessive ROS are considered to accelerate aging and age-related pathologies, inducing cell senescence [10]. In this study we further investigated the activities of HK in regulation of intracellular signaling pathway leading to EPC senescence.

## 2. Materials and methods

### 2.1. Antibodies and reagents

HKa was purchased from Enzyme Research Laboratories. Anti-MnSOD antibody was from BD Transduction Laboratories. Rabbit polyclonal antibody against FOXO4, JNK, phospho-SAPK/JNK (Thr 183/Tyr185), and catalase were from Cell Signaling Technology. Rabbit polyclonal antibody against FOXO4 (T451) was purchased from Abcam. Beta-galactosidase staining kit and hydrogen peroxide assay kit were purchased from BioVision. Reactive oxygen species (ROS) detection reagent ($H_2$DCFDA) was from Invitrogen.

### 2.2. Generation of recombinant heavy chain (HC) and light chain (LC) of human HK

Recombinant HC (19–380aa) and LC (390–644aa) were generated as previously described [11]. In brief, the primers used for polymerase chain reaction (PCR) were as follows: HC, forward 5′-TCCCCCGGGGCCAGGAATCACAGTCCGAG-3′, reverse 5′-ATAAGA ATGCGGCCGCTTATTTCATCAGTGAGATCATT-3′; LC, forward 5′-TCC CCCGGGGCTCATCACGAATAGG-3′, reverse 5′-CCGCTCGAGTTAAGA AAGGCCATCAG-3′. For HC, the sense primer contained a SmaI site and the antisense primer contained a NotI site. For LC, the sense primer contained a SmaI site and the antisense primer contained a XhoI site. The PCR products were digested and ligated to pTriEX-4 Neo vector (Novagen) containing an N-terminal histidine tag. The inserts were confirmed by sequencing. The transformants of *Escherichia coli* strain BL21 (DE3) pLysS (Promega) expressing HC and LC were grown and the proteins were purified using a Ni Sepharose High-Performance column (GE Healthcare) according to the manufacturer's protocol. The resultant proteins were verified by Western blotting.

### 2.3. Preparation of EPCs

In this study the EPCs referred to as endothelial colony-forming cells (ECFCs) were isolated from peripheral blood and their progenitor cell capacities were verified as we previously described [8,9]. To be defined as EPCs, the cells were examined for evidence of clonal proliferative potential, endothelial cell surface phenotype, and in vitro capillary lumen formation [8,9]. After informed consent was obtained in accordance with the Declaration of Helsinki, human blood was collected from healthy volunteer donors. After dilution at 1:1 with Hank's buffered salt solution, blood was overlaid onto Ficoll-Paque (Sigma) and centrifuged at 740*g* for 30 min. Buffy coat mononuclear cells were collected and resuspended in complete endothelial growth culture medium-2 (EGM-2, Cambrex) with additives (Bullet Kit) provided by the manufacturer and 10% fetal bovine serum (FBS). The cells were cultured in a 6-well tissue culture plate precoated with type I collagen (BD Bioscience) at 37 °C. Colonies appearing between 5 and 22 days of culture were identified as a well circumscribed monolayer of cobblestone-appearing cells. EPCs at early passages (passage 2–3) were used for the phenotypic and functional analysis.

### 2.4. Western blotting

EPCs were solubilized using a lysis buffer containing 25 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and protease inhibitor cocktail (Roche Molecular Biochemicals) and centrifuged at 15,000*g* for 20 min. Lysates were assayed for total protein concentration (BCA assay, Pierce), and 30 μg of clarified extract were resolved on a 4–20% gradient gel by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membranes (Millipore). After the membranes were blocked with 5% BSA in Tris-buffered saline (50 mM Tris–HCl pH 7.4, 150 mM NaCl), they were incubated with primary antibodies at 4 °C overnight. Horseradish peroxide (HRP)-conjugated secondary antibodies were used for detection by enhanced chemiluminescence.

### 2.5. Hydrogen peroxide assay

After treatment, EPCs were solubilized using lysis buffer and centrifuged at 1000*g* for 10 min. Supernatants were used for measurement of intracellular hydrogen peroxide levels by the commercial Hydrogen Peroxide Assay Kit according to the manufacturer's protocol.

### 2.6. Senescence-associated β-Galactosidase activity assay

Cell senescence was evaluated by measurement of senescence-associated β-Galactosidase (SA-β-Gal) activity using a β-Galactosidase Staining Kit (BioVision) as we previously described [8]. Briefly, EPCs were washed in PBS and fixed for 15 min at room temperature with fixative solution. After incubation with the staining solution mixture overnight at 37 °C, the cells were observed under a microscope for development of blue color. The absolute numbers of SA-β-Galactosidase-positive cells were counted.

### 2.7. Intracellular ROS production

Intracellular ROS generation was measured using the oxidation-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) (Molecular Probes) as we previously described [8]. After EPCs were incubated with 10 μM $H_2$DCF-DA at 37 °C for 30 min, which was stopped by washing with PBS and FACS analysis was performed immediately on a FACS Calibur flow cytometer (Becton Dickinson).

### 2.8. Measurement of mRNA expression by real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from EPCs using Trizol® reagent (Invitrogen). Quantitative real-time PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix kit (Fermentas) according to the manufacturer's instructions. The mRNA levels were normalized according to levels of β-Actin. The following primers were used: MnSOD, sense 5′-TAGCTCTTCAGCCTGCACTG-3′, antisense 5′-CTTGTCAAAGGAACCAAAGTCAC-3′; β-Actin, sense 5′-AGCGAG-CATCCCCCAAAGTT-3′, antisense 5′-GGGCACGAAGGCTCATCATT-3′.

### 2.9. Data analysis

Unless stated otherwise, the results shown are from a single experiment representative of at least three separate experiments. The data were calculated as average ± SEM from experiments done at least 3 times, and statistically analyzed by Student's *t* test (two

groups only) or One Way Analysis of Variance (ANOVA) and Student–Newman–Keuls test (multiple groups) [7]. Differences with probability values below 0.05 were considered significant.

## 3. Results

### 3.1. HKa increases intracellular $H_2O_2$ level by selective regulation of MnSOD expression

Our previous study has shown that HKa accelerates EPC senescence by induction of intracellular reactive oxygen species (ROS) [8], in this study we determined the underlying mechanisms for the effect of HKa. $H_2O_2$, which is a major component of ROS, can induce endothelial cell senescence [10], we thus examined whether HKa affects the intracellular level of $H_2O_2$. Using an assay by the hydrogen peroxide assay kit, we found that the treatment of EPCs with 100 nM HKa for 72 h significantly increased intracellular $H_2O_2$ level (Fig. 1A), suggesting that HKa regulates the metabolism of $H_2O_2$ and thus increases ROS production in EPCs. Superoxide ($O_2^-$), a toxic free radical, is a cellular by-product of aerobic metabolism, MnSOD is a superoxide dismutases that dismutates $O_2^-$ to $H_2O_2$, which can be subsequently converted to $O_2$ and $H_2O$ by catalase. Our new observation showing HKa enhancement of intracellular $H_2O_2$ levels led us to examine whether HKa modulates the expression of MnSOD and catalase. As shown in Fig. 1B (i), the treatment of EPCs with HKa for 72 h significantly increased the expression of MnSOD at protein level, in a concentration-dependent fashion. Concomitantly, 100 nM HKa also upregulated the mRNA level of MnSOD in EPCs (Fig. 1B, ii). However, HKa at 100 nM did not affect catalase expression (Fig. 1C). These above results suggest that HKa selectively upregulates MnSOD expression, leading to the accumulation of intracellular $H_2O_2$.

### 3.2. HKa stimulates the phosphorylation of JNK and FOXO4

It has been known that MnSOD expression was controlled by Forkhead box-O transcription factors (FOXO), downstream of c-Jun N-terminal kinase (JNK) [12]. FOXO are recently identified as an important family of proteins that modulate the expression of genes involved in cell differentiation, oxidative stress, cell cycle arrest and apoptosis and aging [12]. FOXO4 (AFX1) in human is phosphorylated by JNK at Thr451, resulting in its translocation from the cytosol to the nucleus and the subsequent transcriptional activity [12]. To study the involvement of JNK and FOXO4 in HKa-induced EPC senescence, we measured JNK phosphorylation at Thr183/Tyr185, and FOXO4 phosphorylation at Thr451. As shown in Fig. 2, the treatment of EPCs with HKa stimulated phosphorylation of JNK kinase at Thr183/Tyr185 (A) and phosphorylation of FOXO4 at Thr451 (B). These effects of HKa were concentration-dependent. These results indicate that HKa stimulates activation of JNK and FOXO, which may increase MnSOD expression.

### 3.3. Heavy chain is the functional domain of HKa in induction of ROS production and EPC senescence

HKa is composed of human HK heavy chain (HC) and light chain (LC). To narrow down the functional domain of HKa, we generated recombinant proteins of HC (19–380aa) and LC (390–644aa) (Fig. 3A). We first tested whether HC and LC induce senescence of EPCs. Although in the presence of 50 nM LC, a minimal portion of EPCs were positive for SA-β-Gal staining after culture for 72 h (Fig. 4B), in the presence of 50 nM HKa or HC the majority of EPCs became positive for SA-β-Gal staining (Fig. 4B). Because cell senescence is tightly associated with intracellular ROS production, we tested whether HC exposure increases intracellular ROS in EPCs

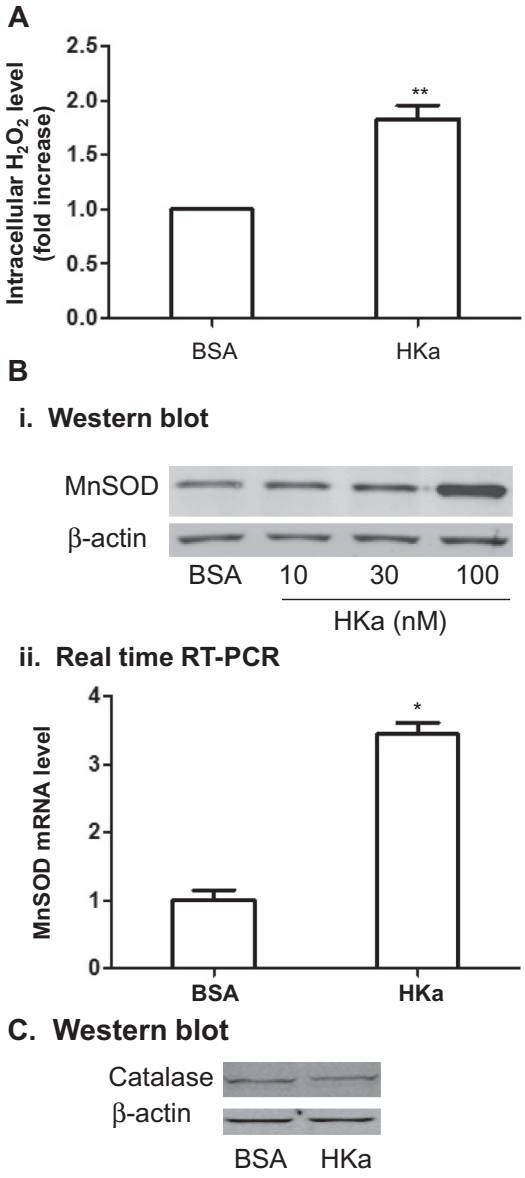


**Fig. 1.** HKa increases intracellular $H_2O_2$ level and upregulates expression of MnSOD in EPCs. (A) EPCs were cultured in the presence of BSA (control) or 100 nM HKa for 72 h. The intracellular $H_2O_2$ level was measured with a hydrogen peroxide assay kit and shown as fold increase compared with the control ($n = 3$). $^{**}p < 0.01$. (B) EPCs were cultured in the presence of 0.1% BSA (lane 1) and HKa at the indicated concentrations for 72 h. MnSOD protein level is analyzed by Western blotting (i). The blot for β-Actin served as loading control. MnSOD mRNA expression level in EPCs cultured with 0.1% BSA or 100 nM HKa for 72 h was quantitated by real time RT-PCR (ii). $^{*}p < 0.01$. (C) After EPCs were cultured in the presence of 0.1% BSA and 100 nM HKa for 72 h, catalase protein level in EPCs was analyzed by Western blotting. The blot for β-Actin served as loading control. The data are representative of three independent experiments.

using the $H_2$DCF-DA labeling assay. As shown in Fig. 4C, similar to HKa, HC, but not LC significantly increased $H_2$DCF-DA oxidation level in exposed EPCs. These observations suggest that HKa-induced EPC senescence and ROS production is mediated by its HC.

### 3.4. HC of HKa increases JNK and FOXO4 phosphorylation and MnSOD expression

We further tested whether HC upregulates JNK and FOXO4 phosphorylation and MnSOD expression. As shown in Fig. 4, HC at 50 nM stimulated the phosphorylation of JNK (Thr183/Tyr1

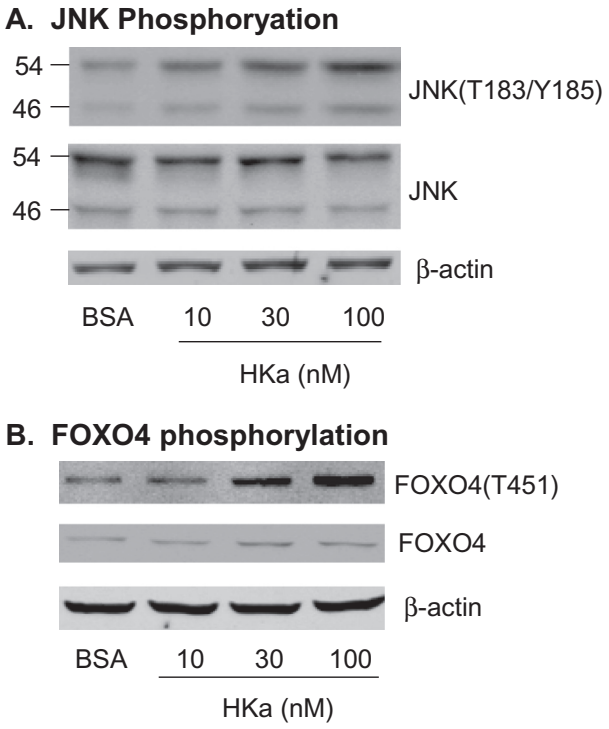


**Fig. 2.** HKa concentration-dependently increases phosphorylation of JNK and FOXO4 in EPCs. EPCs were cultured in the presence of 0.1% BSA and HKa at the indicated concentrations for 72 h. The phosphorylation levels of JNK (A) and FOXO4 (B) were analyzed by Western blot as indicated. The results are representative of three independent experiments.

85) and that FOXO4 (Thr451) to a similar extent with 50 nM HKa. Concomitantly, HC upregulated MnSOD expression at the protein level (Fig. 4). In contrast, LC had no effect on the above events (Fig. 4). These results suggest that HC of HK is responsible for stimulation of intracellular JNK and FOXO4 activation and MnSOD expression.

## 4. Discussion

Our current study demonstrate that (1) Treatment EPC with HKa stimulates JNK phosphorylation at Thr183/Try185, and FOXO4 phosphorylation at Thr451; (2) HKa upregulates MnSOD expression at protein and mRNA level in a concentration-dependent manner, without affecting catalase expression; (3) HKa increases $H_2O_2$ accumulation; (4) HC, like HKa, accelerates the onset of EPC senescence and increases intracellular reactive oxygen species; (5) HC also increases the phosphorylation of JNK at Thr183/Tyr185 and FOXO4 at Thr451, and upregulates MnSOD expression.

Our previous studies have shown that HKa accelerates EPC senescence by induction of ROS and its downstream events [8]. In this study we provide new evidence for the signaling pathway localized upstream of ROS for HKa induction of EPC senescence. JNK is an important member of the mitogen-activated protein kinase superfamily, the members of which are readily phosphorylated and activated by many environmental stimuli, including osmotic shock, UV radiation, heat shock, oxidative stress, cell death ligand FasL, and proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1 [13]. The phosphorylation of JNK plays an important role in controlling diverse cellular functions such as cell proliferation, aging and apoptosis [13]. Our current study demonstrates that HKa enhances JNK phosphorylation at Thr183/Tyr185, indicating that HKa induces EPC senescence via stimulation of JNK-related pathway. FOXO family of transcription factors regulates a variety of cellular programs, including cell cycle arrest, ROS scavenging, and apoptosis, and are of importance in the decision over cell fate [14]. Mammals have four isoforms of the FOXO transcription factor family, FOXO1, FOXO3, FOXO4, and FOXO6. Among of them, FOXO4 expression is the most abundant in EPC and its phosphorylation is the most critical post-translational modification process [15]. Under stress conditions, FOXO4 can be phosphorylated via JNK leading to increased transcriptional activation

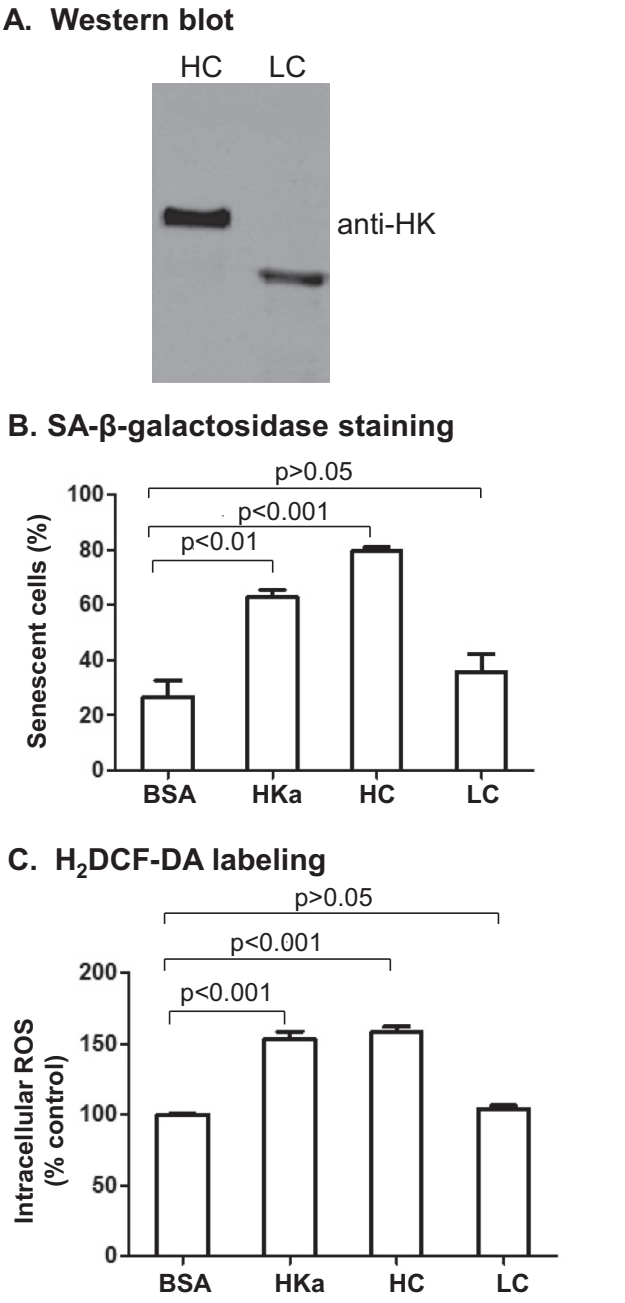


**Fig. 3.** HC of HKa, but not LC, mediates ROS production and senescence of EPCs. (A) Recombinant proteins of human HK HC and LC were verified by Western blot using anti-HK antibody. (B and C) EPCs were cultured in the presence of 0.1% BSA, 50 nM HKa, 50 nM HC or 50 nM LC for 72 h. Senescence of EPCs was analyzed by SA-β-Galactosidase staining and was expressed as a percentage of SA-β-Galactosidase-staining positive cells (B). Intracellular ROS levels were analyzed by $H_2$DCF-DA labeling and indicated as relative levels to BSA treatment which was set as 100% (C). These experiments were done in triplicates.

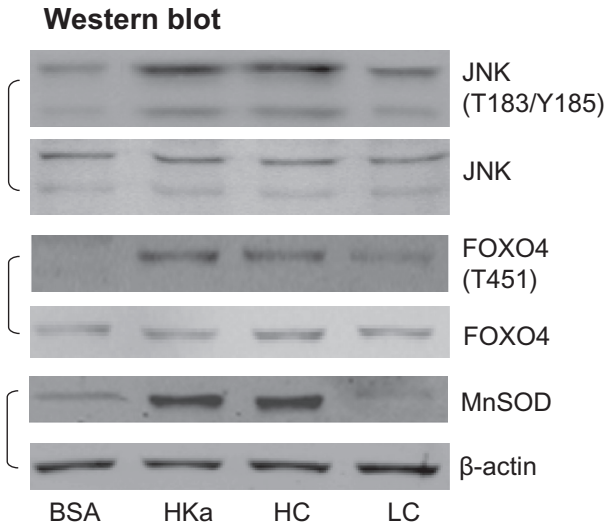


**Fig. 4.** HC increases JNK and FOXO4 phosphorylation and MnSOD expression to the same extent with HKa. EPCs were cultured in the presence of 0.1% BSA (BSA), 50 nM HKa, 50 nM HC or 50 nM LC for 72 h. The samples were analyzed by Western blotting as indicated at the right of each panel. β-Actin served as loading control. These results are representative of three independent experiments.

of the transcription factor [14]. Accordingly, target genes mediating the role of FOXO factors in the various cellular processes include the stress response genes MnSOD [12]. Our observations in this study indicate that HKa-induced JNK activation further phosphorylates its downstream FOXO4 at Thr451, the latter can lead to upregulation of MnSOD expression. In contrast to HKa enhancement of MnSOD expression, HKa does not affect the expression of catalase (Fig. 1C). Therefore, in EPCs treated with HKa, the increased MnSOD expression dismutates $O_2^-$ to $H_2O_2$. When the accumulated $H_2O_2$ is beyond the metabolic capacity of $H_2O_2$-degrading enzyme catalase, the increased $H_2O_2$ will accelerate the senescence of EPC through downstream p38 kinase and $p16^{INK4a}$ pathway. The similar phenomenon has been observed in other cells [16].

In this study, we have found that HC of HKa has a similar activity in stimulation of phosphorylation of JNK and FOXO4, as well as upregulation of MnSOD expression. Thus, HC is the functional domain for HKa. It has been known that endothelial cells express 3 receptors for HKa, uPAR, gC1qR and cytokeratin-1 and HC binds to each receptor [5]. In our future study, we will investigate which receptor is associated with HC and mediates its effects. Because a diminished number or dysfunction in EPC is associated with insufficient repair of damaged vascular walls, our understanding the mechanism of HKa acceleration the onset of EPC senescence is clearly important.

In conclusion, the present study demonstrates that HKa increases the generation of intracellular ROS by the activation of the JNK/FOXO/MnSOD pathway, leading to EPC senescence. This activity of HKa not only provides a new clue to understanding the mechanisms of EPC dysfunction, but also reveals a novel link between KKS activation and vascular dysfunction. Whether KKS-mediated EPC dysfunction is relevant to pathological conditions such as atherosclerosis needs to be noted and studied in the future.

## Author contributions

X. Zhu and X. Zheng performed research, collected data and performed statistical analysis; Y. Wu designed research, analyzed and interpreted data, and wrote the paper.

## Conflicts of interest

The authors declare that they have no conflict of interest.

## Acknowledgments

This study was supported in part by grants from the National Natural Science Foundation of China (81270592, 30971491, 31201058 and 81301534), the Priority Academic Program Development of Jiangsu Higher Education Institutions and the National Institute of Health (AR057542 and AR063290).

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