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Angiotensin-(1-7) Counteracts Angiotensin II-induced Dysfunction in Cerebral Endothelial Cells via Modulating Nox2/ROS and PI3K/NO Pathways

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Abstract

Angiotensin (Ang) II, the main effector of the renin-angiotensin system, has been implicated in the pathogenesis of vascular diseases. Ang-(1-7) binds to the G protein-coupled Mas receptor (MasR) and can exert vasoprotective effects. We investigated the effects and underlying mechanisms of Ang-(1-7) on Ang II-induced dysfunction and oxidative stress in human brain microvascular endothelial cells (HbMECs). The pro-apoptotic activity, reactive oxygen species (ROS) and nitric oxide (NO) productions in HbMECs were measured. The protein expressions of nicotinamide adenine dinucleotide phosphate oxidase 2 (Nox2), serine/threonine kinase (Akt), endothelial nitric oxide synthase (eNOS) and their phosphorylated forms (p-Akt and p-eNOS) were examined by western blot. MasR antagonist and phosphatidylinositol-3-kinase (PI3K) inhibitor were used for receptor/pathway verification. We found that Ang-(1-7) suppressed Ang II-induced pro-apoptotic activity, ROS over-production and NO reduction in HbMECs, which were abolished by MasR antagonist. In addition, Ang-(1-7) down-regulated the expression of Nox2, and up-regulated the ratios of p-Akt/Akt and its downstream p-eNOS/eNOS in HbMECs. Exposure to PI3K inhibitor partially abrogated Ang-(1-7)-mediated protective effects in HbMECs. Our data suggests that Ang-(1-7)/MasR axis protects HbMECs from Ang II-induced dysfunction and oxidative stress via inhibition of Nox2/ROS and activation of PI3K/NO pathways.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Keywords

Cerebral endothelial cells; Ang II; Ang-(1-7); Oxidative stress

Introduction

The renin-angiotensin system (RAS), one of the most important regulatory systems, functions in regulation of blood pressure, tissue perfusion and extracellular volume. Angiotensin (Ang) II is generated from the enzymatic metabolism of Ang I by angiotensin converting enzyme (ACE), which is the major effector peptide of the RAS [1]. Previous studies have reported that Ang II is implicated in the pathogenesis of vascular diseases through activation of the Ang II type 1 receptor (AT₁R) [2, 3]. Pharmacologic interferences by using ACE inhibitors and Ang receptor blockers (ARBs) have achieved some beneficial effects but also resulted in some adverse effects such as hypotension and cough [4, 5]. Ang-(1-7), another important peptide of the RAS, is produced predominately from the degradation of Ang II by ACE2. Ang-(1-7) counteracts the actions of Ang II through activating the G protein-coupled Mas receptor (MasR). The Ang-(1-7)/MasR axis has been found in both systemic circulation and local organs such as the kidney and heart [6]. Ang-(1-7) may counteract the vasoconstrictive, arrhythmogenic and prothrombotic actions of Ang II better than ARBs [7]. Previous studies have shown that Ang-(1-7) attenuates Ang II-stimulated injury in type 2 diabetic nephropathy and prevents Ang II-induced cardiac remodeling [7, 8]. These studies demonstrate the opposing actions of Ang-(1-7) and Ang II.

The endothelium of cerebral microvessels is more complex and dynamic than the endothelium of other blood vessels due to its location and highly specialized structures. For example, cerebral endothelial cells forming the blood-brain barrier express high levels of tight and adherens junctions, and cell adhesion molecules than other endothelial cells [9, 10]. The functional and structural integrity of cerebral endothelium are important in maintaining the homeostasis of the brain vasculature. Its dysfunction is known as an essential step in the pathogenesis of cerebrovascular diseases such as ischemic stroke and subarachnoid hemorrhage [11, 12]. Previous reports have shown that activation of Ang II in endothelial cells triggers reactive oxygen species (ROS) over-production and decrease of nitric oxide (NO) production/bioavailability by inducing multiple downstream signaling pathways, consequently contributing to endothelial dysfunction [13, 14]. The nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (Nox) family is the most prominent source of vascular ROS [15], and its over-expression has been suggested to involve in Ang II-induced endothelial dysfunction, vascular remodeling and hypertension [14, 16]. Ang II-induced ROS over-production and cell apoptosis were inhibited in Nox2 knockout mice [17]. In addition, Ang II has been reported to induce endothelial dysfunction by reducing the production and/or bioactivity of NO, which may result from impaired endothelial NO synthase (eNOS) activity [14, 16]. Recent studies indicate that eNOS is regulated by serine/threonine kinase (Akt)-induced phosphorylation [18, 19]. Therefore, impaired Akt/eNOS signaling and subsequently reduced NO production could be another mechanism underlying the detrimental effects of Ang II in endothelial cells. However, whether Ang-(1-7)

counteracts the effects of Ang II on the specialized cerebral endothelial cells and the underlying mechanisms remains unclear.

In this study, we tested the hypothesis that Ang-(1-7) could protect human brain microvascular endothelial cells (HbMECs) against Ang II-induced pro-apoptotic activity, dysfunction and oxidative stress. We also explored the underlying mechanisms with focus on Nox2- and PI3K-dependent pathways.

Materials and methods

Cell culture

HbMECs and CSC culture medium kits were purchased from Cell Systems, Inc. (Kirkland, WA). The HbMECs were seeded on tissue culture plates with attachment factor and cultured (5% CO₂, 37°C) in CSC complete medium containing 10% serum, 2% CultureBoost-R (human recombinant growth factors) and 0.2% Bac-Off® antibiotic solution. The CSC culture medium was replaced every 48 h. Before experimental interventions, confluent cells were cultured in CSC serum-free medium for 10 h [20].

Concentration-responses of Ang II and Ang-(1-7) on HbMEC pro-apoptotic activity

Ang II (Sigma Aldrich, MO) induced cell injury model was produced as previously reported [20–22]. In brief, confluent HbMECs were incubated with increasing concentrations of Ang II (10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ M) for 24 h. After that, cells were collected for annexin V measurements. In order to determine the effective concentration of Ang-(1-7) (Bachem AG, CH) to suppress Ang II-induced pro-apoptotic activity, HbMECs were pre-treated with different concentrations of Ang-(1-7) (10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ M) for 30 min before addition of Ang II (10⁻⁷ M) for 24 h. After that, HbMECs were collected for annexin V measurements. Upon completion of this study, we chose 10⁻⁷ M of Ang II and Ang-(1-7) for the subsequent experiments.

Experimental groups

The doses of Ang II and Ang-(1-7) were determined by concentration-response studies. The concentrations of MasR specific antagonist A779 (10⁻⁶ M; Bachem Bioscience, PA) and phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (LY; 10 µM; Cayman Chemical, MI) were chosen based on previous reports [23–25]. Unless specified otherwise, confluent HbMECs were assigned to six groups: control, Ang II, Ang-(1-7), Ang II+Ang-(1-7), Ang II +Ang-(1-7)+A779, Ang II+Ang-(1-7)+LY. After incubation for 24 h, HbMECs were harvested for further analyses. For groups treated with both Ang II and Ang-(1-7), Ang-(1-7) was added to HbMECs 30 min prior to Ang II [21]. For receptor/pathway blockade, HbMECs were pre-incubated with A779 or LY for 30 min [23, 24, 26] prior to Ang II and Ang-(1-7).

Pro-apoptotic activity assay

The pro-apoptotic activity in HbMEC was assessed by using an Annexin V Apoptosis Detection Kit (eBioscience, CA). In brief, HbMECs after different treatments were collected by using 0.25% trypsin, and centrifuged at 300g for 8 min. HbMECs were then washed one

time with 1X phosphate buffered saline (PBS) and resuspended in 100 μ l 1X annexin-binding buffer. After that, 5 μ l of FITC-conjugated annexin V and 5 μ l of propidium iodide (PI) were added into cell suspension, and followed by incubation at room temperature (RT) for 15 min in the dark. Pro-apoptotic activities of HbmECs under different treatments were quantified by flow cytometry (Accuri C6 flow cytometer, CA). Both annexin V and PI negative (annexin V⁻/PI⁻) stained cells were considered to be viable cells. The cells stained only with annexin V (annexin V⁺/PI⁻) were considered to be early pro-apoptotic cells, the cells stained with both annexin V and PI (annexin V⁺/PI⁺) were considered to be late pro-apoptotic cells and the cells stained only with PI (annexin V⁻/PI⁺) were considered to be necrotic cells [27, 28]. In this study, we defined the pro-apoptotic cells as annexin V⁺/PI⁻ cells, since the detection of pro-apoptotic signaling at early stages helps to better analyze the pathway of programmed cell death [29].

Measurement of ROS production

Intracellular ROS level was determined by dihydroethidium (DHE; Molecular Probes, OR) staining [30]. To confirm whether Ang II enhances the formation of the superoxide radical in HbmECs, polyethyleneglycol-superoxide dismutase (PEG-SOD), an antioxidant enzyme, was used as a negative control to show superoxide has been produced. HbmECs were pre-treated with PEG-SOD (100 units/ml; Sigma-Aldrich) for 30 min before addition of Ang II [31–33]. Then, HbmECs were stained with DHE working solution (2 μ M) in the dark at 37°C for 2 h, washed one time with 1X PBS and replaced with fresh CSC complete medium. The DHE fluorescence was observed under an inverted fluorescent microscope (EVOS, NY), and the HbmECs were digested by 0.25% trypsin and centrifuged at 300g for 8 min. The DHE fluorescence in ROS measurements was quantified by flow cytometric analysis (Accuri C6 flow cytometer).

Determination of NO generation

The membrane permeable diaminofluorescein-FM diacetate (DAF-FM diacetate; Life Technology, Grand Island, NY) probe was used to assess the production of NO [34]. Measurement of NO using DAF-FM fluorescence in the presence of NG-nitro-arginine methyl ester (L-NAME), a substance widely used to inhibit eNOS, was used as a negative control. HbmECs were pre-treated with L-NAME (100 μ M; Sigma-Aldrich) for 30 min before addition of fresh CSC complete medium [35]. Then, HbmECs were incubated with 5 μ M DAF-FM diacetate probe in CSC serum-free medium (37°C for 45–60 min), washed twice with 1X PBS and incubated with CSC complete medium (37°C for 20 min) for desertification of the DAF-FM diacetate probe. The DAF-FM fluorescence was observed under an inverted fluorescent microscope (EVOS). Five independent images for each well were analyzed using image analysis (ImageJ software, MD) according to previous reports [36, 37].

Western blot analysis

Proteins were extracted from HbmECs with lysis reagent (Thermo Scientific, FL) containing protease inhibitor (1 tablet/10 ml lysis reagent, Roche Diagnostics, Germany). Proteins were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

and transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen, NY). The PVDF membranes were blocked by incubating with 5% non-fat milk for 1 h, and then incubated with antibodies against Nox2 (1:1000; Abcam, MA), Akt (1:500; Cell Signaling Technology, MA), p-Akt (Thr-308, 1:500; Cell Signaling Technology), eNOS (1:500; Cell Signaling Technology), and p-eNOS (ser-1177, 1:500; Cell Signaling Technology), at 4°C overnight. Nox2 inhibition and superoxide catalase were used to test whether Ang-(1-7) induces redox-independent eNOS activation. HbMECs were pre-treated with Nox2 inhibitor gp91ds-tat (1 μ M; Sigma-Aldrich) for 30 min and PEG-catalase (1000 units/ml; Sigma-Aldrich) for 30 min before addition of Ang-(1-7), respectively [33, 38]. The β -actin (1:4000; Sigma-Aldrich) was used to normalize protein loading. The PVDF membranes were incubated with Horseradish peroxidase (HRP)-conjugated IgG (1:40000; Jackson ImmunoResearch, Inc. PA) as secondary antibody for 1 h at RT, after being washed thoroughly with Tris-buffered saline three times (5 min each). Blots were developed with enhanced chemiluminescence developing solution and quantified using the ImageJ software (NIH) [39].

Statistical analysis

All data were expressed as mean $\pm$ SEM of at least three independent experiments. Data were analyzed using one- or two-way ANOVA. For all tests, a value of $P<0.05$ was considered statistically significant. All comparisons were performed using the SPSS statistics 17.0 for Windows.

Results

Concentration-responses of Ang II and Ang-(1-7) on HbMEC pro-apoptotic activity

Incubation of HbMECs with Ang II significantly increased the pro-apoptotic activity in HbMECs. Ang II at the dose of 10^{-9} M did not significantly induce pro-apoptotic activity ($P>0.05$ vs. control; Fig. 1A). When the concentrations increase (10^{-8} - 10^{-6} M), Ang II significantly increased pro-apoptotic activity in HbMECs (Fig. 1A). In order to determine the effective dose of Ang-(1-7) that inhibits Ang II-increased pro-apoptotic activity, HbMECs were pre-treated with different concentrations of Ang-(1-7) (10^{-9} - 10^{-6} M) for 30 min before addition of Ang II (10^{-7} M) for 24 h. Ang-(1-7) at the doses of 10^{-9} and 10^{-8} M were unable to suppress Ang II-induced pro-apoptotic activity (Fig. 1B), whereas Ang-(1-7) at the concentration of 10^{-7} and 10^{-6} M significantly decreased Ang II-induced pro-apoptotic activity (Fig. 1B).

Ang-(1-7)/MasR protects HbMECs from Ang II-increased pro-apoptotic activity via PI3K-dependent pathway

As shown in Fig. 2, no significant difference of pro-apoptotic activity in HbMECs was found between Ang-(1-7) and control groups ($P>0.05$). Ang II significantly increased pro-apoptotic activity when compared with the control ($22\pm1.6\%$ and $8.2\pm1.5\%$, Ang II vs. control, $P<0.05$). Ang-(1-7) suppressed Ang II-induced pro-apoptotic activity ($14\pm1.3\%$ and $22\pm1.6\%$, Ang II+Ang-(1-7) vs. Ang II, $P<0.05$). Furthermore, the anti-apoptotic effects of Ang-(1-7) was almost abolished by A779 ($22.2\pm1.6\%$ and $14\pm1.3\%$, Ang II+Ang-

(1-7)+A779 vs. Ang II+Ang-(1-7), $P<0.05$) and LY ($19.6\pm0.7\%$ and $14\pm1.3\%$, Ang II+Ang-(1-7)+LY vs. Ang II+Ang-(1-7), $P<0.05$).

Ang-(1-7)/MasR suppresses Ang II-induced ROS over-production in HbMECs via PI3K-dependent pathway

As shown in figure 3, the DHE fluorescence indicates ROS production in HbMECs. Ang II significantly increased the ROS production when compared with the control ($P<0.05$). The ROS over-production induced by Ang II was greatly diminished after pre-treatment of PEG-SOD ($P<0.05$), confirming the specificity of DHE fluorescence in ROS measurements. Ang II-induced ROS over-production was attenuated by Ang-(1-7) ($P<0.05$). In addition, pre-treatment of A779 or LY in HbMECs abolished the suppressive effect of Ang-(1-7) on Ang II-induced ROS over-production.

Ang-(1-7)/MasR induces Ang II-induced NO reduction in HbMECs via PI3K-dependent pathway

As shown in figure 4, the DAF-FM fluorescence indicates NO generation in HbMECs (Fig. 4A). Inhibition of DAF fluorescence by using the eNOS blocker L-NAME showed zero fluorescent background, confirming the specificity of DAF fluorescence in NO measurements. The level of NO was nearly undetectable in Ang II-treated HbMECs when compared with the control ($P<0.05$ vs. control; Fig. 4B). However, Ang-(1-7) increased the NO generation by about 1.6 fold of basal level in control group ($P<0.05$ vs. control; Fig. 4B), and induced an increase of NO generation in Ang II-treated group by about 39% ($P<0.05$ vs. Ang II; Fig. 4B). A779 and LY abolished Ang-(1-7)-stimulated NO generation in HbMECs ($P<0.05$; Fig. 4B).

Ang-(1-7) counteracts the effects of Ang II in HbMECs via decreasing Nox2 expression and activating Akt/eNOS signaling cascade

There was no significant difference in Nox2 expression between the control and Ang-(1-7) groups ($P>0.05$). The expression of Nox2 in HbMECs was significantly increased by Ang II ($P<0.05$ vs. control; Fig. 5A), but was ameliorated by about 30% with pretreatment of Ang-(1-7) ($P<0.05$ vs. Ang II; Fig. 5A). The phosphorylated form of Akt (phospho-Akt or p-Akt) and eNOS (phospho-eNOS or p-eNOS) indicates Akt and eNOS activation, respectively. Neither Ang II nor Ang-(1-7) significantly changed the total level of Akt and eNOS, but both of them changed the phosphorylated statuses of Akt and eNOS (Fig. 5B and C). Ang II decreased the ratios of p-Akt/Akt and p-eNOS/eNOS in HbMECs, whereas Ang-(1-7) increased the ratios of p-Akt/Akt and p-eNOS/eNOS in HbMECs. In addition, Nox2 inhibition and hydrogen peroxide scavenge, by using gp91ds-tat and antioxidant enzyme PEG-catalase respectively, didn't affect the eNOS activation afforded by Ang-(1-7).

Discussion

In this study, we demonstrated that Ang-(1-7)/MasR protects HbMECs from Ang II-induced dysfunction and oxidative stress. The underlying mechanisms may rely on modulation of Nox2/ROS and PI3K/NO signaling pathways.

Several previous studies have shown that Ang-(1-7) protects endothelial cells against apoptosis and inflammation [21, 26, 40–43]. To extend these studies, we conducted the experiments in HbMECs, which are commonly used as the in vitro blood-brain barrier model and are much different from the human umbilical vein endothelial cells (HUVECs) used in previous studies. HbMECs display different features from HUVECs under culture condition. For example, HbMECs are characterized by less permissibility, absence of fenestration and high expression of tight and adherens junctions [9, 10, 44]. In addition, HbMECs display selective expression of cell adhesion molecules, higher transendothelial electrical resistance, and elongation resistance to curvature and shear stress [45–48]. Of note, both HbMECs and HUVECs show the characteristic cobblestone appearance which resembles endothelial cell morphology in vivo. It is logical and novel to determine the role of Ang-(1-7) in cerebral endothelial function. In the aspect of mechanisms, previous studies have shown protective effects of Ang-(1-7) on endothelial function by inhibition of LOX-1, JNK and NF-kappaB pathways [21, 40–42] and on cardiac myocytes and pancreatic islet endothelial cells via PI3K/Akt pathway activation [26, 43]. However, it remains unclear whether Ang-(1-7) counteracts the effects of Ang II on HbMECs through the PI3/Akt-dependent pathway.

Accumulating evidence suggests that Ang-(1-7) is a biologically active peptide that counteracts many negative actions of Ang II [21, 49, 50]. The anti-hypertensive effects of Ang-(1-7) could be due to its interaction with vasodilator bradykinin, prostaglandins and NO [50]. Ang-(1-7) may oppose the detrimental actions of Ang II through its anti-oxidative and anti-apoptotic properties [51, 52]. The major vascular source of ROS is the Nox family, from which Nox2 is of special importance in the endothelium of the vascular system [15]. In addition to Nox activation, eNOS uncoupling and/or inactivation could aggravate ROS over-production and decrease antioxidant NO generation [53]. Therefore, both Nox2- and eNOS-mediated signaling pathways are involved in the counteracting effects of Ang-(1-7) and Ang II. To test our hypothesis that Ang-(1-7) could protect HbMECs against Ang II-induced oxidative stress and pro-apoptotic activity, we produced the Ang II-induced injury model in HbMECs as previously reported [21, 22]. As we expected, Ang II at the doses of 10^{-7} and 10^{-6} M increased the pro-apoptotic activity in HbMECs. By using this model, we found that the pro-apoptotic activity induced by Ang II was significantly decreased after co-incubation of Ang-(1-7) at the doses of 10^{-7} and 10^{-6} M.

The underlying mechanisms whereby Ang-(1-7) protects HbMECs against Ang II-induced dysfunction might rely on both Nox2/ROS and PI3K/NO signaling pathways. Firstly, Nox2 over-expression by Ang II could activate multiple downstream signaling pathways to produce potentiate cellular damage or even death [54]. In this study, increased expression of Nox2 protein and over-production of ROS have been observed in Ang II-treated HbMECs. It is essential to know that whether enhanced ROS generation or decreased/diminished ROS scavenge accounts for increased level of ROS [55]. However, since both Nox2 expression and ROS generation were increased in our study, we believe that Ang II-induced oxidative stress is more likely attributed to increased ROS generation than decreased/diminished ROS scavenge. Ang-(1-7) suppressed the Nox2 expression and ROS over-production induced by Ang II, suggesting that Ang-(1-7) counteracts Ang II in cerebral endothelial cells at least in part by suppressing Nox2 expression and subsequently inhibiting ROS over-generation. Secondly, we found that Ang II decreased the activated forms of both Akt and eNOS

proteins, and inhibited the production of NO at the same time. Ang-(1-7) was found to up-regulate the activated forms of both Akt and eNOS proteins, and subsequently induce NO generation which was compromised in the Ang II-induced HbMEC injury model. It is well known that NO is a nature antioxidant and is able to suppress ROS over-production. Our study suggests that activated Akt/eNOS signaling pathway may be one of the protective mechanisms afforded by Ang-(1-7) in endothelial cells. Finally, we applied the MasR antagonist and PI3K inhibitor to verify the involvement of MasR and Akt upstream molecule PI3K in Ang-(1-7)-induced protective effects in HbMECs. We found that the A779 effectively blocked the protective effects of Ang-(1-7) on Ang II-induced dysfunction, suggesting the effects of Ang-(1-7) were mostly mediated by activation of MasR. Application of LY also obviously decreased Ang-(1-7)-induced protective effects on HbMECs. However, application of gp91ds-tat and PEG-catalase didn't significantly affect the eNOS activation attributed to Ang-(1-7). Therefore, Ang-(1-7) may induce redox-independent eNOS activation, which would worth further investigations.

In summary, our results show that Ang-(1-7) can ameliorate Ang II-induced pro-apoptotic activity, ROS over-production and NO reduction in HbMECs, via suppression of Nox2 expression and activation of PI3K/Akt/eNOS signaling cascade.

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Highlights

- We examine the counteracting effects of Ang II and Ang-(1-7) on cerebral endothelial cell function.
- The inhibitory effects of Ang-(1-7) on Ang II-induced endothelial dysfunction and oxidative stress are mediated by Mas receptor.
- Cell pro-apoptotic activity, ROS and NO productions were studied upon stimulation of Ang II and/or Ang-(1-7) in HbMECs.
- The mechanisms rely on suppression of Nox2/ROS and activation of PI3K/Akt/eNOS/NO pathways.

Fig.1.

Ang-(1-7) counteracts the pro-apoptotic activity induced by Ang II in HbmECs. (A) HbmECs were stimulated with increasing doses (10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M) of Ang II for 24 h, and stained with annexin V and PI for flow cytometric analysis. (B) HbmECs were pretreated with increasing doses (10^{-9} , 10^{-8} , 10^{-7} and 10^{-6} M) of Ang-(1-7) for 30 min before addition of Ang II at the indicated dose (10^{-7} M) for 24 h, and stained with annexin V and PI for flow cytometric analysis. Results are mean $\pm$ SEM, n=3/group, # P <0.05 vs. control (con); * P <0.05 vs. 10^{-9} M or 10^{-8} M Ang II; + P <0.05 vs. 10^{-7} M Ang II; & P <0.05 vs. 10^{-7} M Ang II+ 10^{-9} M or 10^{-8} M Ang-(1-7).

Fig. 2. Ang-(1-7)/MasR protects HbMECs from Ang II-increased pro-apoptotic activity via PI3K-dependent pathway. (A) Representative flow cytometric plots of pro-apoptotic activity in HbMECs. Annexin V⁻/PI⁻ cells (the lower left quadrant) were considered viable cells, annexin V⁺/PI⁻ cells (the lower right quadrant) were defined as early pro-apoptotic cells, annexin V⁺/PI⁺ cells (the upper right quadrant) were considered as late pro-apoptotic cells, annexin V⁻/PI⁺ cells (the upper left quadrant) were considered necrosis cells. (B) Summarized data of pro-apoptotic activity in HbMECs. Results are mean±SEM, n=4/group. #P<0.05 vs. con; +P<0.05 vs. Ang II; &P<0.05 vs. Ang II+Ang-(1-7).

Fig. 3.

Ang-(1-7)/MasR suppresses Ang II-induced ROS over-production in HbMECs via PI3K-dependent pathway. (A) Representative DHE fluorescent images of HbMEC ROS production. Scale bar: 400 μ m. (B) Summarized data of HbMEC ROS production. Inhibition of DHE fluorescence with superoxide scavenger PEG-SOD in HbMECs is used to confirm the specificity of DHE fluorescence in ROS measurements. Results are mean $\pm$ SEM, n=5/group. [#] P <0.05 vs. con; ⁺ P <0.05 vs. Ang II; [&] P <0.05 vs. Ang II+Ang-(1-7).

Fig. 4.

Ang-(1-7)/MasR improves Ang II-induced NO reduction in HbmECs via PI3K-dependent pathway. (A) Representative DAF-FM fluorescent images of HbmEC NO production. Scale bar: 400 μ m. (B) Summarized data of HbmEC NO production. Inhibition of DAF-FM fluorescence with eNOS blocker L-NAME in HbmECs is used to confirm the specificity of DAF-FM fluorescence in NO measurements. Results are mean $\pm$ SEM, n=5/group. # P <0.05 vs. con; + P <0.05 vs. Ang II; & P <0.05 vs. Ang II+Ang-(1-7).

Fig. 5.

Ang-(1-7)/MasR counteracts the effects of Ang II in HbMECs via decreasing Nox2 expression and activating Akt/eNOS signaling cascade. (A) Nox2 level in HbMECs. (B) Phospho-Akt (p-Akt) and Akt levels in HbMECs. (C) Phospho-eNOS (p-eNOS) and eNOS levels in HbMECs. Nox2 inhibitor gp91ds-tat and hydrogen peroxide scavenger PEG-catalase are used to test whether Ang-(1-7)/MasR induces redox-independent eNOS activation. Results are mean±SEM, n=5/group. [#]*P*<0.05 vs. con; ⁺*P*<0.05 vs. with Ang II.