



ACE2/ACE imbalance and impaired vasoreparative functions of stem/progenitor cells in aging

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Abstract Aging increases risk for ischemic vascular diseases. Bone marrow–derived hematopoietic stem/progenitor cells (HSPCs) are known to stimulate vascular regeneration. Activation of either the Mas receptor (MasR) by angiotensin-(1-7) (Ang-(1-7)) or angiotensin-converting enzyme-2 (ACE2) stimulates vasoreparative functions in HSPCs. This study tested if aging is associated with decreased ACE2 expression in HSPCs and if Ang-(1-7) restores vasoreparative functions. Flow cytometric enumeration of Lin[−]CD45^{low}CD34⁺ cells was carried out in peripheral blood of male or female individuals (22–83 years of age). Activity of ACE2 or the classical angiotensin-converting enzyme (ACE) was determined in lysates of HSPCs. Lin[−]Sca-1⁺cKit⁺ (LSK) cells were isolated from young (3–5 months) or old (20–22 months) mice, and migration and proliferation were evaluated. Old mice were treated with Ang-(1-7), and mobilization of HSPCs was determined following ischemia induced by femoral ligation. A laser Doppler blood flow meter was used to determine blood flow. Aging was associated with decreased number (Spearman $r = -0.598$, $P < 0.0001$, $n = 56$), decreased ACE2 ($r = -0.677$, $P < 0.0004$), and increased ACE activity ($r = 0.872$, $P < 0.0001$) ($n = 23$) in HSPCs. Migration or proliferation of LSK cells in basal or in response to stromal-derived factor-1 α in old cells is

attenuated compared to young, and these dysfunctions were reversed by Ang-(1-7). Ischemia increased the number of circulating LSK cells in young mice, and blood flow to ischemic areas was recovered. These responses were impaired in old mice but were restored by treatment with Ang-(1-7). These results suggest that activation of ACE2 or MasR would be a promising approach for enhancing ischemic vascular repair in aging.

Keywords Aging · Hematopoietic stem/progenitor cells · ACE2 · Angiotensin-(1-7) · Ischemia

Introduction

Bone marrow–derived hematopoietic stem/progenitor cells (HSPCs) have the potential to stimulate vascular regeneration and therefore accelerate healing of an injured tissue. HSPCs home to the areas of ischemia or vascular injury and accomplish revascularization largely by paracrine mechanisms [1, 2]. In physiological conditions, HSPCs are mobilized from bone marrow into the blood stream in a circadian fashion [3]. Local delivery of enriched HSPCs accelerates vascularization in experimental models of ischemic vascular injury [4, 5]. Adequate number of functional CD34⁺ HSPCs in the circulation has been considered an indication of cardiovascular health [6]. Conversely, either decreased number or attenuated reparative functions increase risk for the development of cardiovascular disease [1, 7]. The therapeutic potential of this cell population for the

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treatment of ischemic vascular disorders has been demonstrated in several clinical studies [8, 9].

Sensitivity to the hypoxia-regulated factors derived from ischemic injury such as vascular endothelial growth factor (VEGF) and stromal-derived factor-1 α (SDF) stimulates the vasoreparative functions of CD34⁺ cells including migration, proliferation, vascular incorporation, and the release of paracrine factors [10]. Optimal expression of CXCR4, VEGFR1, and VEGFR2 enables these cells to respond to the signals of hypoxia [11, 12]. Conversely, hypoxic preconditioning increases the surface expression of CXCR4, VEGFR1, and VEGFR2, which further promotes the vasoreparative functions of progenitor cells [13]. In clinical conditions that are associated with impaired innate vasoreparative potential, hypoxic desensitization of the cells has been observed [10, 14, 15]. We have recently shown that exposure of HSPCs to hypoxia stimulates vascular repair-relevant functions, proliferation, and migration, and potentiates SDF- or VEGF-mediated responses [16].

Aging is associated with increased risk for ischemic vascular disease characterized by vascular endothelial dysfunction and angiogenesis [17]. Several studies showed that impaired reparative or regenerative functions of HSPCs are an underlying mechanism of age-associated risk for cardiovascular disorders. For example, aging is an independent risk factor for reduced number of circulating HSPCs and age-associated functional impairment in the reparative cells correlates with vascular dysfunction [18, 19]. These findings were further corroborated by other studies. Povsic et al. [20] reported that aging decreases the number of circulating CD34⁺ HSPCs, but the number of bone marrow-resident cells is not affected. On the other hand, Woolthuis et al. [21] demonstrated that age has no impact on CD34⁺ HSPCs in steady-state conditions or in the *in vitro* regenerative functions. However the hematopoietic regenerative capacity was found to be impaired following transplantation in individuals who were receiving chemotherapy. A recent large study by Al Mheid et al. [22] in a large cohort of 2792 individuals showed no change in the number of cells in circulation however proportionally decreased with the number of risk factors for cardiovascular disease. Disagreement in the outcomes of different studies is at least in part due to the immunophenotypic markers that were used to identify HSPCs with vasoreparative functions. In a mouse model of skin wounding [23], it was demonstrated that

local delivery of HSPCs derived from young mice accelerates vascularization of injured areas, but cells derived from old mice do not and instead impair the reparative process. Collectively, it is conceivable that impaired ischemic vascular repair with aging is due to both decreased number of circulating cells and vasoreparative dysfunction.

The classical renin angiotensin system (RAS) consists of angiotensin-converting enzyme (ACE), the octapeptide product angiotensin II (Ang II), and AT1 and AT2 receptors. Ang II is known to produce cardiovascular detrimental effects largely by activating AT1 receptor (AT1R). In contrast, an alternative ACE, ACE2, a monocarboxy peptidase, generates the heptapeptide angiotensin-(1-7) (Ang-(1-7)) from Ang II, and Ang-(1-7) produces cardiovascular protective functions by acting largely on the receptor, Mas (MasR) [24]. Previous studies have provided strong evidence for the expression of local RAS in bone marrow and for a regulatory role of angiotensin peptides in the hematopoietic functions in human and murine bone marrow cells [25, 26]. We have previously shown that ACE2 and MasR are expressed in human and murine HSPCs and that the activation of this axis stimulates vasoreparative functions of the cells in health or diseases such as diabetes [27, 28]. Furthermore, we have demonstrated that exposure to hypoxia increases the expression of ACE2 and MasR but not ACE or AT1R in human HSPCs in a HIF1 α -dependent manner at the transcriptional and translational levels and resulted in ADAM17-dependent shedding of ACE2 fragment with catalytic activity [16]. Therefore hypoxia-induced vasoprotective functions are at least in part mediated by upregulation of the vasoprotective axis of RAS in HSPCs. Importantly, hypoxia-regulated factors, SDF1 α or VEGF, also induce ACE2 or MasR independent of HIF1 α in these cells.

Upregulation of RAS with aging has been demonstrated in experimental studies. Aging is associated with increased ACE or AT1R expression or Ang II levels in cardiovascular tissues [29, 30]. As of now, no studies have addressed age-dependent changes in the ACE and ACE2 expressions or activity in human HSPCs. The current study tested the hypothesis that the decreased number of circulating HSPCs with aging is associated with ACE2/ACE imbalance and that pharmacological treatment with Ang-(1-7) reverses aging-associated mobilopathy and restores ischemic vascular repair.

Materials and methods

Characteristics of subjects

This study was approved by the Institutional Biosafety Committee of North Dakota State University. Research involving human subjects has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Institutional Review Board. Human leucocyte samples were derived from healthy Caucasian individuals including both males and females of age ranging from 22 to 83 years at Vitalant (previously United Blood Services) (Fargo, ND). Smokers, diabetics, and individuals on any pharmacotherapy are excluded. Leucocytes were collected in a Leucoreduction chamber (LRS chambers) following apheresis by using the TrimaAccel system (80440). Freshly prepared LRS cones were used for the isolation of HSPCs. Peripheral blood samples were also obtained by phlebotomy in some individuals, where the blood was collected into vacutainer tubes (ACD tubes, BD Biosciences).

Isolation of HSPCs

Peripheral blood mononuclear cells (MNCs) were separated by gradient centrifugation (800g, 30 min) using Ficoll (Ficoll-Paque; GE Healthcare Biosciences). MNCs were collected and freed from plasma by re-suspending the cells in PBS with 2% FBS and 1 mM EDTA, and centrifugation. MNCs were either used for flow cytometry analysis of lineage-negative (Lin^-) or $\text{Lin}^- \text{CD45}^{\text{low}} \text{CD34}^+$ HSPC populations, or for the enrichment of CD34^+ HSPCs. A negative selection kit (StemCell Technologies) was used for the enrichment of Lin^- cells by following supplier's instructions. This kit depletes cells that are positive for CD3, CD14, CD16, CD19, CD20, and CD56, by using magnetic microparticles. Lin^- cells were further enriched for CD34^+ cells by positive selection by using an immunomagnetic separation kit (StemCell Technologies) as per manufacturer's instructions. Cells were either used for experiments involving hypoxia or preserved at -80°C for enzyme activity assays.

Flow cytometry

Flow cytometry was carried out by suspending MNCs in the stain buffer and treated first with FcR blocking

reagent (1:100, Miltenyi Biotec) followed by incubation with the following fluorescent-conjugated antibodies (Biolegend), allophycocyanin (APC) anti-human lineage cocktail (1:500, Biolegend), phycoerythrin (PE) anti-human CD45 (1:500) and fluorescein isothiocyanate (FITC) anti-human CD34 antibodies (1 in 250), or isotype control antibodies (1 in 500) (Biolegend, San Diego, CA, USA) for 45 min at 4°C . Dead cells were excluded using the 7-AAD viability staining solution. Flow cytometry was carried out by using the C6 Accuri cytometer (BD).

Exposure of cells to normoxia or hypoxia

Freshly isolated cells were plated in round-bottom 96-well plates (Nunc) with no more than 30,000 cells per well in StemSpan SFEM (09650; StemCell Technologies). Cells were maintained in a CO_2 incubator at 37°C in 20% O_2 (normoxia) or were exposed to hypoxic conditions (1% O_2 –94% N_2 and 5% CO_2). Hypoxia was accomplished by using the hypoxia chamber (ProOx C21, BioSpherix, Lacona, NY, USA). Hypoxia chamber was maintained at 37°C and was sealed and purged with nitrogen for 1 h to achieve 1% O_2 , 5% CO_2 , and balance N_2 . Cells were exposed to 1% hypoxia for 12h, which was shown to be optimum for detecting maximum change in the expression of RAS components in our previous study [16].

Real-time polymerase chain reaction

RNA was extracted from CD34^+ cells using Trizol, and the concentration and purity of RNAs were determined by absorbance spectroscopy (NanoDrop Technologies). RNA (100 ng) was reverse transcribed using a qScript cDNA synthesis kit (Quantabio) according to manufacturer's protocol. SYBR green was used to detect DNA synthesis in real time. Each sample contained 10 ng DNA, 20 μM of forward and reverse primers, and the iQ SYBR green containing supermix (Bio-Rad). Specific primers for ACE2 and MasR were obtained by Invitrogen (Table 1). β -actin was used as an internal housekeeping gene. The reactions were run on a Stratagene Mx3000P using the following conditions: 10 min at 95°C , followed by 40 cycles (10 s at 95°C (denaturation), 20 s at 60°C (annealing), and 30 s at 72°C (extension)). The Ct values were normalized to β -actin (ΔCt), and the gene expression was expressed as $2^{-\Delta\Delta\text{Ct}}$.

ACE and ACE2 activity assays

ACE and ACE2 activities were determined in the cell lysates or plasma and cell supernatants by using enzyme-specific fluorogenic substrates (ES005 and ES007 for ACE and ACE2, respectively, R&D Systems), as reported before [31]. Enzyme-specific inhibitors, captopril or MLN-4760, were used to define ACE2-specific activity in the assay system, and enzyme-sensitive fluorescence was expressed as arbitrary fluorescence units/ μg of protein/hour.

Animal models

All animal studies were approved by the Institutional Animal Care and Use Committee at North Dakota State University. Young and old C57Bl/6 male mice of ages 3–4 and 20–22 months, respectively, were obtained from Charles River laboratories and NIA-Rodent facility. Mice were maintained on a 12-h light–dark cycle with food and water ad libitum. Where applicable, mice were treated with Ang-(1-7) by using subcutaneous osmotic pumps (Alzet) at a perfusion rate of 1 $\mu\text{g}/\text{kg}/\text{h}$ for 4 weeks. Control treatment was accomplished by using osmotic pumps with saline.

Flow cytometry

RBC-lysed peripheral blood or bone marrow cells were used for flow cytometric enumeration of Lin^- Sca-1⁺ and cKit⁺ (LSK) cells. Cell suspensions were incubated with Trustain (BioLegend) and fluorescent-conjugated antibodies, lineage cocktail-FITC, Sca-1-APC, and cKit-PE (BioLegend) for 45 min at 4 °C in dark.

Table 1 List of primers used for carrying out real-time PCR studies

Gene	Primer sequence
ACE2	Forward 5'-CATTGGAGCAAGTGTGGATCTT-3'
	Reverse 5'-GAGCTAATGCATGCCATTCTCA-3'
Mas receptor	Forward 5'-GCTACAACAGGGCCTCTATCTG-3'
	Reverse 5'-TACTCCATGGTCACCAAGC-3'
β -Actin	Forward 5'-GACAGGATGCAGAAGGAGATTACT-3'
	Reverse 5'-TGATCCACATCTGCTGGAAGGT-3'

Samples were then washed with 1X PBS (Corning Cellgro) and treated with 7-AAD (BD Pharmingen) before performing flow cytometry as described above.

Hind–limb ischemia

Ligation and excision of femoral artery in mice were performed as described by Niiyama et al. [32] under isoflurane anesthesia. Blood perfusion of the limbs was measured by imaging the flux ($\text{blood} \times \text{area}^{-1} \times \text{time}^{-1}$) by using the laser Doppler imaging system (Moor Instruments Inc.) under isoflurane anesthesia, which was expressed relative to the mean blood flux in the contralateral non-ischemic limb. Ischemia-mobilized LSK cells were enumerated before and at different time points following hind–limb ischemia (HLI) by flow cytometry as described above.

Isolation of mouse bone marrow LSK cells

Lin^- cells were isolated by negative selection by using the immunomagnetic enrichment kit (StemCell Technologies Inc.). Briefly, bone marrow monocyte cell suspension was prepared in recommended medium at required dilution and incubated with antibodies for binding CD5-, CD11b-, CD19-, CD45R/B220-, Ly-6G/C (Gr-1)-, and TER119-expressing cells. These cells are then labeled by tetrameric antibody complexes that recognize biotin and dextran-coated magnetic particles, and antibody-bound cells were then separated by using EasySep™ magnet, thus obtaining Lin^- cells. Lin^- cells were then enriched for Sca-1⁺ and cKit⁺ cells by using positive immunomagnetic selection kit (StemCell Technologies) as reported before [33]. Cells were then plated in RPMI1640 (GE Healthcare) in U-bottom, 96-well plate at a low density of 2×10^4 cells/150 μL per well, until they are used for proliferation or migration assay for less than 24 h following isolation.

Migration and proliferation assays

Migration of LSK cells was evaluated by using the QCM™ Chemotaxis cell migration assay kit (EMD Millipore) as per the manufacturer's instructions, as described before [15]. For each treatment, 20,000 cells in duplicate were used in a basal medium, HBSS (Mediatech, Inc.). Migration was performed in response to the treatments for 5 h, and the response was estimated as arbitrary fluorescence units (AFUs) and analyzed as

percentage increase over untreated control of the same treatment group. Proliferation was determined by colorimetry using the cell proliferation BrdU assay kit (Roche Bioscience) as described before [28]. The assay was performed using 10,000 cells treated with or without SDF for 48 h. Proliferation was evaluated by measuring absorbance at 370 nm (Spectramax plate reader) and expressed as fold increase as compared to mitomycin (1 μ M), which is known to inhibit cell proliferation.

Western blotting

Western blotting of ACE and ACE2 was carried out in the cell lysates of CD34+ cells as described before [16]. Cells were lysed in a radioimmuno precipitation assay (RIPA) buffer (Tris 10 mM pH 7.4, containing 140 mM NaCl, 1 mM EDTA, 1 mM NaF, 0.10% SDS, 0.50% sodium deoxycholate 0.1% NP-40, 1% Triton X-100) in the presence of protease inhibitors (Thermo Fisher). Protein concentration in cell lysates and cell supernatants was determined using bicinchoninic acid with bovine serum albumin as a standard (Thermo Fisher). Equal amounts of protein (30 μ g) were loaded and separated by SDS-PAGE using SurePage 10% pre-casted gels (Genescript). Proteins were electro-blotted onto nitrocellulose membranes (Bio-Rad). The blots were blocked using 5% (w/v) milk in Tris-buffered saline containing 0.5% (v/v) Tween-20. The membranes were then incubated with different antibodies. The ACE2 and ACE antibodies that were used are ab87436 and ab28311, respectively, with a β -actin antibody (mab8929; R&D Systems). HRP-conjugated goat anti-mouse (Biolegend) or donkey anti-rabbit (406–401; Biolegend) secondary antibodies were used at 1:20,000 dilution. Enhanced chemiluminescence reagent (ECL, K15045-D50; Advansta) was used to visualize bands and developed on X-ray films (Phoenix research products). ImageJ (NIH) was used for quantification of band intensities.

Biochemical analysis

Protein levels of HIF1 α and activity of prolyl hydroxylase (PHD2) were carried out by Assaygate Inc., by ELISA, and by a custom-developed assay, respectively. Biotinylated peptides derived from the HIF1 α oxygen-dependent degradation domain Biotin-Ahx-DLDLEALAPYIPADDDFQL and a hydroxylated control peptide Biotin-Ahx-

DLDLEALAP[OH]YIPADDDFQL (for hydroxyl validation only) were immobilized on a streptavidin-coated 96-well plate. Peptide hydroxylation of the samples and PHD2 controls was detected using a polyclonal antibody against HIF-1 α [hydroxy Pro564] peptide and HRP-conjugated secondary antibody followed by using TMB substrate. The relative enzyme activity was expressed as arbitrary units/mg protein.

Statistical analysis

Data are presented as mean \pm SEM. Number of experiments “*n*” represents the number of donors used in the experiment or number of mice in the experimental group. Statistical analyses were performed using the software Prism 8.12 (GraphPad Software, Inc., San Diego, CA). Statistical differences among different experimental groups were assessed by using either paired “*t*” test or two-way ANOVA followed by Tukey’s multiple comparisons, where applicable. Data sets were considered significantly different at the level of $P < 0.05$. Correlation between two independent variables was tested by using Spearman correlation. Where applicable x - y data was fit to nonlinear exponential relationship.

Results

In the first set of experiments, we have determined the number of circulating Lin[−]CD45^{low}CD34⁺ HSPCs in the peripheral blood samples from individuals of age ranging from 21 to 87 years by flow cytometry (Fig. 1a). The number of circulating Lin[−]CD45^{low}CD34⁺ cells was decreased age-dependently (Fig. 1b). Spearman correlation analysis detected significant negative correlation, $r = -0.598$, with 95% confidence limits -0.748 to -0.391 ($P < 0.0001$, $n = 56$). Both male ($n = 35$) and female ($n = 21$) cells showed similar decreasing trend in the circulating number of cells in relation to age.

Then, ACE and ACE2 enzyme activities were determined in cell lysates derived from HSPCs from individuals of over the age range. Cells were derived from 23 individuals, 10 females and 13 males that were randomly chosen from the 56 individuals that were studied for circulating cells in the previous set of experiments. ACE2 activity is age-dependently decreased, Spearman $r = -0.677$, with confidence limits -0.356 to -0.855 ($P < 0.0004$, $n = 23$) (Fig. 2a). In contrast, ACE activity

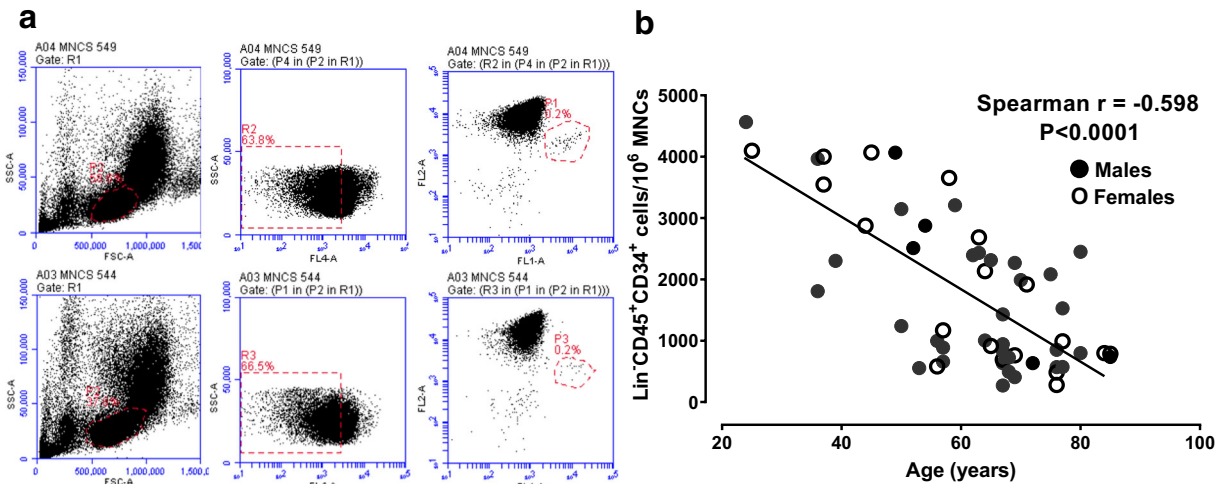


Fig. 1 Aging is associated with decreased circulating hematopoietic stem progenitor cells (HSPCs). **a** Representative dot plots of flow cytometric enumeration of $\text{Lin}^- \text{CD45}^{\text{low}} \text{CD34}^+$ cells in a young (26-year-old male, top panel) or an old (76-year-old male, bottom panel). Shown were selection of monocyte–lymphocyte gate followed by selection of lineage-negative cell population and

is age-dependently increased in cell lysates, Spearman $r = 0.872$, with 95% confidence limits 0.712 to 0.946 ($P < 0.0001$, $n = 23$) (Fig. 2b). As the data indicated no change in ACE activity until advanced age, the data was fit to the exponential model. This analysis showed the following relationship between age (x) and ACE activity (y): $y = 1.208e^{0.048x}$. The simultaneous changes in ACE2 and ACE activities resulted in age-dependent decrease in ACE2/ACE activity ratios (Spearman $r = -0.98$, $P < 0.0001$) (Fig. 2c). Cells derived from both males and females showed similar trend in the activity of ACEs and activity ratio in relation to age. Changes in the ACE and ACE2 activities were further supported by carrying out western blotting for protein expression in lysates of cells derived from both male and female individuals over a range of ages. As shown in Fig. 2d, decreasing and increasing trends in the protein expression of ACE2 and ACE, respectively, were observed.

Previously, we have observed that ACE2 or MasR expression was upregulated by in vitro hypoxic exposure but not ACE or AT1R expression in CD34^+ cells [16]. Similar findings were observed in LSK cells that are mobilized in response to HLI in mice [16]. Therefore, we have checked the sensitivity of cells from the old group to upregulate the expression of ACE2 and MasR in response to hypoxia. This was carried out in a limited number of individuals in two different age groups, 25–40 years (young) ($n = 6$) or > 65 years

then selection of $\text{Lin}^- \text{CD45}^{\text{low}} \text{CD34}^+$ population. Not shown were exclusion of 7AAD-positive cells and doublets prior to monocyte–lymphocyte gating. **b** Scatter plot showing a correlation of number of $\text{Lin}^- \text{CD45}^{\text{low}} \text{CD34}^+$ cells/million of mononuclear cells (MNCs) and age. Data was analyzed by Spearman correlation analysis

(old) ($n = 6$) with equal number of males and females. In normoxic conditions, expression of either ACE2 or MasR showed decreasing trend in old cells compared to young but did not achieve statistical significance. Exposure of cells from the young group to hypoxia (1% O_2) resulted in significant increase in the gene expression of ACE2 ($P < 0.01$, $n = 6$) and MasR ($P < 0.05$, $n = 6$) compared to normoxia (20% O_2) (Fig. 3a). Cells derived from the old group have decreased ACE2 or MasR expression compared to the young group; however, it did not achieve statistical significance. Upon exposure to hypoxia, cells from the old group showed ACE2 expression with an increasing trend but were not significant compared to normoxia ($n = 6$) or compared to that observed in young cells exposed to hypoxia ($P < 0.01$). Similar trend was observed in the MasR expression upon exposure to hypoxia in the old group ($n = 6$) (Fig. 3b).

These findings lead us to hypothesize that in the presence of ACE2 deficiency, administration of the heptapeptide metabolite, Ang-(1-7), would reverse the aging-associated bone marrow mobilopathy via activating MasR. To test this, first, we checked if the dysfunction could be demonstrated in a mouse model of aging. LSK cells in the peripheral blood were enumerated in mice at different age groups. In our preliminary studies, we have observed significant decrease at an age of as early as 9–10 months compared with that at 3 months of

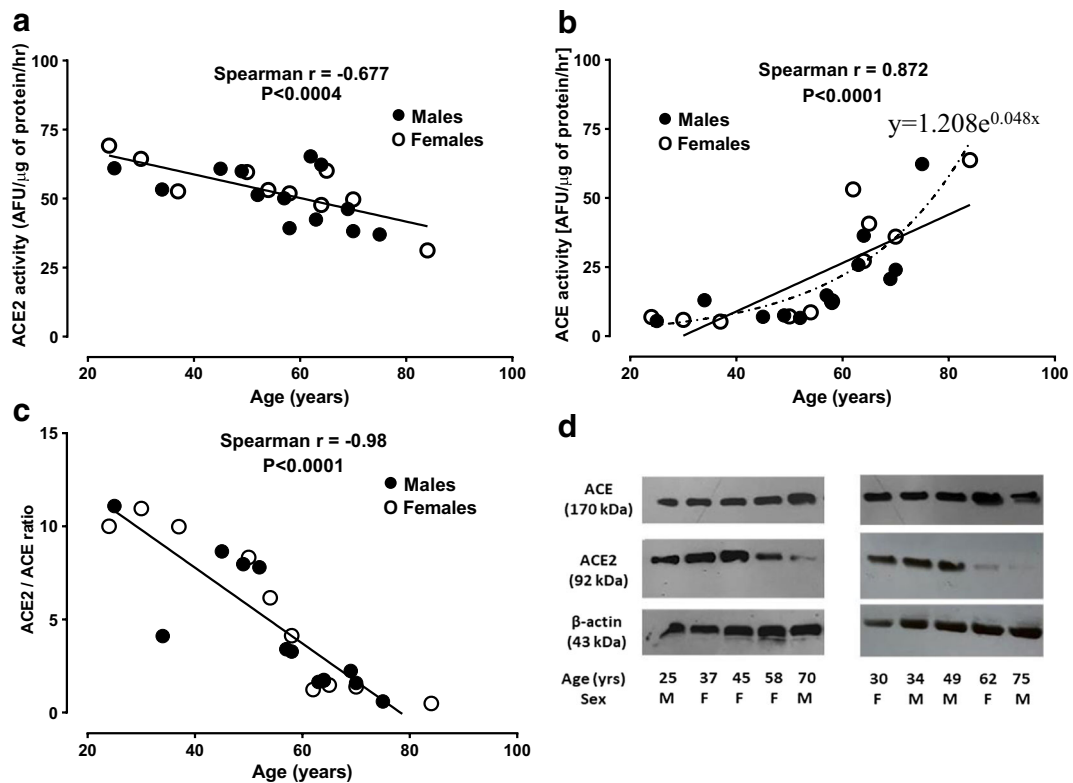


Fig. 2 Aging is associated with decreased ACE2 and increased ACE enzyme activities in the circulating CD34⁺ hematopoietic stem progenitor cells (HSPCs). Age-dependent changes in the activity of ACE2 (a), ACE (b), and ACE2/ACE (c) in lysates of HSPCs derived from individuals over an age range. Data was analyzed by Spearman correlation analysis. Broken line in B

indicates that changes in ACE activity follow a nonlinear exponential model with the relationship, $y = 1.208e^{0.048x}$ (x —age, y —ACE activity). **d** Representative western blots for ACE and ACE2 protein levels in the cell lysates of CD34⁺HSPCs. Age (yrs—years) and sex (M—male; F—female) of the individuals from whom the cells were derived from

age. At the age of 18 months, further reduction was observed in the circulating LSK cells. Therefore, further studies were carried out in two groups of mice, young (3–4 months) and old (18 months of age). Ang-(1-7) treatment by continuous subcutaneous administration for 4 weeks increased the number of LSK cells in the circulation of the old group that were comparable to that observed in the young group ($n = 6$) (Fig. 4a). Isolated cells from peripheral blood derived from the old group have decreased migration response in basal conditions or in response to SDF in vitro ($P < 0.05$, $n = 5$) (Fig. 4b) compared to the young group. This dysfunction was reversed by treatment with Ang-(1-7) (Fig. 4b). Along similar lines, proliferation of cells was attenuated in old cells in basal or in response to SDF compared to the young, and reversal of this dysfunction was accomplished by Ang-(1-7) treatment ($n = 6$) (Fig. 4c).

Then, we asked if bone marrow mobilization of vascular progenitor cells in response to ischemia is

altered with age. To test this, young and old groups of mice were subjected to HLI by femoral artery ligation. Circulating LSK cells were enumerated before and at different time intervals up to 4 weeks following HLI. In the young group, number of LSK cells was increased following HLI achieving a maximum at day 2 by ~ 4 -fold and comes back to pre-ischemic levels by day 7. In the old group, the peak was not observed and the overall mobilization of LSK cells was decreased in response to ischemic injury ($P < 0.05$, $n = 6$) (Fig. 5a). Blood flow recovery was significantly attenuated in old mice compared to the young ($P < 0.05$, $n = 6$) (Fig. 5b and c). In the old group that was treated with Ang-(1-7), the mobilization response following ischemic injury was increased compared to the untreated group ($P < 0.05$, $n = 6$); however, the increase was significant only on the day 2 post-HLI. Accordingly, overall blood flow recovery was increased that was comparable to that observed in the young group (Fig. 5b and c). It is important to note

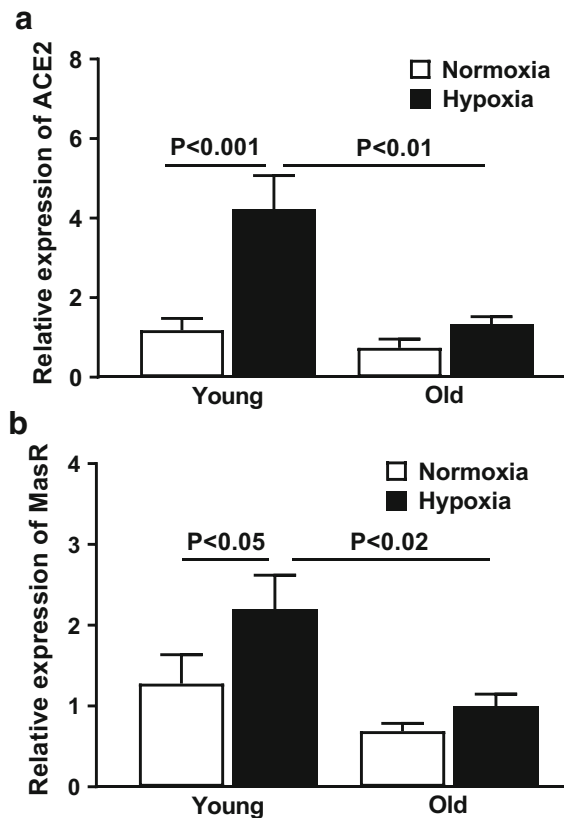


Fig. 3 Upregulation of ACE2 or Mas receptor (MasR) expression by hypoxia in CD34⁺ hematopoietic stem progenitor cells (HSPCs) is decreased in aging. ACE2 (a) or MasR (b) gene expression relative to that of β -actin in HSPCs derived from either young (25–40 years of age) or old (> 65 years of age) individuals in normoxic or hypoxic conditions. Either paired or unpaired “*t*” test was used to determine statistical significance

that either toe or partial foot amputations were observed in the old group (Fig. 5d), which was not observed in either young or Ang-(1-7)-treated old group.

Lastly, we have determined if ischemia-regulated cytokine SDF, which is essential for triggering the mobilization of bone marrow LSK cells into the blood stream, is altered with aging. SDF levels in the circulation following ischemic injury were significantly increased in young mice ($P < 0.01$, $n = 5$) on day 2 post-ischemia, which was not observed in the old group ($n = 5$) (Fig. 6a). In Ang-(1-7)-treated group, ischemic injury significantly increased plasma SDF levels on day 2 post-ischemia (Fig. 6a). Then, we tested if reduced SDF levels in old mice were associated with decreased HIF1 α expression in the skeletal muscle following HLI. In the old group, HIF1 α levels generated in response to ischemic injury were lower than that observed

in young ($P < 0.05$, $n = 5$). Ang-(1-7) treatment increased the accumulation of HIF1 α in the muscle tissue in the old group compared to the untreated ($P < 0.01$, $n = 5$) (Fig. 6b). Then, we asked if this increase is due to changes in the activity of enzyme, prolyl hydroxylase (PHD), which otherwise degrades HIF1 α in the physiological conditions. However, no significant changes were observed in the activity of PHD ($n = 5$) (Fig. 6b). According to this set of experiments, increased HIF1 α expression in Ang-(1-7)-treated mice explain increased SDF levels in response to ischemia.

Discussion

This study reports for the first time an imbalance in the vascular protective and detrimental enzymes of RAS, ACE2/ACE, in the human circulating HSPCs with aging. Evidence for the lack of sensitivity of aging cells to upregulate ACE2 or MasR expression upon hypoxic exposure adds to the novelty. Mobilization of HSPCs in response to ischemic injury and blood flow recovery to ischemic areas are impaired in aging mice, both of which were reversed by pharmacological treatment with Ang-(1-7). Ischemia-induced SDF levels in the circulation were lower in aging mice, which was restored by Ang-(1-7) partly via increasing HIF1 α levels. We have chosen to determine the beneficial effects of Ang-(1-7), an agonist of MasR, instead of activators of ACE2. Small molecule activators of ACE2, diminazene aceturate and xanthenone, have been recently reported, however, the mechanism of activation by these molecules requires further investigation and ACE2-independent mechanisms cannot be ruled out [34, 35].

This study has specifically enumerated Lin⁻CD45^{low}CD34⁺ in the circulation and showed progressive decline with age. This is not in agreement with the largest study, 2792 individuals, that has been reported [22]. This study used ISHAGE criteria for enumeration, a simple method for a rapid enumeration CD34⁺ cells in apheresis products for hematotherapy, and the data acquired is associated with a large variation that likely affected the outcome [36]. ACE2/ACE imbalance in HSPCs points to the upregulation of detrimental axis of local RAS. This is in agreement with previous experimental studies providing evidence for ACE inhibition as a pharmacological approach to increase life span though this study did not evaluate the vascular regenerative potential [37]. Specifically, this study showed that

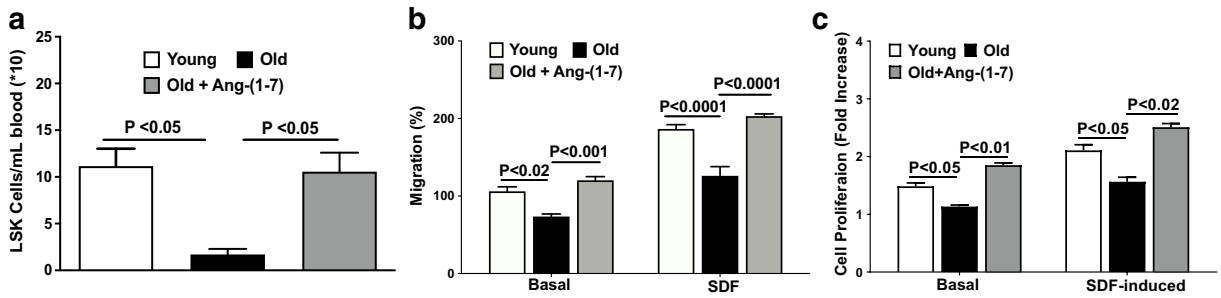


Fig. 4 Treatment with angiotensin-(1-7) (Ang-(1-7)) increases the circulating stem/progenitor cells and vascular repair–relevant functions in the aging mice. **a** Circulating lineage-negative (Lin^-) $\text{Sca-1}^+\text{cKit}^+$ (LSK) cells are lower in old mice compared to the young, and treatment with Ang-(1-7) increased the number of cells that was equivalent to that observed in the young group. Migration (**b**) and proliferation (**c**) of LSK cells in basal or in

response to stromal-derived factor-1 α (100 nM) (SDF) are attenuated in the old compared to the young mice. Ang-(1-7) treatment restored these functions in old cells that were similar to the responses observed in the young group. Where applicable, either paired or unpaired “*t*” test was used to determine statistical significance

long-term treatment with an ACE inhibitor, enalapril, decreased body weight and prolonged life span in rats

fed with high-fat diet, implying that inhibition of the detrimental axis of RAS restores tissue regeneration and

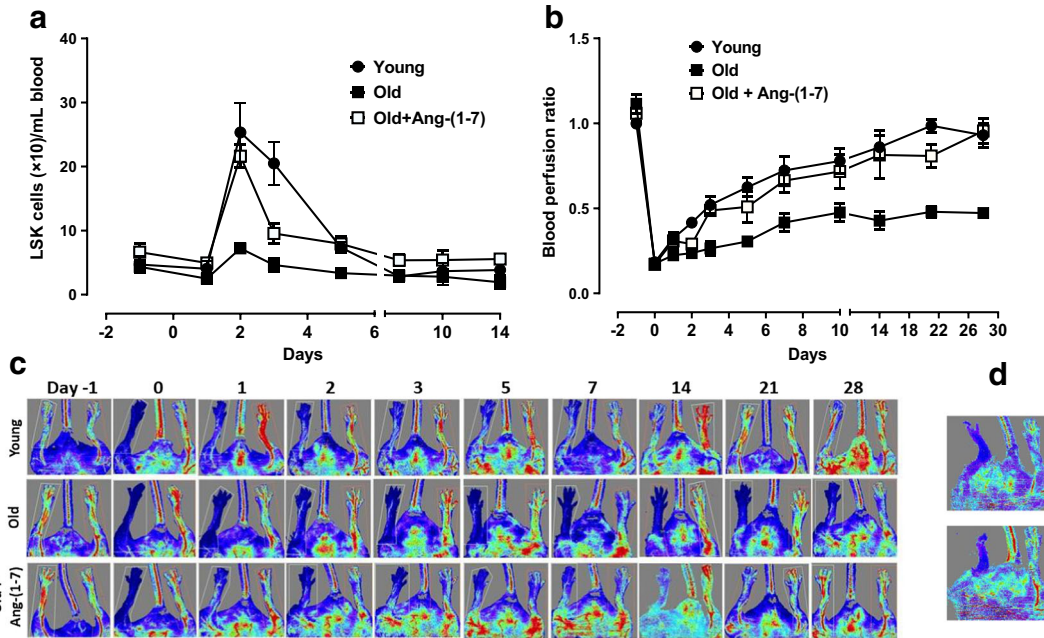


Fig. 5 Angiotensin-(1-7) (Ang(1-7)) treatment increases mobilization of bone marrow stem/progenitor cells in response to ischemia and restores blood flow to ischemic areas in the aging mice. **a** Increase in the circulating lineage-negative (Lin^-) $\text{Sca-1}^+\text{cKit}^+$ (LSK) cells in response to hind-limb ischemia (HLI) is lower in old mice compared to the young. Treatment with Ang-(1-7) restored this mobilization response in the old group (young vs old, and old vs old – Ang-(1-7), $P < 0.05$, two-way ANOVA). Tukey’s multiple comparisons detected significant differences in the following groups: day 2—young vs old ($P < 0.001$), young vs old + Ang-(1-7) (not significant), and old vs old + Ang-(1-7) ($P < 0.01$); day 3—young vs old ($P < 0.001$), young vs old + Ang-(1-7) ($P < 0.05$), and old vs old + Ang-(1-7) ($P < 0.05$). **b** Blood flow

restoration to the areas of ischemia is lower in old mice compare to the young. Treatment with Ang-(1-7) restored blood flow following HLI in the old mice, which was similar to that observed in the young group (young vs old, and old vs old – Ang-(1-7), $P < 0.05$, two-way ANOVA). Tukey’s multiple comparisons detected significant differences in the following groups: day 2—young vs old ($P < 0.05$); day 3—young vs old and old vs old + Ang-(1-7) ($P < 0.01$); days 7 to 28 young vs old ($P < 0.0001$) and Old vs Old + Ang-(1-7) ($P < 0.01$ to 0.001). **c** Representative pseudo-color images obtained by laser Doppler blood flow imaging in different treatment groups. **d** Representative pseudo-color images of blood flow in ischemic limbs of old mice showing either toe or partial foot amputations

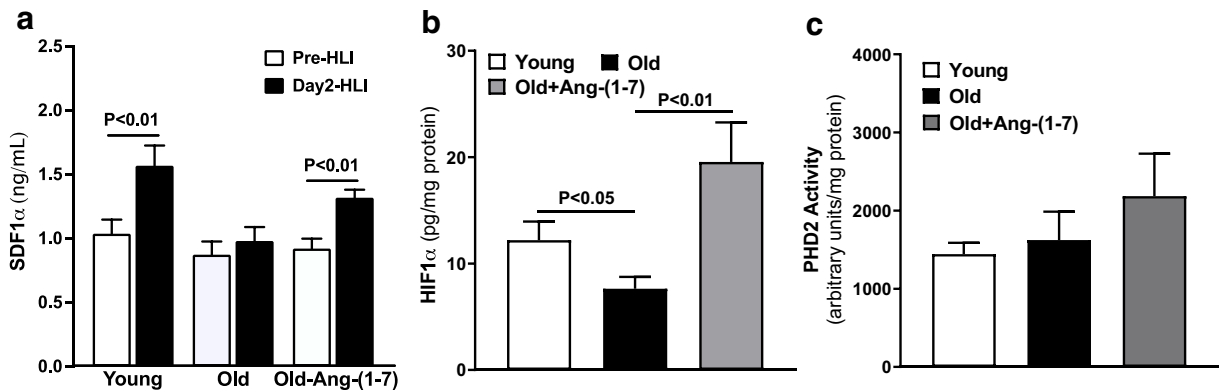


Fig. 6 Angiotensin-(1-7) (Ang-(1-7)) treatment restores hypoxic sensitivity in old mice undergoing ischemic injury. **a** Increase in the circulating levels of stromal-derived factor-1 α (SDF) following ischemic injury is lower in old mice compared to that in young, which was restored in the old group that was treated with Ang-(1-7). **b** Protein levels of hypoxia-inducible factor-1 α (HIF1 α) are lower in the old group compared to that in the young. Ang-(1-7)

increased HIF1 α levels in the old group that are significantly higher than that observed in the untreated old group. **c** Activity of the enzyme, prolyl hydroxylase, PHD, is similar in tissues derived from young, old, or Ang-(1-7)-treated old mice. Where applicable, either paired or unpaired “*t*” test was used to determine statistical significance

maintenance, which were previously shown to be impaired with aging HSPCs [23, 38]. Recent studies have further supported the hypothesis that pharmacological ACE inhibitors are beneficial for modifying body composition and for enhancing physical performance in elderly individuals [39].

While RAS dysregulation is not adequately addressed in HSPCs, evidence for either downregulation of protective axis or upregulation of detrimental axis has been shown in other organs. Recently, Badreh et al. [40] have shown that aging is associated with increased circulating levels of Ang II and high level of AT1aR protein expression in the heart and aorta, while the expression of the protective AT2R is decreased. On the other hand, few studies have addressed the role of ACE2/Ang-(1-7)/MasR axis in aging. ACE2-deficient mice have shown accelerated aging in cerebral vasculature that was associated with increased mitochondrial oxidative stress [41]. Age-associated cardiac myopathy in ACE2-deficient mice was indeed attributed to the Ang II-induced oxidative stress and inflammation [42]. Genetic deficiency of either ACE2 or MasR induced aging of noncardiovascular tissues, bone, and muscle [43]. Pharmacological activation of ACE2 by diminazene aceturate (DIZE) decreased adiposity in young and old rats on high-fat diet that was associated with anorexia [44]. While treatment with DIZE reduced white adipose tissue, the muscle mass was preserved despite strong anorectic effects [44].

ACE2/ACE imbalance was shown to be an underlying mechanism in other disease settings such as cardiac hypertrophy, pulmonary hypertension, and fibrosis [45–48]. However molecular mechanisms of this phenomenon were not yet established. One study in a mouse model of cardiac failure showed that Brahma-related gene-1 (Brg1), chromatin remodeling factor, and forkhead box M1 (FoxM1) transcription factor are activated in cardiac endothelial cells in response to pathological cardiac stress and cooperatively modulate ACE and ACE2 expressions via acting on their promoters [48]. Importantly, pharmacological inhibitor of FoxM1, thiothrepton, eliminated Brg1-dependent ACE promoter activation and repression of ACE2 promoter. This pathway is yet to be explored in other organ systems and pathologies including CD34⁺ cells; however, it is important to note that FoxM1 is required for quiescence and maintenance of hematopoietic stem cells [49, 50]; therefore, it was very unlikely to be a pharmacological target for reversing ACE2/ACE imbalance.

Circulating HSPCs are known to be activated by hypoxic exposure and increased expression of VEGFR2, CXCR4 and their ligands, VEGF, and SDF, a few to list that are HIF1 α -regulated [51]. Our recent findings added ACE2 and MasR to the list of HIF1 α -regulated factors in HSPCs, and this was associated with shedding of soluble ACE2 [16]. Furthermore, LSK cells that were mobilized in response to ischemic injury in mice undergoing HLI increased expression of ACE2,

and MasR was observed [16]. ACE2 shedding would increase the circulating levels of soluble ACE2, which is expected to stimulate cleavage of Ang II to Ang-(1-7), which in turn via acting on MasR would accelerate vascular repair–relevant functions in the HSPCs as well as in the peri-ischemic endothelium. Our study indicates that this beneficial effect is compromised in aging HSPCs. Furthermore, we show that migration and proliferation in response to SDF, functional signatures of migration, and homing of HSPCs in the areas of injury are attenuated with aging in mice. These dysfunctions were reversed by treatment with Ang-(1-7) *in vitro*. *In vivo* studies in aging mice recapitulated the reduction in the circulating HSPCs that was observed in aging individuals. Previously, in the precocious aging model, Klotho mice, vasculogenesis is impaired with reduced number of cKit⁺CD31⁺ cells in the bone marrow, and peripheral blood was observed [52]. Our study further showed reversal of this dysfunction by treatment with Ang-(1-7), which restored the number of circulating HSPCs to that observed in young mice.

In agreement with *in vitro* impairment of migration to SDF, mobilization of HSPCs from bone marrow to the blood stream in response to ischemic injury is decreased in the *in vivo* setting. As predicted, the stem cell mobilopathy is associated with impaired restoration of blood flow to the ischemic areas. This reparative dysfunction is reversed by treatment with Ang-(1-7). Concurrent increase in the circulating SDF levels with ischemic injury is not observed in aging mice compared to the young, which is associated with decreased HIF1 α levels in the ischemic tissue. However, this was not associated with increased enzymatic activity of PHD, a negative regulator of HIF1 α . Treatment with Ang-(1-7) has significantly increased the protein levels of HIF1 α , which partly explains the rise in SDF levels, but no change observed in PHD suggests that the effect is independent of stabilization of HIF1 α from degradation by PHD. In a skin wound model, aging impaired blood flow recovery and mobilization of LSK cells, and these dysfunctions were reversed by stabilization of HIF1 α via inhibiting the PHD [53]. Revascularization potential was shown to be impaired in adipose tissue–derived stromal cells, which was associated with decreased hypoxic sensitivity to induce SDF and VEGF [54]. While the current study determined hypoxia-relevant mechanisms, previous studies have reported that Ang-(1-7) via MasR activation restores NO bioavailability by normalizing ROS generation [55, 56]. Age is known to elevate

ROS generation and reduce vascular protection by NO, which in turn modulate hypoxic signaling in the vasculature, and by restoring NO bioavailability, Ang-(1-7) accelerates revascularization following ischemic insult.

Vasoprotective effects of MasR activation involve both bone marrow–derived progenitor cells as well as vascular endothelium. Angiocrine functions of HSPCs are stimulated by MasR activation in a mouse model of diabetes [57]. Ang-(1-7) treatment restored pro-angiogenic functions of cell supernatants derived from mouse bone marrow HSPCs that were impaired by diabetes in matrigel assay of angiogenesis. At the same time, pro-angiogenic gene expression, SDF, VEGFR1, and Tie 2, is increased in the microvasculature [57]. MasR signaling that mediates vasodilation and angiogenesis in endothelium has been delineated by bioinformatics and proteomics approaches. Major findings of this study point to the importance of p³⁸MAPK and ERK1/2 signaling cascades that lead to upregulation of VEGFR1 and VEGFR2 expressions. In addition, cdc42, RAC, and RAS/RAF were identified as upstream effectors following MasR activation by Ang-(1-7) [58]. PI3K/PRKD1/Akt and MAPK/ERK signaling pathways promote vasodilation as well as angiogenesis via increasing eNOS expression and activation resulting in increased NO bioavailability [58, 59]. However, age-associated alterations in MasR signaling in endothelium or CD34⁺HSPCs are yet to be delineated.

This study has some limitations. Mainly, the study did not evaluate age-dependent changes in the paracrine factors, and angiogenic properties could be determined in CD34⁺ cells. Along similar lines, MasR signaling in either CD34⁺ cells or the peri-ischemic vascular endothelium was not addressed. Age-dependent changes in the circulating angiotensin peptides were not determined, which would directly indicate the impact of ACE2/ACE imbalance. Lastly, while protein or activity of HIF1 α and PHD was determined, immunohistochemistry to identify the localization of these proteins and the co-localization with MasR were not addressed.

In conclusion, our study supports the hypothesis that aging-associated vasoreparative dysfunction in HSPCs is associated with decreased ACE2 activity and that pharmacological treatment with the heptapeptide metabolite of ACE2 would restore reparative functions in HSPCs and ischemic vascular repair. This study warrants further investigations towards development of stable analogs of Ang-(1-7) or novel small molecules that

activate MasR or ACE2 with potential benefits in enhancing vascular regenerative outcomes in elderly individuals.

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