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Effects of Ang 1-7 and Endothelial Microvesicles on Ang II-induced Dysfunction and Apoptosis in Cerebral Endothelial Cells

Xiang Xiao

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EFFECTS OF ANG 1-7 AND ENDOTHELIAL MICROVESICLES ON ANG II-INDUCED DYSFUNCTION AND APOPTOSIS IN CEREBRAL ENDOTHELIAL CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

Xiang Xiao
B.S., University of South China, 2010

2013
Wright State University
WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

August 26, 2013

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Xiang Xiao ENTITLED Effects of Ang 1-7 and Endothelial Microvesicles on Ang II-induced Dysfunction and Apoptosis in Cerebral Endothelial Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Xiang Xiao, M.S. Department of Pharmacology and Toxicology, Wright State University, 2013. Effects of Ang 1-7 and Cellular Vesicles on Ang II-Induced Dysfunction and Apoptosis in Cerebral Endothelial Cells.

Angiotensin II (Ang II) induces endothelial dysfunction and is implicated in the pathogenesis of vascular diseases. Angiotensin 1-7 (Ang 1-7) has been reported to counteract many deleterious effects of Ang II. Endothelial microvesicles (EMVs) are small membrane vesicles released from endothelial cells (ECs) undergoing stress and apoptosis. But their functions are largely unknown. In this study, we investigated the effects of Ang 1-7 and EMVs on apoptosis and dysfunction of human brain microvascular endothelial cells (HbmECs). Reactive oxygen species (ROS) and nitric oxide (NO) production, and Nox2, p-Akt/Akt, p-eNOS/eNOS expression were analyzed. We found that Ang II dose-dependently induced HbmEC apoptosis and that both Ang 1-7 and EMVs can counteract the effects of Ang II. Their protective effects were associated with ROS/NO production which were linked to Nox2, and Akt/eNOS pathways. Our data suggests that both Ang 1-7 and EMVs protect endothelial dysfunction and apoptosis induced by Ang II in HbmECs.
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providing excellent courses and teaching for my masters program study.
I. INTRODUCTION

Part 1: Angiotensin Axis and Endothelial Dysfunction

Renin angiotensin system (RAS)

The renin angiotensin system (RAS) is a hormonal cascade in regulation of cardiovascular, renal, and adrenal function that governs body fluid and electrolyte homeostasis, as well as arterial pressure. It has been extensively studied since the first description of renin by Tigerstedt and Bergmann in 1898. The classical RAS consists of a circulating endocrine system in which the principal effector hormone is Angiotensin (Ang) II. Ang II is a biologically active octapeptide that is produced by the action of renin on angiotensinogen to form Ang I and then subsequently convert to Ang II by Ang-converting enzyme (ACE). The actions of Ang II are mediated via the Ang type 1 receptor (AT1 receptor). However, the current view of RAS system is characterized by an increased complexity, as evidenced by the discoveries of new functional components and actions of the RAS, including local tissue RAS, a (pro)renin receptor, ACE2, Ang 1-7, Mas receptor, the function of the Ang type 2 receptor (AT2 receptor). Studies have demonstrated the importance of a tissue RAS in the brain, heart, blood vessels, adrenal glands, kidney and etc [1-5].
Although some of the components that necessary for the biosynthesis of the active peptide product (Ang II) may be taken up from the circulation into the tissue, the local tissue RAS defines the de novo tissue generation of Ang II and its interaction with Ang II receptor on the same (autocrine) or adjacent (paracrine) cells [6].

The RAS cascade (Fig 1) is activated in response to reduced perfusion which result in renin secretion and release into circulation [6]. Renin cleaves the angiotensinogen to form the inactive decapeptide Ang I. The catabolism of Ang I serves as a point of divergence in the system, leading not only to the production of the bioactive octapeptide Ang II by ACE [7], but also to the formation of the active peptide Ang 1-7 by prolylendopeptidase and neprilysin [8]. ACE2 is a recently identified homologue of ACE, which can catalyze Ang I to Ang 1-9 [9], with subsequent conversion to Ang 1-7 by ACE or neutral-endopeptidase [10]. ACE2 is also highly effective in the conversion of Ang II to Ang 1–7, which has approximately 400-fold more affinity to Ang II than to Ang I [11]. Ang II can further be degraded by aminopeptidase (AMP) and dipeptidyl-aminopeptidase to produce Ang III and Ang IV, respectively [12]. Of note, Ang IV can be produced from Ang III by the activity of AMP. Ang 1-7 can be metabolized by ACE to form the inactive degradation products Ang 1-5 [13].
Figure 1. RAS cascade. AGT: angiotensinogen; Ang: angiotensin; ACE: angiotensin-converting enzyme; AT1 R: Ang II type 1 receptor; AT2 R: Ang II type 2 receptor; Mas R: Ang 1-7 receptor; AMP: aminopeptidase; D-Amp: dipeptidyl-aminopeptidase; PCP: prolyl-carboxypeptidase; PEP: prolylendopeptidase; NEP: neutral-endopeptidase; (P)RR: (pro)renin receptor.

The physiological and pathological roles of RAS

The RAS is a complex regulatory system which plays an important role in mediating both physiological and pathological functions. The main physiological stimuli for RAS activation are low salt intake, blood volume and BP. In turn, Ang II acts to help raise blood volume and BP, thereby prevent hypovolemia and hypotension.
The main physiological roles of RAS are: 1) to prevent life-threatening shrinkage of intravascular volume (rapid actions of Ang II). 2) to help achieve sodium balance without large alterations in BP (slower actions of Ang II). 3) to increase the efficiency of cardiovascular dynamics by promoting the growth of the heart and vessels, and sensitizing blood vessels to vasoconstrictor agents (slowest actions of Ang II). The functions of the RAS from the general point of view is being homeostatic. However it will be harmful if carried to excess. Indeed, increased activity of the RAS, especially in combination with other cardiovascular risks factors, may lead to a cascade of deleterious effects such as hypertension, cardiovascular hypertrophy, oxidative stress with endothelial dysfunction, atherosclerosis and tissue inflammation [14]. In recent years, the pathophysiological implications of the system have been the main focus of attention, inhibitors of the RAS such as ACE inhibitors and Ang II receptor blockers have become important clinical tools in the treatment of cardiovascular and renal diseases such as hypertension, heart failure, and diabetic nephropathy [15].

Two counteracting axes in RAS

There are two counteracting axes that exist in RAS: ACE/Ang II/AT1 receptor axis and ACE2/Ang 1-7/Mas receptor axis. The ACE/Ang II/AT1 receptor is a classical RAS axis. Ang II produced from Ang I by ACE is a well-known bioactive substance in the regulation of blood pressure and is involved in the exaggeration of vascular disease [16]. The major receptor subtypes for Ang II are the AT1 and AT2 receptors. Both the AT1 and the AT2 receptors belong to
the superfamily of G protein-coupled receptors that contains seven transmembrane regions. They share about 34% homology and have distinct signal transduction pathways. The distribution of AT1 receptor covers most organs [17], whereas AT2 receptor expression is observed in only a few organs after birth and is up-regulated in pathological states [18;19]. AT1 receptor stimulation mediates the classical major actions of Ang II and is known to cause vasoconstriction, inflammation, fibrosis, cellular growth/migration and fluid retention (Table 1) [20]. These findings are also closely related to the development of RAS inhibitors, such as ACE inhibitors (ACEi) and AT1 receptor blockers (ARBs), promoting the basic and clinical research in the RAS. Accordingly, They are widely used as antihypertensive, cardiovascular and renoprotective drugs with well-documented effectiveness. Nevertheless, important limitations have been reported related to the use of these drugs. For instance, besides their potential side effects, the responses to ACEi treatment are influenced by gender and ethnic diversity and ARBs have limited efficacy in treatment of end-organ damage [21]. AT2 receptor stimulation appears to antagonize the effects mediated by AT1 receptor stimulation. Therefore, AT2 receptor stimulation could contribute to the effects of ARBs [18;19;22].

The ACE2/Ang 1–7/Mas axis has been highlighted as the counteracting partner of the ACE/Ang II/AT1 receptor axis. Ang 1-7 is now recognized as a biologically active component of RAS since it exerts a vast array of actions, many of them opposite to those attributed to Ang II. It was initially regarded as
an inactive component of the RAS for many years. However, in recent years, two pivotal discoveries clearly established Ang 1-7 as an active RAS mediator. First, the existence and characterization of ACE2, which was established later as the main Ang 1-7 forming enzyme [23;24]. Second, the G-protein coupled Mas receptor was discovered as a functional receptor for its endogenous ligand Ang 1-7 [25]. In mammals, Mas receptor is expressed predominantly in brain and testis with moderate levels of expression in heart and kidney [26;27]. Thus, Ang 1-7 is now considered a biologically active member of the RAS, which binds to Mas receptor to induce many beneficial actions, such as vasodilation, inhibition of cell growth, anti-inflammation, anti-thrombosis and anti-fibrosis [28;29].

<table>
<thead>
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<th>ACE/Ang II/AT1 R</th>
<th>ACE2/Ang 1-7/Mas R</th>
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<tr>
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<td>Ang II</td>
</tr>
<tr>
<td>Enzyme</td>
<td>ACE</td>
<td>ACE2</td>
</tr>
<tr>
<td>Receptor</td>
<td>AT1</td>
<td>Mas</td>
</tr>
<tr>
<td>Effects</td>
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<td>Vasodilatation; anti-inflammation; anti-thrombosis; anti-fibrosis; anti-hypertrophy; antiproliferation</td>
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Table 1. Two counteracting axes in RAS. Ang I: angiotensin I; Ang II: angiotensin II; Ang 1-7: angiotensin 1-7; ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme 2; AT1: angiotensin II type 1 receptor;
Mas: angiotensin 1-7 receptor; AMP: aminopeptidase; D-Amp: dipeptidyl-aminopeptidase; Ang1-5: angiotensin 1-5; Ang III: angiotensin III (2-8).

**Ang II/Ang 1-7 balance regulates endothelial function**

Endothelium is the active inner monolayer of the blood vessels, forming an interface between circulating blood and the vessel wall. It represents the largest organ in the body and plays a critical role in vascular homeostasis. Endothelial cells regulate vascular tone by releasing various contracting and relaxing factors including nitric oxide (NO), arachnoid acid metabolites, reactive oxygen species (ROS), and vasoactive peptides. In addition, the endothelium actively regulates vascular permeability, inflammatory activity, cell proliferation, as well as the balance between coagulation and fibrinolysis. Endothelial dysfunction is referred as impaired endothelium-dependent vasodilation (reduction of the bioavailability of vasodilators and increase of endothelium-derived contracting factors). In addition to that, endothelial dysfunction also comprises a specific state, which is characterized by increased vascular permeability, cell proliferation, and a proinflammatory and prothrombotic phenotype (including leucocyte-endothelial interactions and increased adhesion and aggregation of platelets) [30;31]. Oxidative stress can induce vascular endothelial dysfunction. ROS are generated at sites of inflammation and injury. It can function as signaling molecules that participate in the regulation of fundamental cell activities such as cell growth and cell adaptation responses at low concentrations; whereas, ROS can cause cellular
injury and death at higher concentrations [32]. Exogenous ROS can be produced from pollutants, tobacco, smoke, drugs, xenobiotics, or radiation. What's more, ROS can be produced intracellularly through multiple mechanisms, the major sources being mitochondria, peroxisomes, endoplasmic reticulum, and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in the cell membranes.

Ang II has significant pro-inflammatory actions in the vascular wall, inducing the production of ROS, cytokines, adhesion molecules and chemokines [33]. The endothelial dysfunction caused by Ang II is mainly through the reduction of NO bioavailability and imbalance between the ROS and antioxidant capacity. In endothelial cells (Figure 2), NADPH oxidase catalytic subunit gp91phox (Nox2) is activated by Ang II when it binds to the AT1 receptor [34]; The NADPH oxidase (Nox) regulatory subunit p47phox is phosphorylated and translocated to p22phox. Increased p47phox-p22phox complex formation initiated Nox activation and subsequently ROS over-production [35]. Enhanced production of ROS (mainly O2-) are known to induce programmed cell death in the vascular ECs [36]. The pro-apoptotic effect of endogenous ROS induced by Ang II in ECs seems to involve the disturbance of mitochondrial membrane permeability, which is a prerequisite for cytochrome c release. The cytochrome c is regulated by the Bcl-2 protein family [37]. The Bcl-2 protein family can either promote (e.g., Bax) or inhibit apoptosis (e.g., Bcl-2, Bcl-XL). An imbalance between anti-apoptotic and pro-apoptotic members of Bcl-2 protein family will increase the mitochondrial membrane permeability, and then trigger the release of cytochrome c from the space between the inner and outer
mitochondrial membrane into the cytoplasm [36]. In the cytoplasm, the complex formation of cytochrome c and apoptotic peptidase activating factor 1 (Apaf1) stimulates caspase-9 release, which results in the activation of caspase-3, which eventually leads to EC apoptosis.

Through the detailed mechanism of Ang 1-7 counteracts the actions of Ang II remains unclear, accumulating evidences suggest that Ang 1-7 may oppose the endothelial dysfunction and apoptosis induced by Ang II either through directly inhibiting the activity of Nox or through stimulating NO production and subsequently inhibition of apoptosis executioner proteases (caspases) (Figure 2) [38;39].

![Figure 2. Ang II/Ang 1-7 balance in regulating endothelial cell function: Nox and Akt/eNOS signaling pathways. Ang II: angiotensin II; Ang 1-7: angiotensin](image)

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AT1 R: angiotensin II type 1 receptor; Mas R: Mas receptor; Nox: NAD(P)H oxidase; PI3K: phosphoinositide 3-kinases; p-Akt: protein Kinase B phosphorylation; eNOS: endothelial nitric oxide synthase. ROS: reactive oxygen species; NO: nitric oxide.

**PI3K/Akt/eNOS signaling pathways in endothelial cells**

NO plays a critical role in endothelial function by maintaining vasodilator tone, inhibiting platelet aggregation and adhesion [40]. Many risk factors implicated in cardiovascular diseases seem to be associated with impairment in the NO system [40;41]. Phosphoinositide 3-kinases (PI3K) and its downstream serine/threonine kinase Akt (also known as Protein Kinase B) play a central role in promoting the survival of a wide range of cell types, including ECs [42]. PI3K constitutes a multifunctional family of enzymes activated by receptor tyrosine kinases, G protein coupled receptor and other stimuli. Upon receptor activation, PI3K subunit is recruited to the plasma membrane and binds to inositol lipids. These lipids serve as docking sites for proteins that harbor pleckstrin-homology domains, including Akt and its upstream activator phosphoinositide-dependent kinases 1 (PDK1). Once in the membrane, Akt is phosphorylated by PDK1, leading to the activation of Akt. Akt activation mediates the phosphorylation of endothelial NO synthase (eNOS), and thereby stimulates eNOS activity and increases NO release. Previous studies have found that Ang II had a biphasic effect on Akt phosphorylation [43;44]. It is suggested that low concentration Ang II causes a dose-dependent increase in Akt phosphorylation, while high concentration of Ang II lead to a decrease of
Akt phosphorylation in ECs [43;44]. Recent studies have suggested that Ang 1-7 regulates EC function through functionally active Mas receptor, which stimulates eNOS activity and NO production through Akt-dependent pathways [45;46].

**Part 2: Extracellular Vesicles and Endothelial Dysfunction**

**Extracellular vesicles**

The extracellular space of multicellular organisms contains solutions of metabolites, ions, proteins and polysaccharides. However, it is clear that this extracellular environment also contains a large number of mobile membrane-limited vesicles for which the term of extracellular vesicles is suggested [47]. Extracellular vesicles are shed in response to cell activation, dysfunction, injury, or apoptosis. Major types of extracellular vesicles are exosomes, microvesicles (MVs) and apoptotic bodies (Figure 3). There is an increasing interest in the field on MVs and exosomes, whose release may represent a universal and evolutionarily conserved process.

**Key features of MVs and exosomes**

The key features (table 2) of exosomes include: 1) Exosomes are vesicles surrounded by a phospholipid bilayer (approximately 50–100 nm in diameter), their size range roughly overlaps that of the viruses [47]. 2) Exosomes are an end-product of the endocytic recycling pathway. First, endocytic vesicles form at the plasma membrane and fuse to form early endosomes. They become late endosomes where intraluminal vesicles (ILVs) bud off into the lumen.
These multivesicular bodies (MVBs) then directly fuse with the plasma membrane and release exosomes into the extracellular space. 3) Exosomes have been predominantly characterized in the case of immune cells (dendritic cells, T cell, B cell, macrophages) and tumors. 4) They feature phosphatidylserine (PS) on the outer membrane leaflet and exosomal markers such as CD63, CD81 and CD9 [48;49]. 5) Isolation methods include a slow centrifuge at 1500g for 15 minutes followed by 100 nm gravity-driven filtration. Exosomes are final pellet at 120,000 x g for 70 minutes [47]. For purer preparations, some researchers use sucrose gradient ultracentrifugation. 6) Transmission electron microscopy has been typically used to characterize exosomes due to their small size. Western blot and mass spectroscopy have been used to identify the proteomic profiles of exosomes. Bead-coupled flow cytometry can also be used to detect exosomes [47;50].

The key features (table 2) of MVs include: 1) MVs are structures surrounded by a phospholipid bilayer (approximately 100–1000 nm in diameter). Their size range overlaps that of bacteria and insoluble immune complexes [51]. 2) They are formed by regulated release by budding/blebbing of the plasma membrane. 3) They have been predominantly characterized as products of platelets, red blood cells and ECs. 4) They feature PS on the outer membrane leaflet [52], tissue factor (TF) and cell specific markers. however, some observations also suggest the existence of MVs without PS externalization [52]. 5) Routine isolation methods include a slow centrifuge at 1500g for 15 minutes and followed by centrifuge at 18,000g for 30 min or 100,000g for 60 min [53].
6) Flow cytometry is the commonest method for detecting MVs, transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) were also used to detecting MVs [54;55].

<table>
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<tr>
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<th>Exosomes</th>
<th>MVs</th>
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<tr>
<td><strong>Size Range</strong></td>
<td>30–100 nm in diameter</td>
<td>0.1 -1 µm in diameter</td>
</tr>
<tr>
<td><strong>Biogenesis</strong></td>
<td>Exocytosis of MVBs</td>
<td>Budding from plasma membrane</td>
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<tr>
<td><strong>Markers</strong></td>
<td>CD63, CD81, CD9, and Tsg101</td>
<td>Annexin V binding, TF and cell-specific markers</td>
</tr>
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</table>

Table 2. Key features of exosomes and MVs. MVBs: multivesicular bodies; TF: tissue factor.

**Potential use of endothelial-derived MVs and exosomes as biomarkers and their functional significance**

Both MVs and exosomes are released when cells undergo stress, activation or apoptosis. They can carry the characteristics of their parent cells, which enable them to serve as potential biomarkers for various diseases [47;56;57]. Of note, they also have been shown to mediate cell communications through the ability of transferring membrane proteins, phospholipids and RNAs from their parent cells to distant cells [58]. Recent novel pharmacological approaches have started to reveal the functions of MVs and exosomes.
Endothelial MVs (EMVs) result from endothelial plasma membrane blebbing and carry endothelial proteins such as vascular endothelial cadherin (VE-cadherin or CD144), E-selectin (CD62E), platelet endothelial cell adhesion molecule-1 (CD31) [59], intercellular cell adhesion molecule (ICAM)-1 (CD54), endoglin (CD105), S-endo (CD146) or αv integrin (CD51) [60]. Among these, only CD62E and CD144 are the exclusive markers for EMVs. Like other MVs, EMVs also can transfer functional proteins and nuclear materials such as DNA, mRNA, and miRNA to target cells [58]. Although EMVs represent a sparse population of circulating MVs, their changes in plasma level might predict important clinical information [49]. The level of circulating EMVs are inversely correlated with the amplitude of flow-mediated dilatation in patients presenting endothelial dysfunction, independently of age and pressure [61].

The functional significance of EMVs has been proposed in recent years. Abid Hussein et al. first documented that EMV release could protect EC from apoptosis by diminishing caspase-3 level in cultured ECs [62]. EMVs contribute to the sorting of pro-apoptotic factors and the prevention of cell detachment and apoptosis. Moreover, EMVs carrying endothelial protein C receptor and activated protein C could also promote cell survival by inducting cytoprotective and anti-inflammatory effects [63]. One recent publication has described that the EMVs could protect ECs against apoptosis induced by camptothecin and that inhibition of p38MAPK activity is involved in EMV-mediated anti-apoptotic effect [64]. In most cases, the role of EMV
carried nuclear materials remains to be determined. A pioneering study by Deregibus et al. showed that endothelial progenitor cell derived MVs (EPC-MVs) were able to trigger an angiogenic program to ECs through shuttling mRNAs associated with the PI3K/AKT/eNOS signaling pathway [65]. Recently, Cantaluppi et al. suggested that EPC-MVs carrying miR-126 and miR-296 for inducing the activation of PI3K/Akt/eNOS signaling pathway in islet endothelium that may sustain revascularization and β-cell function [66].

Endothelial exosomes are produced in MVBs during endocytosis and are well-known for antigen presentation [47]. Unlike MVs, they express specific exosomal markers such as CD63, Alix, tetraspanins and tumor susceptibility gene 101 (TSG101) [67]. Endothelial exosomes can serve as biomarkers since they contain functional proteins, mRNAs and miRNAs [68]. A recent study suggests that endothelial miR-214 containing exosomes repress the expression of ataxia telangiectasia in recipient ECs, thereby prevent senescence [59]. This emerging field is still relatively young considering the vast wealth of information,
II. HYPOTHESES AND AIMS

Part 1

Hypothesis:
It is hypothesized that Ang 1-7 protects hbmECs from Ang II-induced HbmEC dysfunction and apoptosis through down-regulation of Nox2 and activation of PI3K/Akt/eNOS signaling pathway.

Specific Aims:

Aim 1: To develop optimal concentration of Ang II for inducing HbmEC apoptosis.

Aim 2: To evaluate the effects of Ang II/Ang 1-7 on HbmEC apoptosis, tube formation ability, ROS and NO production.

Aim 3: To analyze the expression of Nox2, p-Akt/Akt and p-eNOS/eNOS.
Part 2

Hypothesis:
It is hypothesized that EMVs could serve as novel therapeutic resource for protecting HbmEC from dysfunction induced by Ang II.

Specific Aims:

Aim 1: To develop optimal procedures for EMV isolation and characterization.

Aim 2: To analyze the effects of EMVs on HbmEC tube formation ability, ROS and NO production.

Aim 3: To analyze the effects of EMVs on HbmEC expression of p-Akt/Akt and p-eNOS/eNOS.
III. EXPERIMENTAL DESIGNS

Part 1

Aim 1:
HbmECs were cultured with Ang II treatment at different concentration (0, \(10^{-9}\), \(10^{-8}\), \(10^{-7}\), \(10^{-6}\) M) for 24 hours. Then, the cells were collected and labeled with Annexin V/PI for apoptosis assay using flow cytometry. Passages 4-13\(^{th}\) of HbmECs were used for the study.

Aim 2:
HbmECs were pre-treated with different concentrations of Ang 1-7 (0, \(10^{-9}\), \(10^{-8}\), \(10^{-7}\), \(10^{-6}\) M) for 1 hour and followed by Ang II (\(10^{-7}\) M) treatment for 24 hours. Then, the cells were collected and labeled with Annexin V/PI for apoptosis assay using flow cytometry.

After the Ang 1-7 effective concentration (\(10^{-7}\) M) was identified, HbmECs were divided into seven treatment groups: vehicle; Ang II (\(10^{-7}\) M); Ang 1-7 (\(10^{-7}\) M); Ang II + Ang 1-7, Ang II + losartan (\(10^{-6}\) M); Ang II + Ang 1-7 + A779 (\(10^{-7}\) M) or +LY294002 (20 µM). Cells were collected for various assays: tube formation, cell apoptosis, ROS and NO production. Passages 4-13\(^{th}\) of HbmECs were used for the study.

Aim 3:
HbmECs were treated in the same conditions as Aim 2. Then, the proteins were extracted from treated cells and used for analyzing Nox2, p-Akt/Akt and
e-NOS/eNOS expression by western blot. Passages 4-13\textsuperscript{th} of HbmECs were used for the study.

Figure 3: Experiment design for part 1. HbmECs: human brain microvascular endothelial cells; Ang II: angiotensin II; Ang 1-7: Angiotensin 1-7; FC: flow cytometry; ROS: reactive oxygen species; NO: nitric oxide; DHE: dihydroethidium; DAM-FM: 4-amino-5-methylamino- 2',7'-difluorofluorescein; WB: western blot.
**Tube formation Assay Demonstration**

1. **Add 250 μl Matrigel matrix per well of 24-well plate**
2. **Incubate plate for 1 hour at 37°C allowing Matrigel matrix to solidify**
3. **Trypsinize, harvest and count HbmECs**
4. **Add HbmECs (2 x 10⁴ cells/ well) on the top of solidified matrix solution**
5. **Incubate plate for 16 – 24 hours at 37°C**
6. **Label tubes with 8 μg/ml BD calcein AM in HBSS and incubate for 30 minutes at 37°C**
7. **Imaging**
8. **Counting Tubes**

**Note:** Tubes were quantified by counting sprouting capillary-like structures exhibiting lengths four times their width.

---

**Figure 4:** Detailed HbmEC tube formation assay demonstration.
Part 2

Aim 1:
HbmECs were staved under no-serum medium for 24 hours. HbmEC culture medium was collected and centrifuged at 1500 g for 15 min. Supernatant was collected and subjected to 100 nm filter under gravity-driven force. EMVs were retrieved from the up-membrane of that filter using sterile PBS and followed by ultracentrifuged at 100,000 g for 1 hour. EMV surface markers (Annexin V and CD144) were determined by flow cytometry. The morphology was examined by TEM. The size distribution and concentration were determined by NTA.

Aim 2:
HbmECs were divided into three treatment groups: Vehicle, Ang II (10^{-7} M), Ang II + EMVs (10 µg/ml). Cells were collected for various assays: tube formation, cell apoptosis, ROS and NO production. Passages 4-13th of HbmECs were used for the study.

Aim 3:
HbmECs (passages 4-13th) were treated in the same conditions as Aim 2. Then, the proteins were extracted from treated cells and used for analyzing p-Akt/Akt and e-NOS/eNOS expression by western blot.
Figure 5: Experiment design for part 2 (EMV isolation & characterization).

HbmECs: human brain microvascular endothelial cells; EMVs: endothelial cell microvesicles; FC: flow cytometry; TEM: transmission electron microscopy; NTA: Nanosight tracking analysis.
Figure 6: Experiment design for part 2 (EMV function assay). HbmECs: human brain microvascular endothelial cells; Ang II: angiotensin II; EMVs: endothelial cell microvesicles; FC: flow cytometry; ROS: reactive oxygen species; NO: nitric oxide; DHE: dihydroethidium; DAM-FM: 4-amino-5-methylamino-2’,7’-difluorofluorescein; WB: western blot.
IV. MATERIALS AND METHODS

HbmEC Culture

Human brain microvascular endothelial cells (HbmECs) were purchased from Cell Systems Corp. (Kirkland, WA, USA). HbmECs were cultured in CSC complete medium (Cell Systems) containing 10% serum, supplemented with 2% human recombinant growth factors (CSC CultureBoost-R, Cell Systems) and 0.2% Bac-Off\textsuperscript{R} antibiotic solution (Cell Systems) under standard cell culture conditions (37°C, 5% CO\textsubscript{2}). Cells of 4 to 13 passage were used in this study. During cell growth, medium was changed every 2 or 3 days after washed 1 time with phosphate buffer saline (PBS) and replaced with fresh CSC complete medium. Upon confluence, cells were detached using a passage reagent group (Cell Systems) following manufacturer’s protocol, resuspended in CSC complete medium and replated in tissue culture well. Precoated with attachment factor (Cell Systems) is a necessary step before seeding cells. Subsequently, cells were maintained in CSC complete medium prior to the assays.

HbmEC characterization was performed by double staining of cultured cells with 1,1’-dioctadecy1-3,3,3’,3’-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Dil-acLDL) and Lectin from Bandeiraea...
simplicifolia (BS-lectin). Briefly, cells were incubated with Dil-acLDL (10µg/ml; Biomedical Technologies, Stoughton, USA) at 37°C for 1 hour. Cells were then fixed with 0.5% paraformaldehyde (PFA) for 10 minutes, and incubated with FITC-conjugated Bs-lectin (20µg/ml; Sigma-Aldrich, St. Louis, USA) at 4°C for 4 hours. The cells were also stained with nuclear staining dye 4',6-diamidino-2-phenylindole (DAPI; 1 µg/ml; Wako Pure Chemical Industries Ltd, Osaka, Japan) for 20 min at room temperature (RT). After the staining, cells were examined with an inverted fluorescent microscope (EVOS, Washington, USA). Double-stained cells for both Bs-lectin and Dil-acLDL were considered as HbmECs.

**Isolation of EMVs**

Confluent cells were starved by subjecting to CSC serum free medium for 24 hours. After starvation, the supernatant of HbmEC culture was collected and centrifuged at 1500g for 15 minutes to remove cell and cell debris. The supernatant was then subjected to 100 nm syringe filter (CellTreat, Gaithersburg, MD, USA) under gravity-driven force. EMVs (particle size around 100 nm to 1µm) on the upper membrane of filter were retrieved by using syringe pump containing PBS and centrifuged at 100,000g for 60 minutes at 4 °C.

**Characterization of EMVs**

Isolated EMVs were characterized by flow cytometry based on the surface
markers of EMVs. CD144 was used to identify events as EMVs. For analysis of EMVs, freshly isolated MVs were resuspended in PBS and incubated for 30 min at RT in the dark with 5 μl of PE-conjugated anti-mouse CD144 (1:25, eBioscience). Isotype matched PE-labeled nonspecific antibody (Sigma-Aldrich) was served as negative control. After incubation, size calibration of EMVs was performed using 1 and 2 μm flow cytometry beads (Molecular Probes, Invitrogen, Eugene, OR). Labeled EMVs were subjected to flow cytometric analysis. The flow cytometer (BD Accuri C6, San Jose, USA) was set to acquire 100,000 events/sample. The EMVs were defined as particles with size smaller than 1 μm, and CD144+ events, in the gate of MVs. Transmission electron microscopy (TEM) was used to determine the size and morphology of EMVs. The EMVs pellets were fixed with 500 μl 2% glutaraldehyde in 1x PBS for 1 hour at 4°C and centrifuged again at 30,000 × g for 30 min. The samples were washed with 1x PBS for 3 times (1 minute each), fixed with 1% osmium tetroxide (OsO4) in 1x PBS for 2 hours at 4°C and washed another 3 times (1 minute each), then dehydrated with a graded ethanol series (50%, 75%, 95%, 100%) for 5 minutes each. Subsequently, the samples were treated with 100% propylene oxide 2 times (5 minute each), and then treated with 1:2 ratio of spurr's resin and propylene oxide, followed by a 2:1 ratio of spurr's resin and propylene oxide for 2 hours. Finally, the samples were embedded in beam capsules with spurr's resin according to the manufacture’s instruction, baked at 60°C in a hot air oven overnight. Ultrathin sections (60 nm) were prepared with MT7000 and mounted on 300-mesh copper grids, stained with 2% uranyl acetate for 5 minutes, rinsed with double
distilled water for 5 times, and allowed to dry. The specimens were then stained with lead citrate for 10 min (REYNOLDS, 1963), rinsed with double distilled water another 5 times and allowed to air dry. Finally, all specimens were examined with an EM 208 (Philips) transmission electron microscope at an accelerating voltage of 70 KV. EMVs were visible as small (100 - 1000 nm), rounded objects with clear, intact membranes.

NanoSight LM10 instrument (NanoSight, Amesbury, UK) was used to measure the size and concentration of EMVs. When samples contained higher numbers of particles (over $2 \times 10^8$/ml), they were diluted using PBS before analysis, and the relative concentration was then calculated according to the dilution factor. A video of 30 to 60s duration was taken with a frame rate of 30 frames/s, and particle movement was analyzed by NTA software (version 2.2, NanoSight). The NTA software is optimized to first identify and then track each particle on a frame-by-frame basis, and its Brownian movement is tracked and measured from frame to frame. In NTA, the paths of MVs acting as point scatters, undergoing Brownian motion. The velocity of particle movement is used to calculate particle size distribution by applying the two-dimensional Stokes–Einstein equation. Since MVs have a low refractive index, their range of sizes that can be analyzed by NTA using low refractive index, and the smallest detectable size using the NTA system is approximately 50 nm. NTA postacquisition settings were optimized and kept constant between samples, and each video was then analyzed to give the mean, mode, and median vesicle size together with an estimate of the concentration.
Incorporation of EMVs In Target Cells
To study the capacity of EMVs to incorporate into HbmECs, EMVs were labeled with PKH-26 dye (1 x 10^{-6} M, Sigma-Aldrich) for 30 min at 37 °C. After that, EMVs were washed twice with sterile PBS and centrifuged at 100,000g for 60 minutes. After that, EMVs were resuspended and washed in sterile PBS. HbmEC were incubated with PKH26-labeled EMVs for different time frames (0.5, 3, 6, 12, 24 hours). After 3 washing steps with PBS, HbmECs were fixed in 2% paraformaldehyde (PFA) for 20 minutes. Nuclei were stained with DAPI (1 μg/ml, Wako Pure Chemical Industries Ltd) for 10 minutes. Incorporation of EMVs into HbmEC was observed by fluorescence light microscopy.

Apoptosis Assay
For cell apoptosis assay, we used the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, CA, USA). Briefly, the cells after different treatments were collected and washed twice with cold PBS, and then resuspended in 100 μl 1X Annexin-binding buffer. After that, 5 μl FITC-conjugated Annexin V and 5 μl propidium iodide (PI) were added into cell suspension. The samples were then gently vortexed and incubated at RT (~25°C) for 15 min in the dark. The apoptotic cells were recognized as Annexin V+/PI- cells. The percentage of apoptotic cells was analyzed by flow cytometer (BD Accuri C6, San Jose, USA).

Tube Formation Assay
HbmEC tube formation assay was performed by using a tube formation assay
kit (BD Biosciences). Briefly, BD Matrigel matrix solution was thawed on ice overnight, and 250μl of chilled BD Matrigel matrix (10 mg/ml) was place in each well of a 24-well culture plate at 37°C for 1 hour to allow the matrix solution to solidify. HbmECs were replated (2 X 10^4 cells/well) on top of the solidified matrix solution and incubated at 37°C for 24 hours. After that, tubes were labeled by adding 300 μl/well of 8 μg/ml BD calcein AM in Hanks Balanced Salt Solution (HBSS) and incubated for 30 minutes at 37°C. After that, labeling solution was removed and plate was washed twice with HBSS. HbmEC tube formation was evaluated with an inverted fluorescence microscope (EVOS, Washington, USA). Tubes were quantified by counting sprouting capillary-like structures exhibiting lengths four times their width. Five independent fields were assessed for each well, and the average number of tubes per field was determined.

**Measurement of ROS Generation**

Intracellular ROS production in HbmECs was determined by dihydroethidium (DHE) (Invitrogen Molecular Probes, Eugene, OR, USA) staining and followed by flow cytometric analysis. DHE is a cell-permeable compound that upon entering the cells, it interacts with O2- to form oxyethidium, which in turn interacts with nucleic acids to emit a bright red color detectable qualitatively by fluorescent microscope. Briefly, HbmECs were incubated with DHE (2 μmol/L) in dark for 30 min and rinse off excess DHE with PBS twice. Red fluorescence was visualized by an fluorescence microscopy. After that, HbmECs were trypsinized and collected by centrifuge at 300g, 8 minutes, 4°C. Fluorescence
intensity was analyzed by a flow cytometer (BD Accuri C6, San Jose, USA). For each sample, 10,000 events were collected.

**Determination of Nitric Oxide Production**

The membrane-permeable indicator diaminofluorescein diacetate (DAF-FM DA) (Life technology, Grand Island, NY, USA) was used to assess NO production released by HbmECs. This FAF-FM DA is converted by intracellular esterases to form the DAF-FM, which reacts with NO to form green fluorescent product. Briefly, cells were loaded with 10 μM DAF-FM DA in CSC serum free medium (37°C for 60 minutes, in dark), washed twice with PBS and incubated with CSC serum free medium (37°C for 30min, in dark) for de-esterification of the intracellular diacetates. DAF-FM DA fluorescence was visualized by inverted fluorescence microscopy and measured using a spectrofluorometer.

**Western Blot Analysis**

The levels of Nox2, Akt/p-Akt and eNOS/p-eNOS in HbmECs were determined by western blot analysis. Cells were harvested after different treatments and resuspended in Lysis-M reagent containing complete protease inhibitor tablets (1 tablet/10 ml lysis reagent, Roche Diagnostics, Germany). Pipette thoroughly, followed by vortex-mixing, keep it on ice and sonicated for 3 times. Centrifuge the sample at 13,000g for 5 minutes at RT, transfer the supernatant (protein) to a new tube. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12% Bis-Tris Novex mini-gel, Invitrogen) and transferred onto polyvinylidene difluoride (PVDF) membrane (Invitrogen). The
PVDF membrane was blocked by incubating with 5% non-fat dry milk and Tris-buffered saline with Tween 20 (TBS-T; 1X TBS with 0.1% Tween-20) for 1 hour and then incubated with antibodies against Nox2 (1:1000; Abcam), p-Akt (1:1000; Cell Signaling Technology), p-eNOS (1:1000; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), eNOS (1:1000; Cell Signaling Technology) at 4°C overnight. β-actin (1:4000, Sigma, MO) was used to normalize protein loading. After being washed with TBS-T for 3 times (5 minutes each), membranes were incubated with horseradish peroxidase (HRP) conjugated IgG (1:40000, Jackson Lab) for 1 hour at RT. Blots were then developed with enhanced chemiluminescence developing solutions and quantified.

**Statistical Analysis**

Results are expressed as means ± SEM of at least four experiments. Differences between 2 groups were performed by the Student t-test. Multiple comparisons were analyzed by 1- or 2-way ANOVA. For all tests, a P<0.05 was considered significant. All comparisons were performed using the statistical package SPSS 16.0 for Window.
V. RESULTS

1. Characterization of HbmECs

The cultured HbmECs showed a typical, cobblestone morphology of ECs (Figure 7A). When cultured under standard conditions (37°C, 5% CO2). HbmECs were defined as cells binding with Bs-Lectin (Figure 7B) and up-taking Di-acLDL (Figure 6C).

![Figure 7. Characterization of HbmECs by Dil-acLDL and Bs-lectin double staining. A: HbmECs without staining, cells showed a typical, cobblestone morphology of ECs; B: Bs-Lectin staining (Green); C: Di-acLDL up-taking](image-url)
(Red); D: DAPI nuclear staining (Blue); E: Di-acLDL and Bs-Lectin positive cells (Yellow); scale bar: 200 µm.

2. The dose-response effect of Ang II on HbmEC apoptosis

To determine whether Ang II is involved in programmed cell death, HbmECs were treated with various concentrations (0, 10-9, 10-8, 10-7, 10-6 M) of Ang II for 24 hours. Flow cytometric assay of Annexin V/PI staining showed that Ang II dose-dependently induced apoptosis in HbmECs (Figure 8). HbmECs treatment with 10-8 M, 10-7 M and 10-6 M of Ang II increased the apoptotic rate by about 2.2-, 2.6- and 2.5-fold when compared to control (basal medium) (P<0.01; n=4). There was no significant difference between the apoptotic rate in cells cultured with 10-9 M Ang II and control, whereas the apoptotic rate is significant higher in cells treated with Ang II at the concentration of 10-7 M than 10-9 M (P<0.05, n=4). Thus, the concentration of Ang II was established by using 10-7 M for further experiments.
Figure 8. The dose-dependent effect of Ang II on HbmEC apoptosis. HbmECs were treated with Ang II at different concentration (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) for 24 hours, and then cells were collected and subjected to cell apoptosis analysis. n = 4. #P<0.05 vs vehicle; * P<0.05 vs 10^{-9} M Ang II.
3. Ang 1-7 improves the tube formation ability compromised by Ang II in HbmECs

As shown in Figure 8, the ability of HbmEC tube formation was attenuated in Ang II-treated group by about 36% when compared with control (P<0.01). Pretreatment with Ang 1-7 enhanced tube formation ability of HbmECs by 42% (P<0.05) when compared with Ang II alone. Moreover, losartan and A779 blocked Ang II-induced impairment and Ang 1-7-induced enhancement of tube formation in HbmECs, respectively (P<0.01).

A: HbmEC tube formation
Figure 9. Ang 1-7 improves the tube formation ability of HbmECs compromised by Ang II. A: Representative tube formation figures. A1: vehicle; A2: Ang II; A3: Ang 1-7; A4: Ang II + Ang 1-7; A5: Ang II + losartan; A6: Ang II + Ang 1-7 + A779; scale bar: 200 µm. B: Summarized data. Data are the mean ± SEM, n = 4. ## P<0.01 vs Control, ++P<0.01 vs Ang II, &&P<0.01 vs Ang II + Ang 1-7.

4. The dose-response effect of Ang 1-7 on cell apoptosis induced by Ang II in HbmECs

To quantify the anti-apoptotic effects of Ang 1–7 on Ang II-induced apoptosis on bmECs, flow cytometry analysis after Annexin V/PI staining was conducted (Figure 10A). The percentage of Annexin V-positive stained cells was enhanced by exposure to Ang II (10-7 M) alone compared to control cells (P<0.01). Pretreatment with Ang 1-7 markedly reduced the apoptotic rate caused by Ang II, and the degree of suppression was significantly increased at
higher concentrations (10-7 and 10-6 M) of Ang 1-7. Thus, the concentration of Ang 1-7 was established by using 10-7 M for further experiments.

We used receptor/pathway blockers to further determine the mechanism in apoptotic effect of Ang II and anti-apoptotic effect of Ang 1-7, respectively (Figure 10B). Measurement of annexin V/PI staining by flow cytometry also showed that Ang II mainly induced early apoptosis (Annexin V+/PI- events).

10-7 M Ang II significantly increased the rate of apoptosis to 23.8 ± 7% (P<0.01), 10-7 M Ang 1–7 alone had no effect on the rate of apoptosis of HbmECs compared with the control group (P>0.05). Pre-incubation with Ang 1-7 attenuated Ang II-induced apoptosis (P<0.01). Ang II-induced apoptosis was inhibited by AT1 receptor antagonist losartan (10-7 M) (P<0.01). The Mas receptor antagonist A-779 (10-6 M) almost completely abolished the protective effects of Ang 1–7 on HbmEC apoptosis (P< 0.01). The PI3K inhibitor LY294002 (20 μM) partially abolished the protective effects of Ang1–7 on HbmEC apoptosis (P<0.01).
AngII (10^{-7} M) Induced Apoptosis (%)

A

B

LY294002

Ang1-7

Losartan

A779

LY294002

AngII

Ang1-7

Losartan

A779

LY294002
Figure 10. Ang 1-7 decreases apoptosis induced by Ang II in HbmECs. A: HbmECs were pre-treated with different concentrations of Ang 1-7 (0, 10-9, 10-8, 10-7, 10-6 M) for 1 hour and followed by Ang II (10-7 M) treatment for 24 hours. B: Data summary for HbmEC apoptosis under different treatment groups. Data are the mean ± SEM, n = 4. ##P<0.01 vs Control, ++P<0.01 vs Ang II, &P<0.05, &&P<0.01 vs Ang II + Ang 1-7.

5. Ang 1-7 decreases the production of ROS induced by Ang II in HbmECs

DHE fluorescent dye and flow cytometric analysis were used to investigate the effect of Ang 1-7 on Ang II-induced ROS production. Results showed that DHE fluorescence (red) was increased by about 2.7-fold after 24 hours stimulation of Ang II (Figure 11A). Pretreatment with Ang 1-7 reduced the intensity of DHE fluorescence by about 37% when compared with Ang II, reflecting a reduction in ROS generation. Flow cytometric analysis (Figure 11B) showed that the peak of the signal moved to the right, corresponding to increased DHE fluorescence after incubation with Ang II. This increase in fluorescence intensity was markedly reduced, as evidenced by a leftward shift after treatment with Ang 1-7 in addition to Ang II, which reflected a reduction in ROS generation. The effects that induced by Ang II and Ang 1-7 were neutralized by their receptor antagonist losartan and A779, respectively. The protective effect of Ang 1-7 is partially blocked by the PI3K inhibitor (LY294002).
A: Microscope (DHE staining)

B: Flow cytometry
Figure 11. Ang 1-7 decreases Ang II-induced ROS production. A:
Representative DHE staining images observed under microscope. A1: vehicle; A2: Ang II; A3: Ang 1-7; A4: Ang II + Ang 1-7; A5: Ang II + losartan; A6: Ang II + Ang 1-7 + A779; A7: Ang II + Ang 1-7 + LY294002; scale bar: 400 µm. B:
Representative flow cytometric figures. B1: analysis control; B2: vehicle; B3: Ang II; B4: Ang 1-7; B5: Ang II + Ang 1-7; B6: Ang II + losartan; B7: Ang II + Ang 1-7 + A779; B8: Ang II + Ang 1-7 + LY294002. C: Summarized data. Data are the mean ± SEM, n = 4. ## P<0.01 vs Control, ++P<0.01 vs Ang II, &P<0.05, && P<0.01 vs Ang II + Ang 1-7.
6. Ang 1-7 increases the production of NO compromised by Ang II in HbmECs

The NO production was lower (vs control; P<0.01; Figure 12) in the Ang II-treated HbmECs. Ang 1-7 treatment increased the NO production in HbmECs by about 1.8-fold when compared to control. Pre-treatment of Ang 1-7 and losartan induced 1.72- and 1.62-fold of NO production in Ang II-treated group (P<0.01). In addition, A779 blocked those effects induced by Ang 1-7 (P<0.01), and LY294002 partially blocked the NO production induced by Ang 1-7 treatment (P<0.01).

A: Microscope (DAF-FM staining)
Figure 12. Ang 1-7 increases NO production of HbmECs compromised by Ang II. A: Representative DAM-FM staining figures. A1: vehicle; A2: Ang II; A3: Ang 1-7; A4: Ang II + Ang 1-7; A5: Ang II + losartan; A6: Ang II + Ang 1-7 + A779; A7: Ang II + Ang 1-7 + LY294002; scale bar: 200 µm. B: Summarized data. Data are the mean ± SEM, n = 4. # P<0.05, ## P<0.01 vs Control, ++P<0.01 vs Ang II, &P<0.01 vs Ang II + Ang 1-7.

7. Ang 1-7 counteracts Ang II on Nox2, p-Akt/Akt and p-eNOS/eNOS expression in HbmECs

Ang II has been shown to increase Nox2 expression (P<0.01; Figure 13B), treatment with Ang 1-7 decreased Nox2 expression when compared with Ang II treatment (P<0.01; Figure 13B). Ang 1-7 has been shown to increase eNOS activity in an EC culture model, and to also induce NO production in human studies. However, the mechanism through which Ang 1-7 improves NO production in ECs has not been well defined. We hypothesized that Ang 1-7
could activate the phosphorylation of Akt (p-Akt) and eNOS (p-eNOS), and ultimately increase NO production. Treatment with Ang II decreased the expression of p-Akt and p-eNOS (P<0.05 or 0.01; Figure 13C and D). Whereas, treatment with Ang 1-7 increased the expression of p-Akt and p-eNOS (P<0.05 or 0.01; Figure 13C and D). Co-treatment of Ang 1-7 with Ang II increased p-Akt and p-eNOS expression in HbmEC. In addition, losartan (AT1 receptor antagonist) was able to block those effects of Ang II-induced on HbmECs. A779 (Mas receptor antagonist) and LY294002 (PI3K inhibitor) were able to block those effects of Ang 1-7-induced on HbmECs. Our results also showed that addition of Ang II, Ang 1-7 or both have no changes in Akt or eNOS protein levels. The Ang II impairment of p-Akt and p-eNOS are not equivalent, with the reduction of p-Akt being greater. This suggests that another serine kinase may also be important in the phosphorylation of eNOS.

A

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<td>+</td>
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**B**

Nox2/β-actin (fold of control)

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**C**

P-Akt/Akt

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Figure 13: Ang 1-7 counteracting the effects of Ang II on Nox2, p-Akt/Akt and p-eNOS/eNOS expression in HbmECs. A: Representative western blot bands for Nox2, p-Akt/Akt and p-eNOS/eNOS. B: Summarized data for Nox2. C: Summarized data for p-Akt/Akt. D: Summarized data for p-eNOS/eNOS. Data are the mean ± SEM, n = 4. #P<0.05 vs control; +P<0.05, ++P<0.05 vs Ang II; &&P<0.01 vs Ang II + Ang 1-7.

8. EMV characterization

Flow cytometric analysis of EMVs was performed to determine the size and phenotype of EMV. Isolated EMVs were analyzed by using anti-CD144. The EMVs were defined as CD144 + (Figure 14 A3) MVs, at gate R1 with size small than 1.0 µm (Figure 14A1). Isotype antibody was used as negative control (Figure 14A2).
Transmission electron microscopy (TEM) showed that EMV had a spheroid morphology with size $\geq 100$ nm (Figure 14B), and they were heterogeneous in size. Nanoparticle tracking analysis (NTA) was used to analyze the size and concentration of EMVs. The NTA gave a vesicle size distribution from 40 to 653 nm with two peaks around 152 and 214 nm. ~ 80% of vesicles were larger than 100nm and the vesicle count was $3.54 \times 10^{10}$ per ml (Figure 14C1). The screenshot of the same vesicles analyzed on the NanoSight LM10 showing a heterogeneous size population of vesicles (Figure 14C2).

**A: EMVs examined by FC**

**B: EMVs examined by TEM**

**C: EMVs examined by NTA**

Figure 14. Characterization of EMVs. Representative flow cytometric analysis of endothelial EMVs (A). A1: the position of 1- (blue circle) and 2-µm (yellow square) calibration beads and the R1 gate for MVs. A2: PE-conjugated isotype
control. A3: PE-conjugated CD144. EMVs were defined as CD144 + events in the gate of MVs. B: Isolated EMVs were observed by TEM; scale bar: 100 nm. C: Measurement of EMV by NTA (C). C1 and C2: Size curve (white line) and screen shot of video from NanoSight LM10 showing optimal light scatter from EMVs that were retrieved from the up-membrane of 100 nm filter.

9. EMVs uptaken by target cells after co-incubation

Previous report indicated the possibility that protein and/or nucleic acids (mRNAs, miRNA) are packaged into MVs and exosomes and can be taken up by cells, acting as biomolecules. To elucidate whether EMVs can be uptaken by target cell, we incubated HbmECs with PKH26-labeled EMVs and analyzed after different time points. Immunofluorescence staining revealed that EMVs were detectable in the cytoplasm of target cells after 0.5 hour treatment, and that uptake in target cell was time dependent. To confirm that EMVs were incorporated into the cytoplasm of cells, DAPI nuclear staining was also applied. Fluorescence images showed that EMVs were internalized by HbmECs (Figure 15A).

**A: Microscope (HbmECs with PKH26-labeled EMVs)**
EMVs are incorporated by HbmECs in a time-dependent manner. Representative figures for HbmECs incubated with PKH26-labeled EMVs for 24 hours (A). A1: DAPI staining (Blue). A2: PKH26-labeled EMVs (red). A3: Merged image for DAPI/PKH26-labeled EMVs; scale bar: 200 µm. B: Summarized data. Data are the mean ± SEM, n = 3.

10. EMVs improve tube formation ability of HbmECs compromised by Ang II

The ability of HbmEC tube formation was attenuated in Ang II-treated group by about 36% when compared with control (P<0.01; Figure 16B). Pretreatment with EMVs enhanced tube formation ability of HbmECs by 43% (P<0.01; Figure 16B) when compared with treatment with Ang II alone.
Figure 16. EMVs improve tube formation ability of HbmEC compromised by Ang II. A: Representative tube formation pictures. A1: Vehicle; A2: $10^{-7}$ M Ang II; A3: $10^{-7}$ M Ang II + 10 µg/ml EMVs; scale bar: 200 µm. B: Summarized data. Data are the mean ± SEM, n = 4. **P < 0.01 vs control; +++P < 0.01 vs $10^{-7}$ M Ang II.

11. EMVs reduce ROS production of HbmECs induced by Ang II

DHE fluorescent dye and flow cytometric analysis were used to investigate the effect of Ang 1-7 on Ang II-induced ROS production. Results showed that DHE fluorescence (red) was increased by about 2.7-fold after 24 hours stimulation.
of Ang II (Figure 17). Pretreatment with Ang 1-7 reduced the intensity of DHE fluorescence by about 37% (P<0.01) when compared with Ang II, reflecting a reduction in ROS generation. The effects that induced by Ang II and Ang 1-7 were neutralized by their receptor antagonist losartan and A779, respectively. The protective effect of Ang 1-7 also was partially blocked by the PI3K inhibitor (LY294002). Flow cytometric analysis showed that the peak of the signal moved to the right, corresponding to increased DHE fluorescence, after incubation with Ang II. This increase in fluorescence intensity was markedly reduced, as evidenced by a leftward shift after treatment with Ang 1-7 in addition to Ang II, which reflected a reduction in ROS generation.

A: Microscopy (DHE staining)
Figure 17. EMVs decrease Ang II-induced ROS production. A: Representative DHE staining figures. A1: vehicle; A2: Ang II; A3: Ang II + EMVs; scale bar: 400 μm. B: Representative flow cytometric figures. B1: analysis control; B2: vehicle; B3: Ang II; B4: Ang II + EMVs. C: Summarized data. Data are the mean ± SEM, n = 4. ## P<0.01 vs Control, ++P<0.01 vs Ang II.
12. EMVs increase the production of NO compromised by Ang II in HbmECs

The NO production was lower (vs control; P<0.01; Figure 18) in the Ang II-treated HbmECs. Pre-treatment of EMVs and endothelial exosomes induced 1.9- and 1.79-fold of NO production in Ang II-treated group, respectively (P<0.01).

**A: Microscopy (DAF-FM staining)**

![Microscopy images](Figure 18)

**B**

![Bar graph](Figure 18)

Figure 18. EMVs increase NO production of HbmECs compromised by Ang II.

A: Representative DAM-FM staining figures. A1: vehicle; A2: Ang II; A3: Ang II + EMVs; scale bar: 400 µm. B: Summarized data. Data are the mean ± SEM, n = 4. ## P<0.01 vs Control, ++ P<0.01 vs Ang II.
13. EMVs up-regulate the expression of p-Akt/Akt and p-eNOS/eNOS inhibited by Ang II in HbmECs

As we found that EMV could inhibit endothelial dysfunction and apoptosis induced by Ang II, we hypothesized that EMVs might protect HbmEC dysfunction and apoptosis by activating the Akt/eNOS activity. The western blot analysis on p-Akt/Akt and p-eNOS/eNOS expression showed that Ang II-induced down-regulation of Akt and eNOS were activated after EMV treatment (Figure 19A). Treatment with Ang II decreased the expression of p-Akt and p-eNOS (P<0.05). Whereas, pre-treatment with EMVs increased the expression of p-Akt and p-eNOS expression in HbmECs, in the absence of changes in Akt and eNOS protein levels (P<0.05; Figure 19A). This data suggest that the protective effects of EMVs may be through activating Akt/eNOS signaling pathway.

A

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<tr>
<th>Treatment</th>
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Figure 19. EMVs up-regulate the expression of p-Akt/Akt and p-eNOS/eNOS inhibited by Ang II in HbmECs. A: Representative western blot bands showing p-Akt/Akt and p-eNOS/eNOS expression in HbmECs. B Summarized data for p-Akt/Akt and p-eNOS/eNOS expression. Values are the mean ± SEM, n = 4. 

#P< 0.05 vs control; ++P < 0.05 vs Ang II.
VI. DISCUSSION

The major findings of this study are as follows: 1) Ang 1-7 counteracts the deleterious effects of Ang II-induced endothelial dysfunction and apoptosis through direct inhibition of Nox2 activity and activation of the PI3K/Akt/eNOS signaling pathway. 2) EMVs can also protect endothelial cells against Ang II-induced dysfunction and apoptosis by decreasing ROS production and activating the Akt/eNOS/NO pathway.

Endothelial dysfunction is a condition in which the endothelium of blood vessels does not function normally. It is characterized by reduced vasodilation, proinflammatory state and prothrombic properties. Endothelial dysfunction is an important early event in the pathogenesis of atherosclerosis, contributing to plaque initiation and progression. The severity of endothelial dysfunction has been shown to have prognostic value for cardiovascular events. Correction of endothelial dysfunction may be associated with reduced cardiovascular risk. Mechanisms that participate in the reduced vasodilatory responses in endothelial dysfunction include reduced NO, increased oxidative stress. Apoptosis (early programmed cell death) of ECs is associated phenomenon. It has been documented that upregulation of vasoactive peptides such as Ang II induces endothelial dysfunction in vivo and in vitro. In addition, studies indicated that ACE inhibitors and AT-1 receptor blockers have protective
effects on ECs. Ang 1-7 is a vasodilator peptide counteracting many deleterious effects of Ang II induced. However, the detailed mechanism is not clear.

In this study, we demonstrated that the ability of HbmEC tube formation is impaired by Ang II. We also found that Ang II decreases the expression levels of p-Akt and p-eNOS, and NO production in HbmECs; however, increases Nox2 expression and ROS production in the HbmECs. These findings are supported by previous evidence demonstrating that the RAS plays a key role in modulating EC function through regulating NO and ROS production [16,22]. Recently, it has been suggested that Ang II inhibits Akt-induced eNOS activation and subsequent NO release in ECs [69] and that Ang II infusion decreases NO production in aorta by causing eNOS uncoupling.

One of the major and interesting findings of the present study is that pretreatment with Ang 1-7 enhances HbmEC tube formation ability decreased by Ang II. Those effects of Ang 1-7 can be partially abolished by Mas receptor antagonist (A779), suggesting that Ang 1-7 counteract the effect of Ang II via Mas receptor dependent pathway.

Previous studies have suggested that Ang1–7 could against Ang II-induced apoptosis in other types of ECs. Consistent with previous reports, we found that Ang II enhances apoptosis and that Ang 1-7 inhibits Ang II-induced apoptosis in HbmECs. Alone, Ang1–7 had no effect on HbmEC apoptosis. The AT-1 receptor antagonist losartan almost completely abolished the Ang II
induced apoptosis. The Mas receptor antagonist A-779 almost completely abolished the suppression by Ang1–7 of Ang II-induced apoptosis, suggesting that Ang1–7 specifically ameliorates Ang II-induced apoptosis. The data suggest that Ang 1–7 may be used to improve Ang II-induced apoptosis and consequently improve Ang II-associated diseases. Interestingly, preincubation of PI3K inhibitor LY294002 partially abolished the suppression by Ang 1-7 on Ang II-induced apoptosis, suggesting the PI3K activation is also involved in the antiapoptotic effects of Ang 1-7 induced. In contrast with its anti-apoptotic effects, Ang1–7 has also been reported to induce apoptosis in circulating fibrocytes and human lung cancer cells. Thus, the anti-apoptotic effect of Ang 1–7 is dependent on the cell type. We are not certain that the anti-apoptotic effects of Ang 1-7 will be applicable to all types of ECs.

Oxidative stress is one of the important factors that increase endothelial permeability. ROS play an important role in EC dysfunction and a family of NADPH oxidases (Noxs) is the major source of ROS involved in redox signaling. Previous studies have also proven that Ang II increase ROS production through activates Nox pathway in various cell types, including vascular smooth muscle, fibroblasts, ECs and cardiomyocytes. However, vascular Noxs are expressed in a cell-specific manner, with ECs expressing mainly Nox2 and Nox4; vascular smooth muscle cells, Nox1, Nox2, and Nox4; and fibroblasts, Nox2 and Nox4. Our study demonstrated that Ang II was able to induce overproduction of ROS and activation of Nox2 activity in cultured HbmECs. Whereas, preincubation of Ang 1-7 can block Ang II-induced ROS
production, through down-regulation of Nox2 activity. The effects of Ang II and Ang 1-7 induced were abolished by their selective receptor antagonist losartan and A779, respectively. Thus, Ang 1-7 attenuates Ang II-induced oxidative stress through Mas receptor.

Ang II induced overproduction of ROS seem also to be involved in the mediation of endothelial injury leading to programmed cell death or apoptosis. Recently, it has been suggested that Ang II inhibits Akt-induced eNOS activation and NO release in ECs. Of note, in addition to activation of NADPH oxidase and inhibition of Akt/eNOS signaling cascades, other cellular signaling also involved in Ang II-induced deleterious effects, such as protein kinase C activation, calcium (Ca2+) loading and phosphorylation of mitogen-activated protein kinase (MAPK). In this study, we evaluated the possible mechanisms by which Ang 1-7 may positively interfere in the RAS signaling in HbmECs. Our data demonstrates that the exposure of HbmECs to Ang 1-7 was directly able to activate the signaling pathway producing NO, a molecule that possesses some vasoprotective effects. Akt and eNOS are two crucial enzymes in this signaling pathway. Their activation is related to a serine phosphorylation, which is significantly inhibited by Ang II through the AT1R stimulation. On the contrary, Ang 1-7 counteracts the inhibitory effects of Ang II on them. The use of receptor antagonists indicates that the effects of Ang 1–7 are largely mediated by Mas receptor. Moreover, the PI3K activation is an important step for the Akt recruitment that it is necessary to activate the eNOS signaling and subsequently NO production. Our study confirms that the
PI3K/Akt axis is also crucial in the effects mediated by Ang1–7. In fact, Ang1–7 is able to promote the Akt and eNOS serine phosphorylation that is inhibited by the LY294002, a selective PI3K inhibitor. Our data suggest that the exposure of HbmECs to Ang1–7 is directly able to activate the signaling pathway producing NO, a key biological molecule involved in the preservation of endothelial function and vascular integrity. The biological relevance of this finding is supported by the demonstration that Ang1–7 is able to induce an endothelium-dependent vasodilation in mice arteries, similarly to that exerted by muscarinic receptor stimulation. This data add new information to previous reports showing the protective role of Ang 1-7 in cells. Our results highlight the importance of the Ang 1-7/Mas axis as a potential regulator of endothelial function.

The most exciting finding of this study is that we demonstrate that EMVs can be uptaken by target HbmECs and the incorporation of EMV inhibits Ang II-induced dysfunction and apoptosis in HbmECs. Activation of Akt/eNOS activity is possibly involved in EMV-mediated protection against apoptosis. We use flow cytometric analysis to assess the surface markers of EMVs, we found EMVs were carry their parent proteins and express the markers as their parent cells (CD144 for endothelial cells). We examined the size and morphology of EMV using the TEM, we found the EMVs are membrane-bound vesicles and had a spheroid morphology. The size is around 100 nm or bigger, that's consistent with other's reports. The NTA reveal that EMVs have a heterogenous size population. Since this is only one time experiment, we did
not adopt this EMV concentration for our later experiments but use the protein concentration of 10µg/ml to our further experiments. In this study, we also demonstrate that EMVs can be uptaken by target cell in a time-dependent manner and we apply for the time point 24 hours as our further experiment according to others reports.

EMVs have been shown to act as a paracrine mediator as they can merge with target cells for exerting their functions. Several studies have demonstrated that EPC-MVs can protect ECs from cell dysfunction and apoptosis by Ang II and other risk factors. But not much data is available for the role of EMVs in regulating endothelial function. In this study, we generated MVs from HbmECs which starvation and examined its effects of on HbmEC treated with Ang II. We found the tube formation ability and NO production that were compromised by Ang II were increased after EMV treatment, accompany with decreased ROS production. More interestingly, the expression of Akt and eNOS were up-regulated in HbmECs treated with EMVs when compared to Ang II treatment. This data indicates that EMVs could protect HbmEC dysfunction through activating NO pathway and decreasing ROS production.

We demonstrated that inhibition of Akt/eNOS signaling pathway was involved in endothelial dysfunction induced by Ang II. EMVs activated the Ang II-induced Akt/eNOS inhibition after Ang II treatment. These findings, in accordance with other data, MVs regulate Akt/eNOS activity and thereby mediate protective effects. Nevertheless, the detailed mechanisms of their roles need further investigation.
VII. CONCLUSION

Our data demonstrate that Ang 1-7 could protect cerebral endothelial cell dysfunction and apoptosis induced by Ang II, this functional role might rely on Nox/ROS and PI3K/Akt/eNOS pathway. Also, our data suggests that EMVs could protect Ang II-induced cerebral endothelial dysfunction, their functional role might rely on in the control of ROS production and Akt/eNOS/NO pathway in the target cells. This finding indicates that both Ang 1-7 and EMVs could be used to treat Ang II-induced endothelial dysfunction.
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