Aus dem Max-Delbrück-Centrum für Molekulare Medizin
in der Helmholtz-Gemeinschaft

DISSERTATION

Angiotensin-Converting Enzyme Inhibitor and Angiotensin II Receptor Blocker in Cerebral Arteriogenesis

Angiotensin-Konversionsenzym-Hemmer und Angiotensin-II-Rezeptorblocker in der zerebralen Arteriogenese

zur Erlangung des akademischen Grades
Medical Doctor - Doctor of Philosophy (MD/PhD)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 23. März 2024

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List of Abbreviations

AA: arachidonic acid
ACE: angiotensin-converting enzyme
ACE2: angiotensin-converting enzyme 2
ACEis: angiotensin-converting enzyme inhibitors
AGTR1: angiotensin II receptor type 1
AGTR2: angiotensin II receptor type 2
AKt: protein kinase B
Ang I: angiotensin I
Ang II: angiotensin II
Ang 1-7: angiotensin 1-7
ARBs: angiotensin II receptor blockers
BA: basilar artery
BDKRB: bradykinin receptor
BDKRB1: bradykinin receptor 1
BDKRB2: bradykinin receptor 2
BK: bradykinin
Ca: calcium
CAM: calmodulin
CBF: cerebral blood flow
CCA: common carotid artery
CeVD: cerebrovascular disease
CVD: cardiovascular disease
CVRC: cerebrovascular reserve capacity
DABK: desArg^{9}-bradykinin
DAG: diacylglycerol
DAKD: desArg^{10}-kallidin
ECs: endothelial cells
eNOS: endothelial nitric oxide synthase
EPCs: endothelial progenitor cells
ERK: extracellular signal-regulated kinase
FMD: flow-mediated vasodilatation
G_{α}: G-protein alpha subunit
$G_{\beta\gamma}$: G-protein beta-gamma complex
HMWK: high-molecular-weight kininogen
ICA: internal carotid artery
IMT: intima-media-thickness
iNOS: inducible nitric oxide synthase
IP3: inositol trisphosphate
KD: kallidin
KKS: kallikrein-kinin system
LMWK: low-molecular-weight kininogen
MAS1: MAS1 proto-oncogene
MCA: middle cerebral artery
MEK: mitogen-activated protein kinase
MHEC5-T: murine heart endothelial cells
MKP-1: mitogen-activated protein kinase phosphatase 1
NO_x: nitrite/nitrate
PBMCs: peripheral blood mononuclear cells
P: phosphorus
PGs: prostaglandins
PCA: posterior cerebral artery
PI3K: phosphoinositide 3-kinases
PKC: protein kinase C
PLA2: phospholipase A2
PLC-β: phospholipase C beta
PP2A: protein phosphatase 2A
RAS: renin-angiotensin system
SHP-1: phosphatase-1
VA: vertebral artery
3-VO: 3-vessel occlusion
$Ø_{PCA}$: diameter of posterior cerebral artery

Abstract

Aim: This project aims 1) to investigate the role of angiotensin-converting enzyme inhibitors (ACEis) and angiotensin II receptor blockers (ARBs) on cerebral arteriogenesis *in vivo*. 2) To investigate the pro-arteriogenic effects of ACEis and ARBs by examining a particular role of the bradykinin receptor (BDKRB) signaling *in vitro.* 3) To investigate the roles of ACEis and ARBs on nitric oxide-dependent endothelial function and leukocytic BDKRB mRNA expression in patients with cardiovascular disease (CVD).

Methods: 1) A rat model of cerebral arteriogenesis (3-VO) was established. The diameter of the posterior cerebral artery ($Ø_{PCA}$) was assessed by using an angioarchitecture technique, and cerebrovascular reserve capacity (CVRC) was measured by using the laser Doppler flowmetry. 2) Murine heart endothelial cells (MHEC5-T) were treated with an ACEi or ARB alone, or in combination with a BDKRB antagonist. Cell proliferation and migration were analyzed. 3) 177 patients with CVD were divided into three groups (ACEi group, ARB group, non-ACEi/ARB group) according to their medication histories. Plasma nitrite/nitrate (NO_x) was measured by using the Griess reagent method, flow-mediated vasodilatation (FMD) was assessed by using a data analysis algorithm, carotid intima-media-thickness (IMT) was measured by using the B-mode ultrasound, and BDKRB mRNA expression levels in peripheral blood mononuclear cells (PBMCs) were analyzed by using the quantitative polymerase chain reaction.

Results: 1) ACEis significantly increased $Ø_{PCA}$ and improved CVRC after 3-VO. 2) ACEis significantly promoted MHEC5-T cell proliferation, which was abolished by antagonists of BDKRBs. 3) Plasma NO_x was significantly higher in the ACEi group than in the ARB group and non-ACEi/ARB group. mRNA expression level of BDKRB1 was significantly higher, but mRNA expression level of BDKRB2 was significantly lower in the ACEi group compared to the non-ACEi/ARB group.

Conclusion: 1) ACEis stimulate cerebral arteriogenesis *in vivo*. 2) ACEis exert pro-arteriogenic effects via the BDKRB signaling pathway *in vitro*. 3). ACEis increase plasma NO_x production and regulate BDKRB mRNA expression in PBMCs from patients with CVD.

Zusammenfassung

Zielsetzung: Das Ziel dieses Projekt ist: 1) die Rolle von Angiotensin-Konversionsenzym-Hemmer (ACEi) und Angiotensin-II-Rezeptorblocker (ARB) auf die zerebrale Arteriogenese *in vivo* zu untersuchen. 2) Die Untersuchung einer pro-arteriogenen Wirkung von ACEi und ARB *in vitro*, mit funktioneller Analyse der Rolle der Bradykinin-Rezeptor (BDKRB)-Signalwirkung. 3) Die Untersuchung der Wirkung einer ACEi- und ARB-Gabe auf die Stickoxid-abhängige Endothelfunktion und die leukozytische BDKRB-mRNA-Expression von Patienten mit Herz-Kreislauf-Erkrankungen (HKE).

Methoden: 1) Ein Rattenmodell der zerebralen Arteriogenese (3-VO) wurde etabliert. Der Durchmesser der Arteria cerebri posterior ($Ø_{PCA}$) wurde unter Verwendung einer Angioarchitekturtechnik bestimmt, und die zerebrovaskuläre Reservekapazität (CVRC) wurde unter Verwendung einer Laser-Doppler-Durchflussmetrie gemessen. 2) Murine Herz-Endothelzellen (MHEC5-T) wurden jeweils mit einem ACEi oder ARB allein oder in Kombination mit einem BDKRB-Antagonisten behandelt. Anschließend wurden Zellproliferation und Migration analysiert. 3) 177 Patienten mit HKE wurden entsprechend ihrer Medikamentenanamnese in drei Gruppen (ACEi-Gruppe, ARB-Gruppe, Nicht-ACEi/ARB-Gruppe) aufgeteilt. Die Plasma Nitrit/Nitrat (NO_x) Konzentration wurde mit der Griess-Reagenz-Methode gemessen und die Flußvermittelte Vasodilatation (FMD) mit einem Datenanalysealgorithmus bewertet. Die Karotis-Intima-Media-Dicke (IMT) wurde unter Verwendung eines B-Mode-Ultraschalls gemessen und die BDKRB-mRNA-Expressionsniveaus in peripheren mononukleären Blutzellen (PBMCs) unter Verwendung der quantitativen Polymerase-Kettenreaktion analysiert.

Ergebnisse: 1) ACEi erhöhte signifikant $Ø_{PCA}$ und CVRC nach 3-VO. 2) ACEi förderte signifikant die MHEC5-T-Zellproliferation, welche durch die Gabe mit einem BDKRB-Antagonisten inhibiert werden konnte. 3) Die Plasma-NO_x Konzentration war in der ACEi-Gruppe signifikant höher als in der ARB-Gruppe und der Nicht-ACEi/ARB-Gruppe. Die mRNA-Expression von BDKRB1 war in der ACEi-Gruppe signifikant höher, die mRNA-Expression von BDKRB2 jedoch signifikant niedriger als in der Nicht-ACEi/ARB-Gruppe.

Schlussfolgerung: 1) ACEi stimuliert die zerebrale Arteriogenese *in vivo*. 2) ACEi übt *in vitro* eine pro-arteriogene Wirkungen über den BDKRB-Signalweg aus. 3) ACEi erhöht die Plasma-NO_x Konzentration und moduliert BDKRB-Expression in PBMCs bei Patienten mit HKE.

1 Introduction

1.1 Angiogenesis and arteriogenesis

Angiogenesis and arteriogenesis are two mechanisms of vascular growth [1]. Angiogenesis is defined as the formation of further capillaries sprouting from pre-existing vessels. It has been well-accepted that hypoxia is the trigger of angiogenesis. In more detail, hypoxia-inducible factor, epidermal growth factor, and platelet-derived growth factor, coordinate with vascular endothelial growth factor to regulate angiogenesis in multiple steps [2] (Figure 1).

By contrast, arteriogenesis is defined as the remodeling of preexisting arterioles into functional conduit arteries upon stenosis or occlusion of a major artery [1]. An arterial stenosis or occlusion leads to a steep blood pressure gradient in a major artery, and an increased blood flow in the corresponding collateral arteries concurrently. Here, the frictional force of blood fluid shear stress has been regarded as the trigger of arteriogenesis [1]. It has been demonstrated that altered shear stress can be detected by mechanosensors, such as integrins and caveolae, on the abluminal side of endothelial cells (ECs) [3]. EC hypertrophy is one of the signs of the initial phase of arteriogenesis, in which activation of volume-regulated endothelial chloride channels plays a key role [4]. Simultaneously, a number of cell adhesion protein genes and shear-stress sensitive genes are modulated in response to the altered fluid shear stress [5]. Subsequently, monocytes are attracted and migrate into the perivascular space and differentiate into macrophages, which release numerous growth factors and cytokines, and significantly promote vascular cell proliferation via paracrine signaling [6].

In summary, it signifies that cerebral angiogenesis and cerebral arteriogenesis are two rescue mechanisms of vascular growth after ischemic stroke. However, it is pointed out that a large artery cannot be replaced by an emerging capillary immediately, because the volume of blood flowing through capillaries is negligible compared to a sudden decrease of cerebral blood flow after ischemic stroke. In contrast, collateral arteries (arterioles) can be dynamically recruited in a short period, because they are pre-existing rather than newly formed. In other words, cerebral collateral circulation is recruited immediately in the case of cerebral artery occlusion, thereby maintaining blood perfusion and protecting against further ischemic damage. Therefore, cerebral arteriogenesis can be regarded as the primary compensatory mechanism after ischemic stroke.

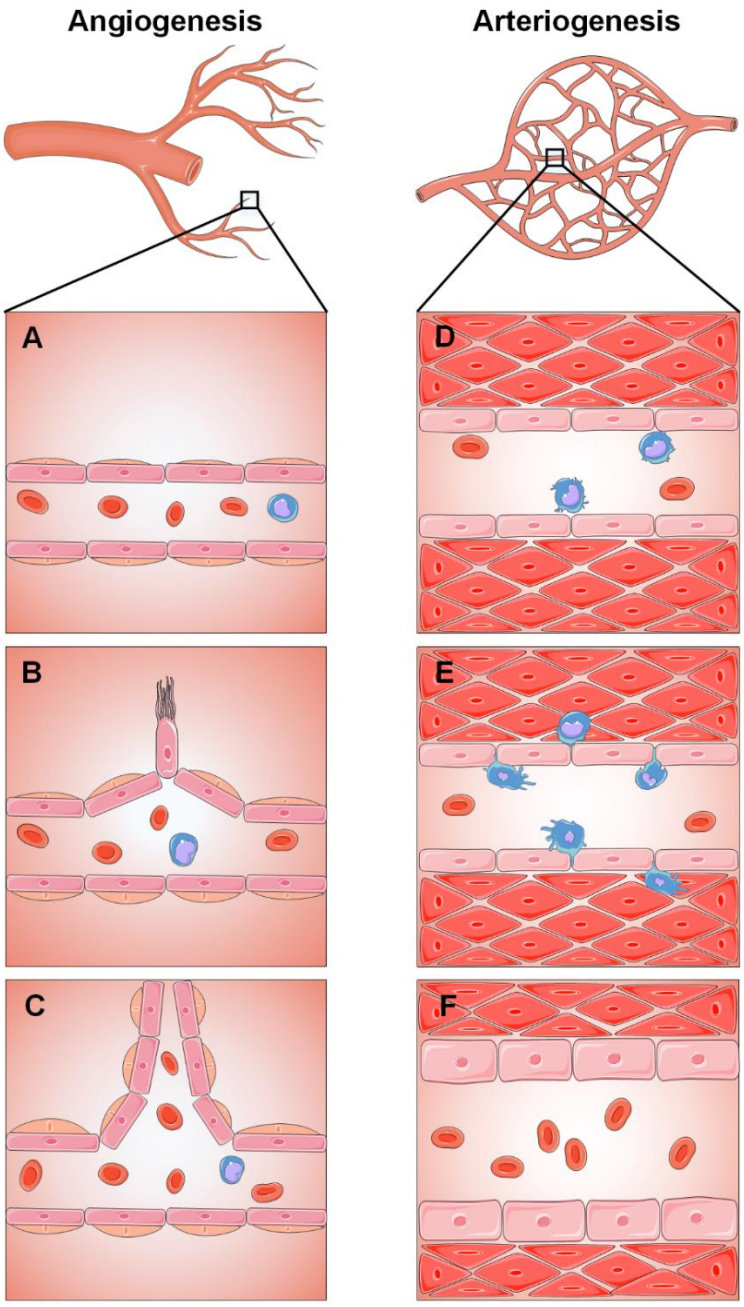

Figure 1. Mechanisms of angiogenesis and arteriogenesis.

Figure (A-C): Mechanisms of angiogenesis. **Figure A:** Degradation of basement membrane and extracellular matrix [7]. **Figure B:** ECs migration and tube formation. ECs proliferate and migrate towards the higher gradient concentration of vascular endothelial growth factor, forming a tube-like structure [8]. **Figure C:** Maturation of nascent blood vessels.

Figure (D-F): Mechanisms of arteriogenesis. **Figure D:** Activation of ECs. **Figure E:** Migration and differentiation of monocytes. **Figure F:** Maturation of the collateral arteries.

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1.2 Kallikrein-kinin system and renin-angiotensin system

The kallikrein-kinin system (KKS) is an endogenous metabolic cascade regulating blood pressure, vascular permeability, and cellular morphogenesis. Bradykinin (BK) and kallidin (KD) are cleaved from the precursor molecules of high-molecular-weight kininogen (HMWK) and low-molecular-weight kininogen (LMWK) by plasma kallikrein and tissue kallikrein, respectively. BK and KD are the ligands of bradykinin receptor 2 (BDKRB2). After the removal of C-terminal arginine from BK and KD, desArg^{9}-bradykinin (DABK) and desArg^{10}-kallidin (DAKD) are formed. DABK and DAKD are the ligands of bradykinin receptor 1 (BDKRB1). In general, BDKRB2 is ubiquitous and constitutively expressed in various cell types, whereas BDKRB1 is expressed at very low levels under physiological conditions [9] (Figure 2).

Beyond that, KKS plays an important role in vascular growth [10]. Here, we first reported that kininogen is a molecular marker for early-phase cerebral arteriogenesis [11]. We later demonstrated that cerebral arteriogenesis is modulated by the BDKRB signaling pathway [12]. In particular, the expression of BDKRB1 on circulating immune cells seems to be a pivotal determinant in leucocyte transmigration and cytokine production, which are critical for the paracrine signaling mechanism during arteriogenesis [6].

In addition, the renin-angiotensin system (RAS) is an enzymatic cascade regulating blood pressure, fluid balance, and vascular resistance. Plasma renin converts angiotensinogen to angiotensin I (Ang I), which is subsequently converted by angiotensin-converting enzyme (ACE) to angiotensin II (Ang II). Both Ang I and Ang II can be metabolized by angiotensin-converting enzyme 2 (ACE2) to angiotensin 1-7 (Ang 1-7). Ang II is the ligand of angiotensin II receptor type 1 (AGTR1) and angiotensin II receptor type 2 (AGTR2), and Ang 1-7 is the ligand of MAS1 proto-oncogene (MAS1). AGTR1 action leads to vasoconstriction, while both AGTR2 and MAS1 actions lead to vasodilation [13] (Figure 2).

Intriguingly, RAS is also playing a critical role in vascular growth beyond its hypertensive effect. It has been shown that Ang II restores blood flow by promoting collateral development [14]. In addition, *in vivo* studies demonstrated that mesenteric arterial collaterals formation was facilitated by the AGTR2-mediated immune cell activation [15], whereas suppressed by the AGTR1-induced excess superoxide production [16]. Yet, the role of RAS in arteriogenesis is controversially discussed.

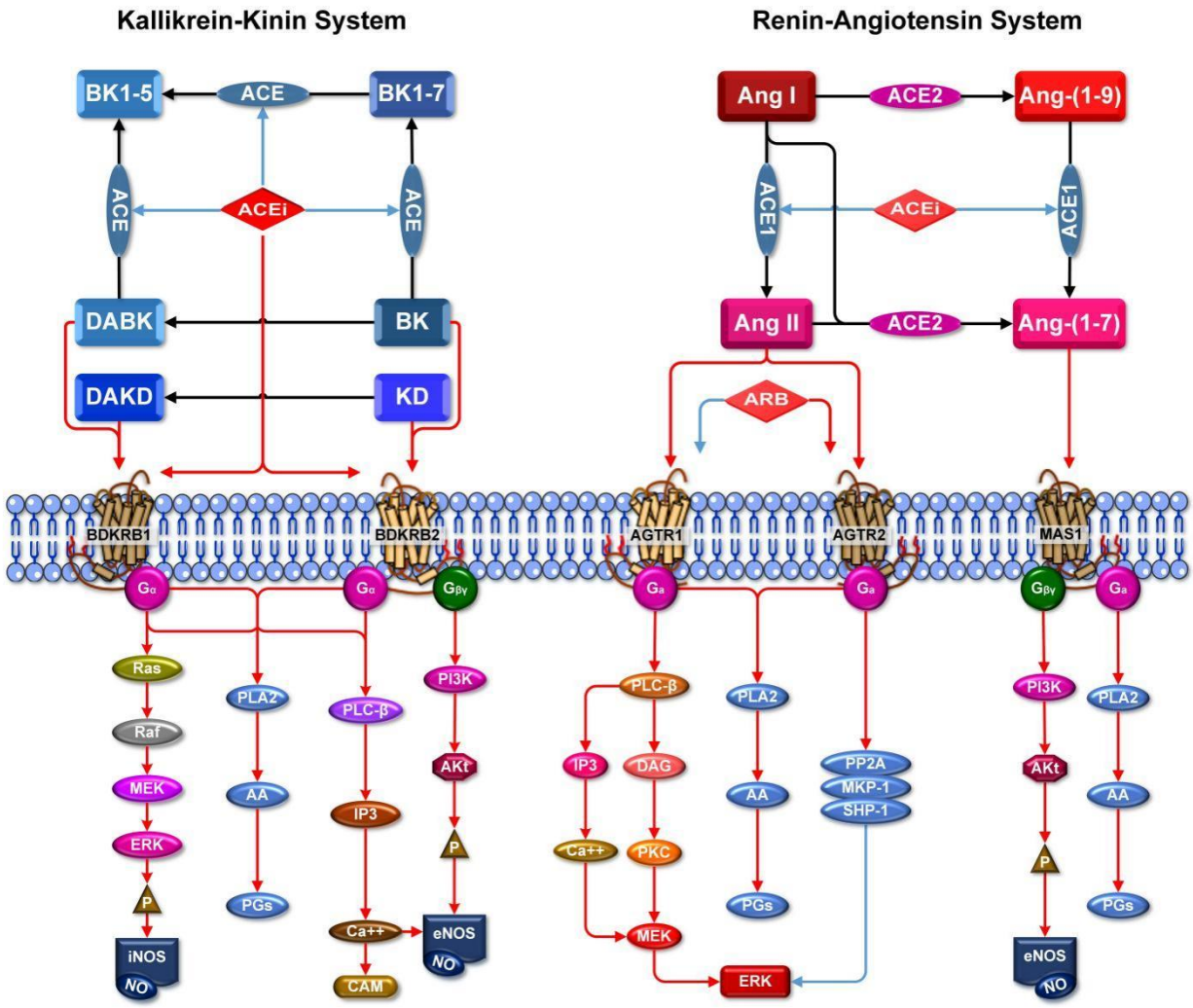

Figure 2. Signal transductions in KKS and RAS.

DABK and DAKD are the ligands of BDKRB1, while BK and KD are the ligands of BDKRB2. Because ACE degrades kinins in multiple steps, inhibition of ACE increases the concentration level of kinins. In addition, ACEis stimulate BDKRB1 at a Zn-binding sequence of the second extracellular loop, and resensitize BDKRB2 by altering the conformation of ACE domains of ACE-BDKRB2 receptor heterodimer. BDKRB1 signal transduction cascades activate iNOS through the Ras-Raf-MEK-ERK pathway, while BDKRB2 signal transduction cascades activate eNOS through the PI3K-Akt and the PLC-β-IP3-Ca^{2+} signaling pathways [17].

Ang I is converted by ACE to Ang II, and both Ang I and Ang II are converted by ACE2 to Ang 1-7. Ang II is the ligand of AGTRs. ARBs selectively block AGTR1, thereby shifting Ang II binds to AGTR2. AGTR1 signal transduction cascades mediate vasoconstriction through the PLC-β-IP3-Ca^{2+}/DAG-PKC signaling pathway [18], while AGTR2 signal transduction cascades mediate vasodilation by activating several phosphatases (e.g., PP2A, MKP-1, SHP-1) [19].

Blue lines indicate inhibitory or blocking effects; red lines indicate activating or stimulating effects. Figure 2 was originally made by the author Kangbo Li.

1.3 Angiotensin-converting enzyme inhibitor and angiotensin II receptor blocker

ACE is a membrane-bound peptidyldipeptide hydrolase with two catalytic domains [20]. Physiologically, ACE not only catalyzes the conversion of Ang I into Ang II, but also degrades BK in multiple steps, therefore, ACE represents a central bridge between KKS and RAS [21]. Inhibition of ACE accumulates the vasodilator BK, but suppresses the vasoconstrictor Ang II, which are the cardiovascular protective mechanisms of ACE inhibitors (ACEis). More interestingly, recent research has shown that ACEis can be regarded as allosteric enhancers of BDKRBs [22]. On the one hand, as direct agonists, ACEis bind to BDKRB1 at a Zn-binding sequence of the second extracellular loop; on the other hand, as allosteric enhancers, ACEis alter the conformation of ACE domains of ACE-BDKRB2 receptor heterodimer of the cell membrane, thereby resensitizing BDKRB2 [22]. Therefore, we hypothesized that ACEis could therapeutically stimulate arteriogenesis by regulating the BDKRB signaling pathway.

In addition to ACEis, angiotensin II receptor blockers (ARBs) are commonly used as cornerstones in cardiovascular disease (CVD) management. ARBs selectively block AGTR1, while enhance the cardiovascular protective effects mediated by AGTR2 [23]. More surprisingly, it was shown that BDKRB2 can associate with either AGTR1 or AGTR2 to be a heterodimer [24, 25]. In this regard, it has been reported that the AGTR1-BDKRB2 receptor heterodimer contributed to Ang II hyperresponsiveness in spontaneously hypertensive rats [26], whereas the AGTR2-BDKRB2 receptor heterodimer contributed to nitric oxide (NO) production in rat pheochromocytoma cells [25]. Here, because both AGTR2 signaling and BDKRB2 signaling promote collateral development, and heterodimers normally enhance peptide ligand binding and receptor activation, we hypothesized that ARBs might also stimulate arteriogenesis via the AGTR2 signaling or (and) BDKRB2 signaling.

In order to verify our hypotheses that both ACEis and ARBs might stimulate arteriogenesis, the following three studies were conducted in the current project. (1) *In vivo* study, we investigated the roles of ACEis and ARBs in a rat model of cerebral arteriogenesis. (2) *In vitro* study, we investigated the pro-arteriogenic effects of ACEis and ARBs on murine ECs and analyzed whether the putative pro-arteriogenic effects were mediated by a BDKRB signaling pathway. (3) Observational study, we investigated the roles of ACEis and ARBs on plasma NO production, flow-mediated vasodilatation, carotid intima-media thickness, and leukocytic BDKRBs expression from patients with CVD. Comprehensive analyses were conducted to uncover the roles of ACEis and ARBs in arteriogenesis.

2 Methods

2.1 *In vivo* study

2.1.1 Procedure of 3-VO surgery

3-vessel occlusion (3-VO) surgical procedure was carried out on male Sprague-Dawley rats (300-350 g) as previously described [6]. 3-VO was named because three vessels supplying blood to the rat brain were ligated to induce cerebral arteriogenesis. In brief, the bilateral vertebral arteries (VAs) were sealed by electrocoagulation technology through the paravertebral access. Subsequently, the unilateral common carotid artery (CCA) was ligated through the ventral midline incision (Figure 3).

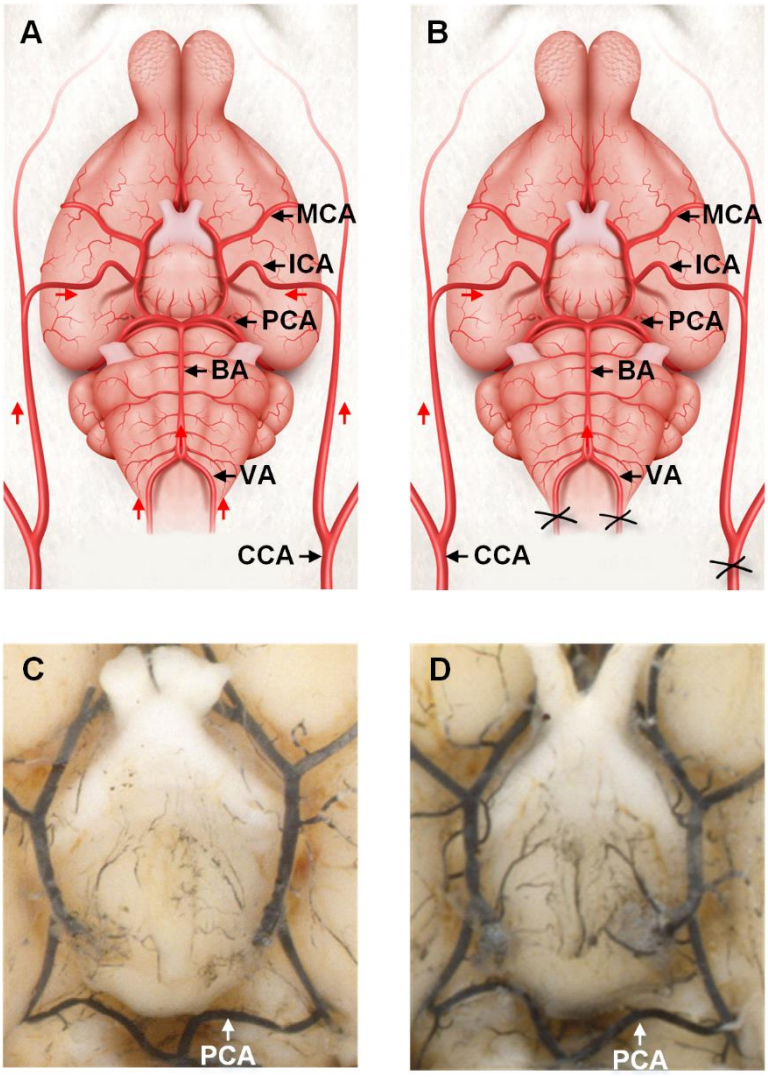

Figure 3. Schematic representation of the 3-VO model.

Figure (A, C): The brain receives blood from two pairs of large blood vessels, i.e., the ICAs and the VAs, under physiological conditions. **Figure (B, D):** In the case of 3-VO, blood in the brain is only supplied by the unligated ICA, circulation direction of arterial blood is changed towards the

pressure gradients, flowing through the paired PCAs, and then into the ligated ipsilateral hypoperfused cerebral hemisphere. Therefore, PCAs are recruited as collateral pathways and regarded as the target vessels to analyze the active vascular remodeling.

Figure 3A and Figure 3B were modified based on Figure 3 of the manuscript published by Crumrine et al. [27], licensed under the Creative Commons Attribution 4.0 International License. Figure 3C and Figure 3D were offered by Prof. Ivo Buschmann, copyright all rights reserved.

2.1.2 Pharmacological interventions and grouping

All experimental animals were randomly assigned to the following groups (n=10-30/group): (1) Non-surgical (NS) group: without 3-VO procedure and any treatment. (2) 3-VO (7D) group: 3-VO followed by a 7-day administration of distilled water (0.5 ml/day, i.g.). (3) 3-VO (21D) group: 3-VO followed by a 21-day administration of 0.5% Methyl cellulose solution (Charité - Universitätsmedizin Berlin) (0.5 ml/day, i.g.). (4). 3-VO+ACEi (7D) group: 3-VO followed by a 7-day administration of Ramipril (CT Arzneimittel GmbH) (0.2 mg/kg/day, dissolved in 0.5 ml distilled water, i.g.). (5). 3-VO+ARB (21D) group: 3-VO followed by a 21-day administration of Candesartancilexetil (AstraZeneca GmbH) (2 mg/kg/day, dissolved in 0.5 ml 0.5% Methyl Cellulose Solution, i.g.).

2.1.3 Visualization of cerebral angioarchitecture

Cerebral angioarchitecture was visualized by using a post-mortem latex perfusion method [6]. In brief, a lethal dose of papaverine hydrochloride (50 mg/kg, i.a.) (Linden Arzneimittel-Vertrieb-GmbH) was injected into the CCA to induce maximal vasodilation, followed by a pressure-controlled perfusion with the Indian ink (Établissements LALO) colorized latex milk (Spartan Products, Inc.) (6 mL/kg, i.a.). External diameters of the posterior cerebral artery (PCAs) ($Ø_{PCA}$) in both the ligated ipsilateral (IPS) and the ligated contralateral (CON) sides were measured by using a stereo-zoom microscope (Leica Microsystems GmbH), and presented as $Ø_{PCA(IPS)}$ and $Ø_{PCA(CON)}$, respectively.

2.1.4 Measurement of cerebrovascular reserve capacity

Cerebrovascular reserve capacity (CVRC) is a hemodynamic parameter that indicates the ability of vessels to regulate cerebral blood flow (CBF) and cerebral blood volume. It has been demonstrated that the magnitude and response time of CVRC correlate variably to stroke risk and symptomatology [28]. In brief, a transcranial laser probe was placed directly on the skull surface covering the frontoparietal cortex. Blood was drawn from one of the femoral arteries for blood gas analysis. As an exogenous vasodilator agent - acetazolamide (30 mg/kg, i.v.) (Goldshield Pharmaceuticals Ltd) was applied to induce

changes in CBF. CBF was measured by the PeriFlux System 5000 Laser Doppler Perfusion Monitor (Perimed AB). Relative CBF was calculated by normalizing against the baseline value, and expressed as a percentage, CVRC was expressed as the percent deviation of relative CBF from 100%.

2.1.5 Statistical analysis

Data were given as mean ± standard deviation (SD). $Ø_{PCA}$ and CVRC between two groups were analyzed by unpaired *t*-test. $Ø_{PCA}$ and CVRC between three groups were analyzed by one-way analysis of variance (ANOVA). Relative CBF in different groups over different measurement time points were analyzed by two-way repeated measures ANOVA. A *P*-value less than 0.05 was considered statistically significant.

2.2 *In vitro* study

2.2.1 Cell culture

Murine heart endothelial cells (MHEC5-T) (Leibniz Institute DSMZ GmbH) were grown in RPMI 1640 medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH) at 37°C in a humid atmosphere with 5% CO_2.

2.2.2 Cell proliferation assay

Around 4000 cells per well were seeded into a 96-well plate, followed by an 8-hour incubation of experimental drugs. Then, cell proliferation was assessed by using the WST-1 Assay Kit (Abcam) according to the stand manufacturer's instructions. Absorbance was measured at 450 nm by using a multimode microplate reader (Tecan Group AG).

Here, two sub-experiments were conducted. First, MHEC5-T were treated with different concentrations (0.01 μM, 0.1 μM, 1 μM, 10 μM, and 20 μM) of the following experimental drugs: Ramipril (ACEi) (Sigma-Aldrich Chemie GmbH), Candesartancilexetil (ARB) (Sigma-Aldrich Chemie GmbH), R715 [antagonist of BDKRB1 (BDKRB1i)] (Tocris Bioscience) and HOE 140 [antagonist of BDKRB2 (BDKRB2i)] (Enzo Life Sciences GmbH). RPMI 1640 medium was used as a negative control.

Second, MHEC5-T were treated with a fixed concentration (0.01 μM) of the following experimental drugs: Ramipril (ACEi), Ramipril (ACEi) + R715 (BDKRB1i), Ramipril (ACEi) + HOE 140 (BDKRB2i), Ramipril (ACEi) + R715 (BDKRB1i) + HOE 140 (BDKRB2i); Candesartancilexetil (ARB), Candesartancilexetil (ARB) + R715 (BDKRB1i), Candesartancilexetil (ARB) + HOE 140 (BDKRB2i), Candesartancilexetil (ARB) + R715 (BDKRB1i) + HOE 140 (BDKRB2i); and control (RPMI 1640 medium).

2.2.3 Cell transwell migration assay

Cell migration was assessed by using the 6.5 mm Transwell® with 8.0 μm Pore Polyester Membrane Insert (Corning Incorporated) according to the stand manufacturer's instructions. In brief, MHEC5-T were diluted (1×10^5 cells/mL) by using RPMI 1640 medium alone, or combined with the experimental drugs (as per the second sub-experiment of 2.2.2.). 100 μL medium containing MHEC5-T was added into the upper compartment, while 600 μL Endothelial Growth Medium (Angio-Proteomie) was added into the lower compartment. Thereafter, MHEC5-T were allowed to migrate from the upper compartment into the lower compartment through the pores of the membrane. The total number of migrated MHEC5-T in the lower compartment was counted after 48-hour incubation.

2.2.4 Statistical analysis

Data were given as mean ± SD. The optical density (O.D.) value of cell proliferation and the total number of migrated cells between groups were analyzed by using one-way ANOVA. A *P*-value less than 0.05 was considered statistically significant.

2.3 Observational study

2.3.1 Study population and grouping

The WalkByLab registry study aims at screening for early detection of CVD in the non-metropolitan region of Brandenburg state in Germany. 177 patients with CVD were randomly selected from the study database, and subsequently divided into three groups (ACEi group, ARB group, or non-ACEi/ARB group) according to their medication histories.

2.3.2 Extraction of plasma and peripheral blood mononuclear cells

Venous blood was collected by the routine venipuncture procedure, then diluted with an equal volume of phosphate buffered saline (Thermo Fisher Scientific), and layered on Ficoll-Paque density gradient media (GE Healthcare) (ratio: 4:3), followed by 25 min centrifugation (400 × g, no brake). The upper layer of plasma and the middle layer of peripheral blood mononuclear cells (PBMCs) were collected for further plasma nitrite/nitrate (NO_x) determination and RNA isolation, respectively.

2.3.3 Determination of plasma NO_x

Nitrite and nitrate are the final metabolites of NO in a physiological system, therefore, plasma NO_x concentration was measured to assess the NO production. First, plasma samples were ultrafiltered by using the Amicon Ultra-0.5 Centrifugal Filter Unit (Merck

Millipore) to remove hemoglobin. Then, plasma NO_x was measured using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical) based on the Griess method [29], absorbance was measured at 548 nm using a multimode microplate reader (Tecan Group AG).

2.3.4 Assessment of flow-mediated vasodilatation

Flow-mediated vasodilatation (FMD) is defined as the dilation or widening of an artery in response to reactive hyperemia. It has been demonstrated that the magnitude of FMD correlates to the production of NO released by ECs [30]. The classical gold standard to assess brachial FMD is the ultrasound-based diagnostic imaging technique, while a novel AngioDefender™ device (Everist Health) was used in our study, the equivalence of the FMD values determined by the above two methods has been verified [31].

2.3.5 Assessment of carotid intima-media thickness

Intima-media thickness (IMT) is defined as the thickness of the innermost two layers (tunica intima and tunica) of an arterial wall, which can be visualized as a double-line pattern in a longitudinal view [32]. In the current study, carotid IMT was measured by using a high-resolution B-mode ultrasound (Koninklijke Philips N.V.) with electronic calipers to measure on a longitudinal scan of the bilateral CCAs, at a point of 10 mm proximal to the beginning of the dilation of each carotid artery bulb.

2.3.6 Analysis of BDKRBs mRNA expression

Total RNA was extracted from PBMCs using the Trizol reagent (Thermo Fisher Scientific). 1 μg total RNA was used to yield the first strand cDNA by using the QuantiTect Reverse Transcription Kit (QIAGEN). mRNA expression levels of BDKRBs were analyzed by using the LightCycler® 96 Real-Time PCR System (Roche). The specific primers used were as follows: BDKRB1 (Forward): ATTCTCCCACCTCAGCCTCT and (Reverse) CTCTGGTTGGAGGATTGGAG; BDKRB2 (Forward): CTTCATGGCCTACAGCAACA and (Reverse) GCACACTCCCTGGTACACCT; RPLPO (Forward): ACGGGTACAAACGAGTCCTG, (Reverse) AGCCACAAAGGCAGATGGAT. Experiments were run in duplicate.

2.3.7 Statistical analysis

mRNA expression levels were given as mean ± standard error of the mean (SEM), and other data were expressed as mean ± SD. Normally distributed data were analyzed by one-way ANOVA, and abnormally distributed data were analyzed by Kruskal-Wallis test. Correlation between variables was analyzed by using Spearman correlation coefficient. A *P*-value less than 0.05 was considered statistically significant.

3 Results

3.1 *In vivo* study

3.1.1 $Ø_{PCA}$

$Ø_{PCA}$ in the 3-VO (7D) group or 3-VO (21D) group was significantly larger than in the NS group, $Ø_{PCA(IPS)}$: [3-VO (7D) vs. NS: $P \leq 0.001$)], [3-VO (21D) vs. NS: $P \leq 0.0001$)]; $Ø_{PCA(CON)}$: [3-VO (7D) vs. NS: $P \leq 0.001$)], [3-VO (21D) vs. NS: $P \leq 0.0001$)]. In addition, $Ø_{PCA}$ in the 3-VO (21D) group was significantly larger than in 3-VO (7D) group, $Ø_{PCA(IPS)}$: [3-VO (21D) vs. 3-VO (7D): $P \leq 0.0001$)], $Ø_{PCA(CON)}$: [3-VO (21D) vs. 3-VO (7D): $P \leq 0.05$] (Figure 4A). Beyond that, $Ø_{PCA(IPS)}$ in the 3-VO+ACEi (7D) group was significantly larger than in the 3-VO (7D) group ($P \leq 0.001$) (Figure 4B). $Ø_{PCA}$ was unchanged between the 3-VO (21D) and 3-VO+ARB (21D) groups (Figure 4C) [33].

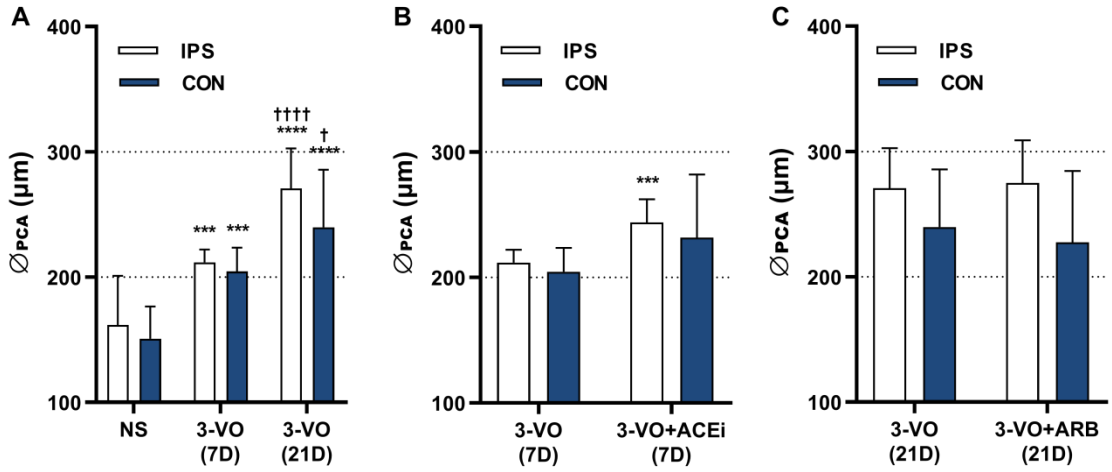

Figure 4. $Ø_{PCA}$.

Figure A: *** $P \leq 0.001$, **** $P \leq 0.0001$, compared to the NS group; † $P \leq 0.05$, †††† $P \leq 0.0001$, compared to the 3-VO (7D) group. Figure B: *** $P \leq 0.001$, compared to the 3-VO (7D) group. Figures were made based on the raw data from the manuscript published by Hillmeister and Nagorka et al. [33].

3.1.2 CBF and CVRC

When comparing the NS, 3-VO (7D), and 3-VO (21D) groups, results indicated that either the grouping factor ($P \leq 0.05$) or the measurement time point factor ($P \leq 0.0001$) had a significant effect on relative CBF (Figure 5A). In addition, CVRC in the NS group was significantly higher than in the 3-VO (7D) group ($P \leq 0.05$), whereas it remained unchanged between the NS and 3-VO (21D) groups (Figure 5D).

When comparing the 3-VO (7D) and 3-VO+ACEi (7D) groups, results showed that again either the grouping factor ($P \leq 0.05$) or the measurement time point factor ($P \leq 0.001$) had a significant effect on relative CBF (Figure 5B). In addition, CVRC in the 3-VO+ACEi (7D) group was significantly higher than in the 3-VO(7D) group ($P \leq 0.05$) (Figure 5E).

When comparing the 3-VO (21D) and 3-VO+ARB (21D) groups, results demonstrated that only the measurement time point factor had a significant effect on relative CBF ($P \leq 0.0001$) (Figure 5C). In addition, CVRC was unchanged between groups (Figure 5F) [33].

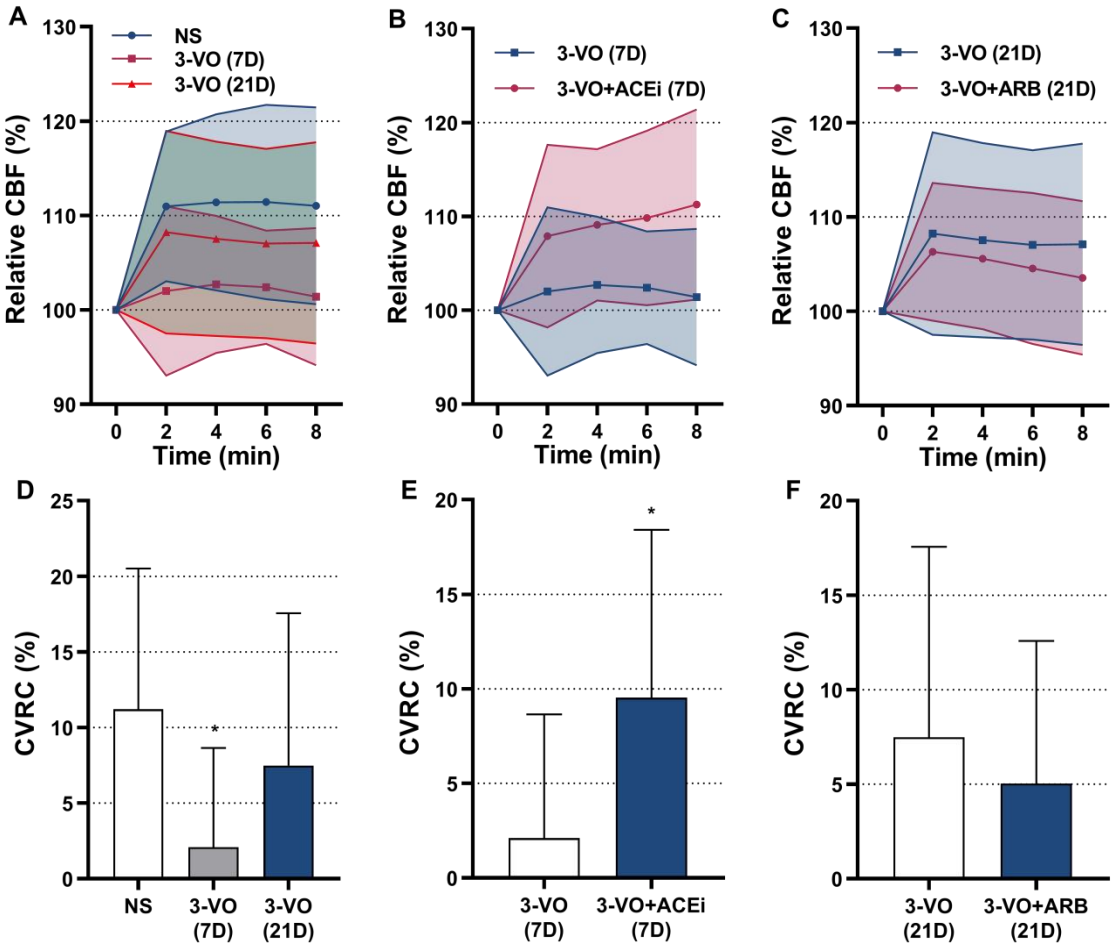

Figure 5. Relative CBF and CVRC.

Figure (A-C): Shaded areas indicated error bands. Figure D: $^{*}P \leq 0.05$, compared to the NS group. Figure E: $^{*}P \leq 0.05$, compared to the 3-VO (7D) group. Figures were made based on the raw data of the manuscript published by Hillmeister and Nagorka et al. [33].

3.2 *In vitro* study

3.2.1 MHEC5-T proliferation

0.01 µM ACEi significantly promoted MHEC5-T proliferation compared to control ($P \leq 0.05$) (Figure 6A). Whereas 1 µM, 10 µM, and 20 µM ARB significantly inhibited MHEC5-

T proliferation compared to control ($P \leq 0.001$, $P \leq 0.001$, $P \leq 0.01$, respectively) (Figure 6B). In addition, 10 μM and 20 μM BDKRB1i inhibited MHEC5-T proliferation, however without significant differences (Figure 6C). 0.1 μM, 1 μM, 10 μM, and 20 μM BDKRB2i significantly inhibited MHEC5-T proliferation in a concentration-dependent manner ($P \leq 0.0001$, $P \leq 0.001$, $P \leq 0.01$, $P \leq 0.0001$, respectively) (Figure 6D).

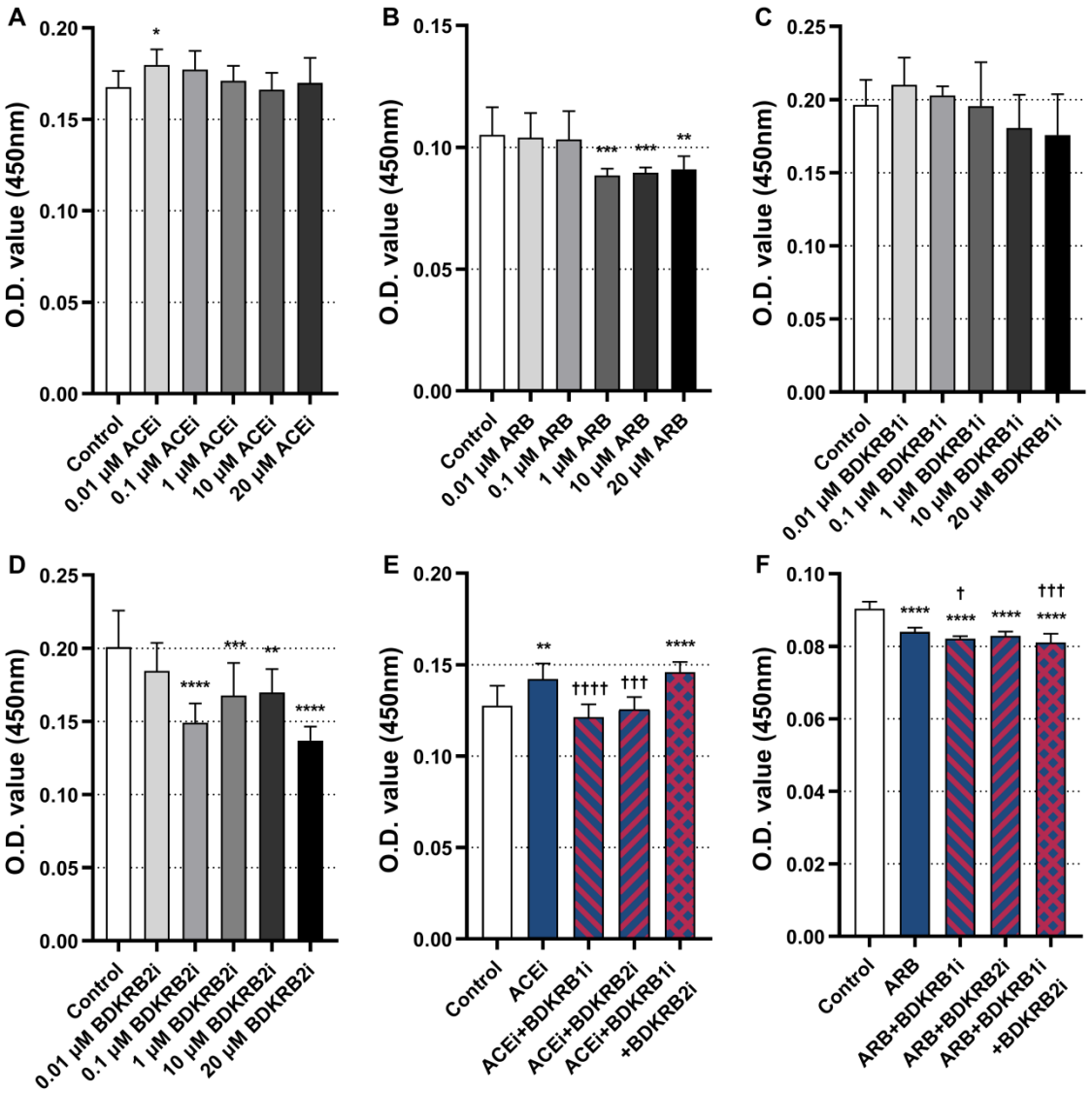

Figure 6. MHEC5-T proliferation.

Figure (A-D), $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$, compared to control. Figure (E), $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$, compared to control, $^{\dagger\dagger\dagger}P \leq 0.001$, $^{\dagger\dagger\dagger\dagger}P \leq 0.0001$, compared to ACEi. Figure (F), $^{****}P \leq 0.0001$, compared to control, $^{\dagger}P \leq 0.05$, $^{\dagger\dagger\dagger}P \leq 0.001$, compared to ARB.

Figure 6A and Figure 6E were adapted based on Figure 6A and Figure 6E from the manuscript published by Li et al. [34]. Figure 6B, C, D, F were originally made by the author Kangbo Li.

0.01 μM ACEi significantly promoted MHEC5-T proliferation compared to control ($P \leq 0.01$), 0.01 μM ACEi combined with 0.01 μM BDKRB1i or BDKRB2i significantly inhibited MHEC5-T proliferation compared to 0.01 μM ACEi alone ($P \leq 0.0001$, $P \leq 0.001$, respectively). However, 0.01 μM ACEi combined with 0.01 μM BDKRB1i and BDKRB2i significantly promoted MHEC5-T proliferation compared to control ($P = 0.0001$) (Figure 6E).

Furthermore, 0.01 μM ARB alone or combined with 0.01 μM BDKRB1i or (and) BDKRB2i significantly inhibited MHEC5-T proliferation compared to control ($P \leq 0.0001$). 0.01 μM ARB combined with 0.01 μM BDKRB1i, or combined with 0.01 μM BDKRB1i and BDKRB2i significantly inhibited MHEC5-T proliferation compared to 0.01 μM ARB alone ($P \leq 0.05$, $P \leq 0.001$, respectively) (Figure 6F).

3.2.2 MHEC5-T migration

0.01 μM ACEi showed no significant effects on MHEC5-T migration when compared to control. 0.01 μM ACEi combined with 0.01 μM BDKRB1i or (and) BDKRB2i inhibited MHEC5-T migration compared to control or 0.01 μM ACEi alone, however, no significant difference was detected (Figure 7A).

In contrast, 0.01 μM ARB, 0.01 μM ARB combined with 0.01 μM BDKRB1i, and 0.01 μM ARB combined with 0.01 μM BDKRB1i and BDKRB2i, significantly inhibited MHEC5-T migration compared to control ($P \leq 0.05$, $P \leq 0.05$, $P \leq 0.05$, respectively). In addition, 0.01 μM ARB combined with 0.01 μM BDKRB1i slightly inhibited MHEC5-T migration compared to 0.01 μM ARB alone, but there was no statistical significance (Figure 7B).

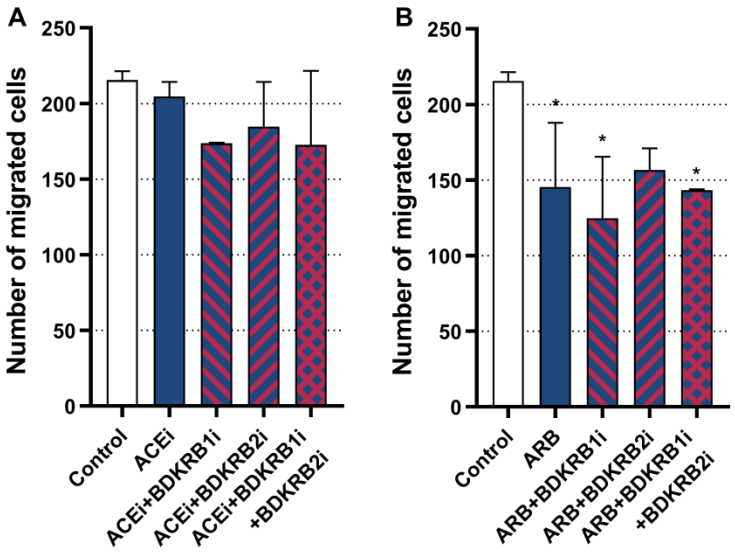

Figure 7. MHEC5-T migration.

* $P \leq 0.05$, compared to control. Figure 7 was originally made by the author Kangbo Li.

3.3 Observational study

3.3.1 Plasma NO_x, FMD, and carotid IMT

Plasma NO_x concentration was significantly higher in the ACEi group than in the non-ACEi/ARB group ($P \leq 0.05$) and ARB group ($P \leq 0.01$). In addition, plasma NO_x concentration was slightly higher in the ARB group than in the non-ACEi/ARB group, but no significant difference was detected (Figure 8A). Besides, FMD in the ACEi group was higher than in the ARB group and non-ACEi/ARB group, but there were no significant differences [35] (Figure 8B). Carotid IMT was unchanged between groups (Figure 8C).

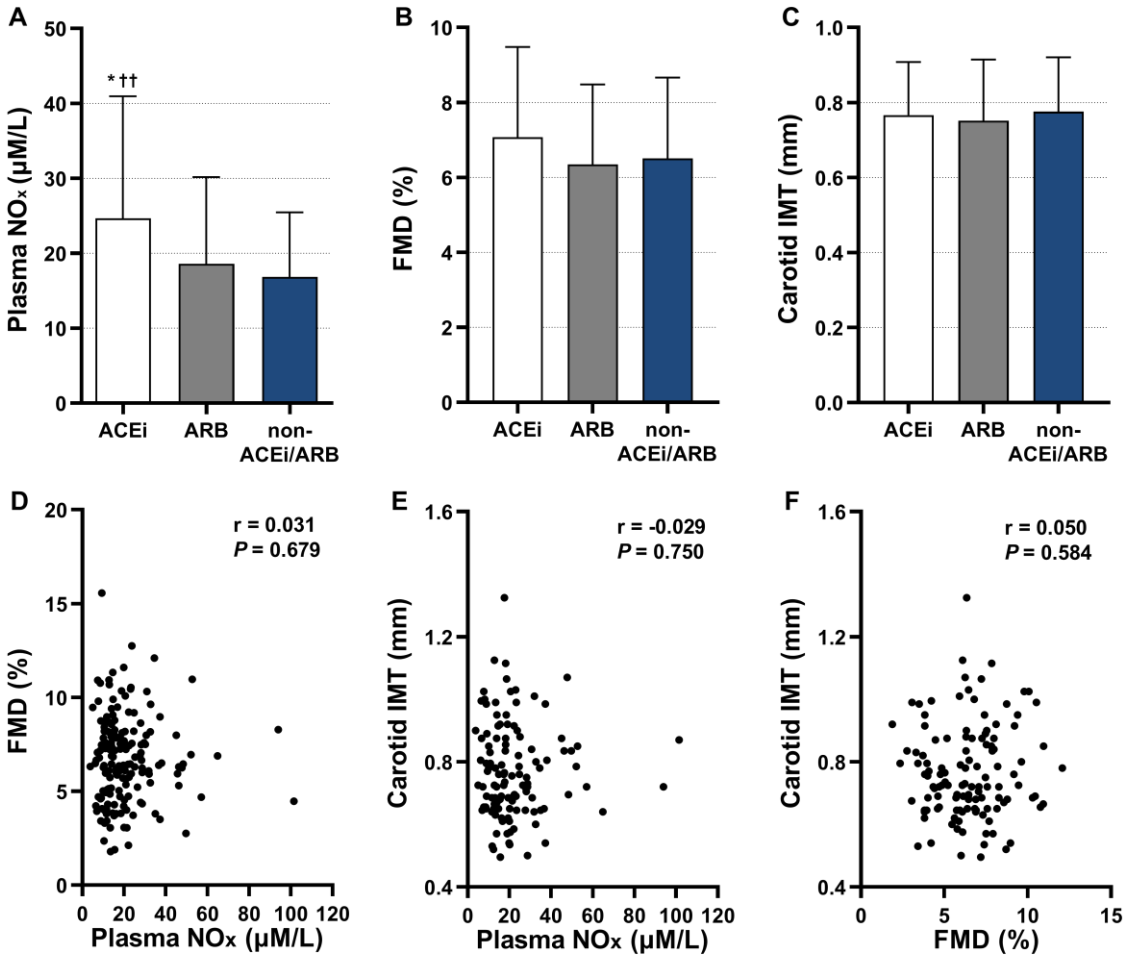

Figure 8. Plasma NO_x, FMD, and carotid IMT.

Figure 8A: Plasma NO_x in three groups. **Figure 8B:** FMD in three groups. **Figure 8C:** carotid IMT in three groups. $^{*}P \leq 0.05$, compared to the non-ACEi/ARB group, $^{††}P \leq 0.01$, compared to the ARB group. **Figure 8D:** Correlation between plasma NO_x and FMD. **Figure 8E:** Correlation between plasma NO_x and carotid IMT. **Figure 8F:** Correlation between FMD and carotid IMT.

Figure 8A and Figure 8B were adapted based on Figure 1A and Figure 2A respectively from the manuscript published by Li et al. [35]. Figure 8(C-F) were originally made by the author Kangbo Li.

Correlation analyses showed that plasma NO_x concentration positively correlated to FMD value (r = 0.031) (Figure 8D), but negatively correlated to carotid IMT (r = −0.029) (Figure 8E). In addition, FMD value positively correlated to carotid IMT (r = 0.050) (Figure 8F). However, these results were statistically non-significant.

3.3.2 mRNA expression of BDKRBs

mRNA expression levels of BDKRB1 in the ACEi group or ARB group were significantly higher than in the non-ACEi/ARB group ($P \leq 0.0001$, $P \leq 0.001$, respectively). BDKRB1 mRNA expression levels were slightly higher in the ACEi group than in the ARB group, but without reaching a significant difference (Figure 9A). mRNA expression levels of BDKRB2 in either the ACEi group or ARB group were significantly lower than in the non-ACEi/ARB group ($P \leq 0.05$, $P \leq 0.05$, respectively).

BDKRB2 mRNA expression levels were slightly lower in the ACEi group than in the ARB group, but without reaching a significant difference [35] (Figure 9B). Consequently, the relative mRNA expression ratios of BDKRB1/BDKRB2 in either the ACEi group or ARB group were significantly higher than in the non-ACE/ARB group ($P \leq 0.0001$) (Figure 9C).

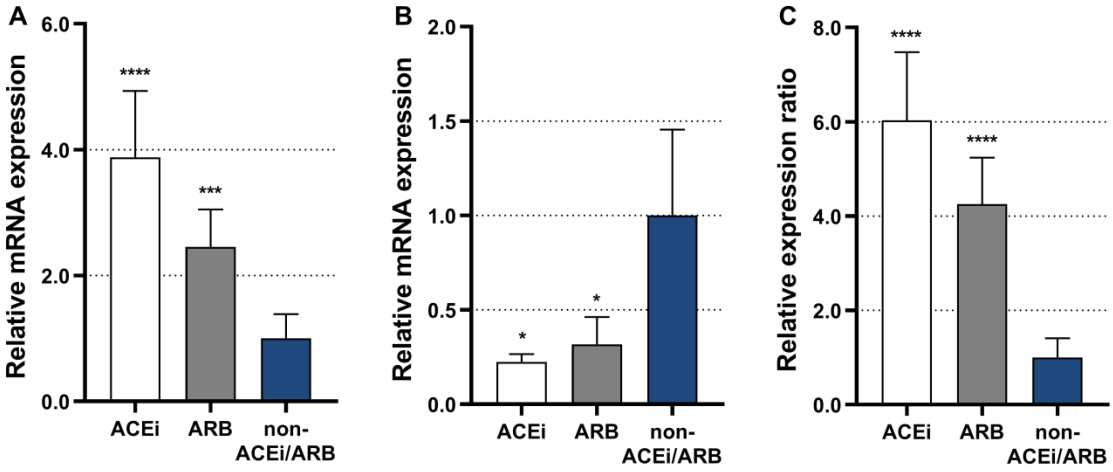

Figure 9. Relative mRNA expression levels of BDKRBs.

Figure 9A: Relative mRNA expression levels of BDKRB1**. Figure 9B:** Relative mRNA expression levels of BDKRB2. **Figure 9C:** Relative mRNA expression ratios of BDKRB1/BDKRB2. $^{*}P \leq 0.05$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$, compared to the non-ACEi/ARB group.

Figure 9A, B were adapted based on Figure 4A and Figure 4B respectively from the manuscript published by Li et al. [35]. Figure 9C was originally made by the author Kangbo Li.

4 Discussion

4.1 3-VO induces cerebral arteriogenesis

Firstly, in this project, we validated again that 3-VO is an ideal approach to stimulate cerebral arteriogenesis. Our results showed that both PCA enlargement and CVRC restoration were time-dependent in the 3-VO groups [33], which indicated that structural [36] and functional [37] adaptations of cerebral collateral circulation were effectively stimulated experimentally.

In particular, it is well known that the cerebral collateral circulation includes extracranial sources and intracranial routes, the latter again consist of the primary collateral pathways (i.e., circle of Willis) and the secondary collateral pathways (i.e., the ophthalmic artery and leptomeningeal vessels) [38]. Dynamic recruitments of anastomotic junctions occurred in the primary collateral pathways (circle of Willis) as a result of 3-VO. In this situation, CBF is redistributed from the unligated ICA, through the paired PCAs, and finally into the ligated ipsilateral hypoperfused cerebral hemisphere. Obviously, the paired PCAs were recruited as collateral pathways. Therefore, arterial remodeling (lumen enlargement) was very remarkable in the PCAs, which were regarded as the target vessels to investigate the structural adaptations of cerebral arteriogenesis.

On the other hand, CVRC is defined as the ability of cerebral vessels to regulate CBF and cerebral blood volume depending on the vasoactive stimuli [28]. It has been demonstrated that insufficient cerebral collateralization may lead to a significant reduction of CBF, therefore, the magnitude and response time of CVRC indicate the efficiency of cerebral collateralization to overcome ischemia [39]. In the case of 3-VO, CVRC was measured on the frontoparietal region, which receives the blood supply from the branches of the ICA (i.e., the anterior cerebral arteries and middle cerebral arteries), whereas it indirectly represents the efficiency of the primary collateral pathways (circle of Willis).

4.2 ACEis increase Ø$_{PCA}$ and improve CVRC after 3-VO

In the first part of this project, we investigated the role of ACEis and ARBs on cerebral arteriogenesis *in vivo*. To the best of our knowledge, it was the first work to demonstrate that cerebral arteriogenesis can be therapeutically stimulated by the administration of ACEis. Here, our results showed that ACEis, rather than ARBs, significantly increased Ø$_{PCA}$ and improved CVRC [33]. Consistent with our finding, regarding the structural adaptation of target vessels, it has been reported that ACEis, rather than ARBs, significantly

promoted luminal expansion of mesenteric collaterals in spontaneously hypertensive rats [16]. In addition, clinical research also indicated that ACEis treatment, rather than ARBs treatment, positively correlated to coronary collateral grades [40]. Secondly, regarding the functional adaptation of CVRC, studies have shown that long-term ACEi treatment improved cerebellar and basal ganglia CVRC in patients with stroke [41]. Furthermore, it has been reported that ARB treatment improved the lower limit of cerebral autoregulation *in vivo* [42]. Yet, an increased CVRC induced by ARBs was not observed in our current study. Indeed, our recent *in vivo* study also demonstrated that ARBs had no effect on collateral perfusion in a rat model of coronary arteriogenesis [34].

4.3 ACEis promote MHEC5-T proliferation

In the second part of this project, we investigated the roles of ACEis and ARBs on EC proliferation. Here, the MHEC5-T was used because it is the one of most commonly used cell lines in vascular research [43]. Our results showed that 0.01 μM ACEi rather than ARB significantly promoted MHEC5-T proliferation. Consistent with our finding, it has been reported that ACEis increased the number of endothelial progenitor cells (EPCs) in both peripheral blood and bone marrow during cardiac pressure overload [44], and promoted ECs survival by upregulating the gene expression of eNOS and fibroblast growth factor-2 [45]. Similarly, it has been reported that ARBs also promoted the proliferation of EPCs [46], and exerted a proangiogenic effect in ECs [47], which were not observed in our current study. Paradoxically, many studies have shown that both ACEis and ARBs also exert antiangiogenic effects on ECs in various disease models [48, 49]. Therefore, it is plausible that the effects of ACEis and ARBs differ, depending on the disease model and the cell type used in different experimental conditions.

4.4 ACEis modulate pro-arteriogenic effects via the BDKRB signaling pathway

In addition, we also investigated whether the ACEi-modulated pro-arteriogenic effects via the BDKRB signaling pathway. Here, our results showed that ACEi-induced MHEC5-T proliferation was abolished by either BDKRB1i or BDKRB2i. This indicated that ACEis modulated pro-arteriogenic effects via the BDKRB signaling pathway. Indeed, it is known that ACEis increase the concentration of the ligands of BDKRBs by preventing their degradation in multiple steps. Furthermore, ACEis directly active BDKRB1 on the second extracellular loop without an intermediate peptide ligand, while indirectly resensitize BDKRB2 by altering the conformation of ACE domains of the ACE-BDKRB2 receptor heterodimer [22].

Indeed, both BDKRB1 and BDKRB2 were implicated in cell proliferation during vascular growth, however, BDKRB2 owns more credits than BDKRB1. In this regard, Yau et al. demonstrated that BK stimulated DNA synthesis in smooth muscle cells from porcine or human coronary arteries, which can be potentiated or abolished by an agonist or antagonist of BDKRB2, respectively [50]. Yang et al. demonstrated that BK-induced bovine corneal EC proliferation was attenuated by an antagonist of BDKRB2 [51]. In addition, Nurmi et al. showed that BDKRB2 played a critical role in cardiac EC proliferation [52]. Regarding BDKRB1, Morbidelli et al. reported that only BDKRB1 was responsible for the BK-induced mitogenic activity of ECs from postcapillary veins [53]. Moreover, Hu et al. observed that BK enhanced interleukin-1-induced angiogenesis *in vivo*, which was abolished by an antagonist of BDKRB1 [54].

Additionally, an unexpected result was that the combination of ACEi with BDKRB1i and BDKRB2i significantly increased MHEC5-T proliferation, which was against our expectations. However, this controversial result was consistent with the results from our former *in vivo* and *in vitro* studies [12, 33]. The possible mechanism for this paradoxical phenomenon is still unclear, but it warrants further research, as a stimulatory effect after inhibition of both BDKRBs was confirmed again in this work.

On the other hand, given that BDKRB2 heterodimerizes with either AGTR1 [24] or AGTR2 [25], we speculated that combination of ARB and BDKRB1i would block AGTR1 and inhibit BDKRB1, and thereby potentiate the biological function of the AGTR2-BDKRB2 receptor heterodimer [25]. In this context, it has been reported that AGTR2 action inhibited EC proliferation [55], whereas BDKRB2 action promoted EC proliferation [56]. Yet, only inhibition of MHEC5-T proliferation was observed when combining ARB with BDKRB1i in our current study.

4.5 ACEis enhance plasma NO_x concentration

Atherosclerosis is a widespread, chronic progressive disease that is not limited to a single arterial territory [57]. Therefore, coexistence of CVD and cerebrovascular disease (CeVD) is very common [58]. In addition, CVD also includes CeVD in broad terms [59]. Therefore, patients with different types of CVD were randomly selected in the third part of this project.

First, the roles of ACEis and ARBs on plasma NO_x concentration, FMD value, and carotid IMT in patients with CVD were analyzed. NO is not only a potent vasodilator, but also a pro-arteriogenic factor [60]. It has been shown that the endogenous endothelium-derived

NO contributed to EC proliferation [61] and migration [62]. Here, our results indicated ACEis were superior to ARBs in increasing plasma NO_x concentration. Consistent with our finding, an *in vivo* study showed that ACEis induced a greater increase in plasma NO concentration than ARBs [63]. In addition, it has been reported that ACE knockout mice were resistant to develop hypertension induced by a NO synthesis (NOS) inhibitor [64]. Besides, administration of ACEis decreased an NOS inhibitor serum level in patients with hypertension [65]. Therefore, ACE plays a critical role in NO metabolism.

Beyond that, our results showed that FMD in the ACEi group was higher than that in the ARB group and non-ACEi/ARB group, but without a significant difference. Indeed, previous research has shown that both ACEis and ARBs prevented endothelial dysfunction, whereas the protective effect of ACEis was more profound in the healthy volunteers [66]. In addition, a positive correlation between reduction of pulse pressure and improvement of endothelial function was only shown in the ACEi treatment group rather than the ARB treatment group [67]. However, those were not confirmed in our current study.

NO is synthesized by NOS, which is a family of enzymes consisting of eNOS, iNOS, and neuronal NOS [68]. Previous research suggested that both ACEis and ARBs were involved in NOS stimulation [69, 70]. Here, given ACEis can be regarded as allosteric enhancers of BDKRBs [22], it can be assumed that ACEis could modulate BDKRB2, and lead to Ca^{2+}-calmodulin-dependent activation of eNOS through the PLC-β-IP3-Ca^{2+} signaling pathway, and phosphorylation of eNOS at Ser^{1177} through the BDKRB2-PI3K-Akt signaling pathway. Secondly, ACEis could also modulate BDKRB1, and lead to phosphorylation of iNOS at Ser^{745} through the Ras-Raf-MEK-ERK pathway [17] (Figure 2). On the other hand, ARBs also have effects on NO production. ARBs selectively block AGTR1 and shift the Ang II actions to the AGTR2, which induces the phosphorylation of eNOS at Ser^{633} and Ser^{1177} through the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) signaling pathway [71].

Regarding carotid IMT, previous research has demonstrated that carotid IMT can be regarded as a predictor for ischemic stroke [72]. Beyond that, it has been reported that the RAS inhibition therapy reduced IMT by enhancing NO production [73]. Yet, the superiority of ACEis over ARBs in reducing carotid IMT was not determined in our current study.

In addition, correlation analyses showed that plasma NO_x concentration positively correlated to FMD value, but inversely correlated to carotid IMT. Although significance was not

reached, these results are consistent with other studies [74, 75]. Regarding the correlation analysis between FMD and IMT, no correlation was confirmed in our current study. Still, studies investigating their relationship were heterogeneous in this context. Some studies demonstrated that FMD and IMT were independent parameters [76, 77], but other studies showed significant correlations in particular patients [78, 79].

4.6 ACEis modulate BDKRBs mRNA expression in PBMCs

Additionally, we also investigated the roles of ACEis and ARBs on mRNA expression of BDKRBs in PBMCs from patients with CVD. Our results also showed that ACEis upregulated BDKRB1 mRNA expression but downregulated BDKRB2 mRNA expression, resulting in increased BDKRB1/BDKRB2 gene expression ratios. In summary, here, we demonstrated for the first time that the administration of ACEis indeed modulated the expression of BDKRBs of circulating PBMCs systemically.

ACEis not only increase the concentration of BDKRB1 ligands by preventing their degradation in multiple steps, but also directly bind to BDKRB1 on the second extracellular loop, which together result in a superimposed effect. Yet, accumulating evidence suggested that BDKRB1 is amplified and upregulated in PBMCs in pathological processes, and in turn, BDKRB1 accelerates inflammatory processes [80].

Upregulation or imbalance of BDKRB1/BDKRB2 ratios has been considered a hallmark of cardiac decompensation or inflammatory response [80]. However, BDKRB1 also causes many cardiovascular protective functions and maintains cardiovascular homeostasis [81]. It is pointed out that inflammatory activation is a double-edged sword, and arteriogenesis is modulated by immune activation and inflammatory activation [34]. In this regard, our previous studies showed that cerebral arteriogenesis was therapeutically stimulated or inhibited by an agonist or antagonist of BDKRB1. In addition, a stronger reduction in peripheral collateral blood flow was shown in BDKRB1 knockout ($BDKRB1^{-/-}$) mice than BDKRB2 knockout ($BDKRB2^{-/-}$) mice. Beyond that, bone marrow grafting from wild type to $BDKRB1^{-/-}$ mice showed a stronger recovery of blood flow after femoral arterial occlusion surgery [12]. Here, BDKRB1 signaling may interact with the paracrine signaling between immune cells and vascular cells, thereby facilitating arteriogenesis [6].

Further studies are needed to reveal the underlying molecular mechanisms, and shed light on the future therapeutic perspective of BDKRBs targeted therapy in ischemic vascular disease.

5 Conclusion

This project demonstrates that 1) ACEis stimulate cerebral arteriogenesis *in vivo*. 2) ACEis modulate pro-arteriogenic effects through the BDKRB signaling pathway *in vitro*. 3) ACEis increase plasma NO_x production and regulate BDKRBs mRNA expression level in PBMCs from the patients with CVD.

It is the first time to investigate the role of ACEis in cerebral arteriogenesis *in vivo* and *in vitro*. The results of this project extend our knowledge of the relationship between KKS and RAS in cerebrovascular growth, and provide original evidence for the pleiotropic pro-arteriogenic effects of ACEis in ischemic vascular disease.

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Statutory Declaration

“I, Kangbo Li, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic ‘Angiotensin-Converting Enzyme Inhibitor and Angiotensin II Receptor Blocker in Cerebral Arteriogenesis (German translation: Angiotensin-Konversionsenzym-Hemmer und Angiotensin-II-Rezeptorblocker in der zerebralen Arteriogenese)’, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of Contribution to the Publications

Publication 1: Hillmeister P, Nagorka S, Gatzke N, Dülsner A, **Li K**, Dai M, Bondke Persson A, Lauxmann MA, Jaurigue J, Ritter O, Bramlage P, Buschmann E, Buschmann I. Angiotensin-converting enzyme inhibitors stimulate cerebral arteriogenesis. Acta Physiol (Oxf). 2022.

Contribution: as the co-author, Kangbo Li participated in the protocol design, data analysis, and interpretation. Kangbo Li made Figure 1, A-F. Dr. Stephanie Nagorka performed the animal experiments and collected data. Dr. Philipp Hillmeister wrote and revised the manuscript.

Publication 2: Li K, Kratzmann V, Dai M, Gatzke N, Rocic P, Bramlage P, Grisk O, Lubomirov LT, Hoffmeister M, Lauxmann MA, Ritter O, Buschmann E, Bader M, Persson AB, Buschmann I, Hillmeister P. Angiotensin receptor-neprilysin inhibitor improves coronary collateral perfusion. Front Cardiovasc Med. 2022.

Contribution: as the co-first author, Kangbo Li designed and performed the *in vitro* experiments, collected, analyzed, and interpreted data. Kangbo Li made all the tables and figures. Kangbo Li wrote and revised the manuscript. Dr. Petra Rocic, Dr. Nora Gatzke, and Kangbo Li performed the animal experiments and collected data.

Publication 3: Li K, Zemmrich C, Bramlage P, Persson AB, Sacirovic M, Ritter O, Buschmann E, Buschmann I, Hillmeister P. Effect of ACEI and ARB treatment on nitric oxide-dependent endothelial function. Vasa. 2021.

Contribution: as the first author, Kangbo Li designed the cross-sectional study, conducted all the experiments, and collected, analyzed, and interpreted data. Kangbo Li made Table II, Table III, Table IV, Figure 1A, Figure 2A, and Figure 4. Kangbo Li wrote and revised the manuscript.

Signature, date and stamp of supervising university professor

Signature of doctoral candidate

Journal Data Filtered By: **Selected JCR Year: 2019** Selected Editions: SCIE, SSCI
Selected Categories: "**PHYSIOLOGY**" Selected Category Scheme: WoS

Gesamtanzahl: 81 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	PHYSIOLOGICAL REVIEWS	28,712	25.588	0.024010
2	Annual Review of Physiology	9,466	19.556	0.010190
3	JOURNAL OF PINEAL RESEARCH	10,537	14.528	0.009430
4	PHYSIOLOGY	3,583	7.212	0.005380
5	International Journal of Behavioral Nutrition and Physical Activity	11,154	6.714	0.018870
6	Comprehensive Physiology	4,877	6.604	0.009170
7	JOURNAL OF CELLULAR PHYSIOLOGY	26,456	5.546	0.024290
8	Acta Physiologica	5,106	5.542	0.008320
9	EXERCISE AND SPORT SCIENCES REVIEWS	3,290	4.915	0.002720
10	Reviews of Physiology Biochemistry and Pharmacology	805	4.700	0.000670
11	JOURNAL OF PHYSIOLOGY-LONDON	50,045	4.547	0.037090
12	AMERICAN JOURNAL OF PHYSIOLOGY-LUNG CELLULAR AND MOLECULAR PHYSIOLOGY	13,085	4.406	0.015510
13	AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY	26,114	3.864	0.020400
14	AMERICAN JOURNAL OF PHYSIOLOGY-GASTROINTESTINAL AND LIVER PHYSIOLOGY	14,186	3.725	0.012280
15	PSYCHOPHYSIOLOGY	14,586	3.692	0.012670
16	JOURNAL OF GENERAL PHYSIOLOGY	7,476	3.628	0.007380
17	International Journal of Sports Physiology and Performance	5,072	3.528	0.009760

Publication 1: Hillmeister P, Nagorka S, Gatzke N, Dülsner A, **Li K**, Dai M, Bondke Persson A, Lauxmann MA, Jaurigue J, Ritter O, Bramlage P, Buschmann E, Buschmann I. Angiotensin-converting enzyme inhibitors stimulate cerebral arteriogenesis. Acta Physiol (Oxf). 2022 Feb;234(2):e13732.
https://doi.org/10.1111/apha.13732

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE, SSCI

Selected Categories: **"CARDIAC and CARDIOVASCULAR SYSTEMS"**

Selected Category Scheme: WoS

Gesamtanzahl: 141 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	Nature Reviews Cardiology	11,539	32.419	0.022990
2	EUROPEAN HEART JOURNAL	81,447	29.983	0.141060
3	CIRCULATION	190,210	29.690	0.200030
4	JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY	125,873	24.094	0.177000
5	CIRCULATION RESEARCH	65,425	17.367	0.068900
6	BASIC RESEARCH IN CARDIOLOGY	5,629	17.165	0.005290
7	EUROPEAN JOURNAL OF HEART FAILURE	17,114	15.534	0.027740
8	JACC-Cardiovascular Imaging	14,398	14.805	0.032190
9	JAMA Cardiology	11,453	14.676	0.036150
10	JACC-Heart Failure	6,212	12.035	0.019320
11	JACC-Cardiovascular Interventions	15,448	11.195	0.035240
12	CARDIOVASCULAR RESEARCH	26,765	10.787	0.019070
13	JOURNAL OF HEART AND LUNG TRANSPLANTATION	15,107	10.247	0.021660
14	Cardiovascular Diabetology	8,782	9.951	0.012280
15	Circulation-Heart Failure	9,005	8.790	0.016490
16	JACC-Basic to Translational Science	1,842	8.648	0.005140
17	PROGRESS IN CARDIOVASCULAR DISEASES	5,799	8.194	0.006870
18	European Journal of Preventive Cardiology	8,287	7.804	0.014250
19	Circulation- Cardio-vascular Imaging	7,640	7.792	0.015510
20	European Heart Journal- Cardiovascu-lar Imaging	8,423	6.875	0.020790

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	TRENDS IN CARDIOVASCULAR MEDICINE	3,542	6.677	0.004230
22	European Heart Journal-Cardiovascular Pharmacotherapy	919	6.617	0.002480
23	Circulation-Arrhythmia and Electrophysiology	8,834	6.568	0.015230
24	Circulation-Cardiovascular Interventions	6,530	6.546	0.014270
25	EuroIntervention	7,160	6.534	0.014970
26	JACC-Clinical Electrophysiology	2,801	6.375	0.009790
27	HEART RHYTHM	16,459	6.343	0.025740
28	JACC: CardioOncology	267	6.250	0.000230
29	Circulation-Genomic and Precision Medicine	858	6.054	0.003910
30	Frontiers in Cardiovascular Medicine	3,172	6.050	0.007630
31	HEART	22,182	5.994	0.028490
32	JOURNAL OF NUCLEAR CARDIOLOGY	5,882	5.952	0.005390
33	Circulation-Cardiovascular Quality and Outcomes	6,318	5.882	0.013260
34	JOURNAL OF CARDIAC FAILURE	6,604	5.712	0.008690
35	Journal of the American Heart Association	26,960	5.501	0.075700
36	Clinical Research in Cardiology	5,218	5.460	0.007940
37	JOURNAL OF CARDIOVASCULAR MAGNETIC RESONANCE	6,935	5.364	0.010460
38	JOURNAL OF THE AMERICAN SOCIETY OF ECHOCARDIOGRAPHY	14,150	5.251	0.018650
39	CANADIAN JOURNAL OF CARDIOLOGY	8,782	5.223	0.015090
40	EUROPACE	12,468	5.214	0.021850

Publication 2: Li K, Kratzmann V, Dai M, Gatzke N, Rocic P, Bramlage P, Grisk O, Lubomirov LT, Hoffmeister M, Lauxmann MA, Ritter O, Buschmann E, Bader M, Persson AB, Buschmann I, Hillmeister P. Angiotensin receptor-neprilysin inhibitor improves coronary collateral perfusion. Front Cardiovasc Med. 2023 Feb 3;9:981333. https://doi.org/10.3389/fcvm.2022.981333

Journal Data Filtered By: **Selected JCR Year: 2019** Selected Editions: SCIE, SSCI

Selected Categories: **“PERIPHERAL VASCULAR DISEASE”**

Selected Category Scheme: WoS

Gesamtanzahl: 65 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	CIRCULATION	158,218	23.603	0.205020
2	CIRCULATION RESEARCH	51,539	14.467	0.071470
3	ANGIOGENESIS	3,571	9.780	0.005480
4	HYPERTENSION	36,242	7.713	0.046840
5	Journal of Stroke	1,247	7.470	0.004240
6	STROKE	66,466	7.190	0.078010
7	ARTERIOSCLEROSIS THROMBOSIS AND VASCULAR BIOLOGY	32,385	6.604	0.032080
8	EUROPEAN JOURNAL OF VASCULAR AND ENDOVASCULAR SURGERY	9,932	5.328	0.013510
9	International Journal of Stroke	4,853	4.882	0.015560
10	Current Atherosclerosis Reports	2,586	4.608	0.004550
11	THROMBOSIS AND HAEMOSTASIS	15,589	4.379	0.020570
12	CURRENT OPINIONIN LIPIDOLOGY	4,151	4.254	0.005450
13	JOURNAL OF HYPERTENSION	16,940	4.171	0.020170
14	JOURNAL OF THROMBOSIS AND HAEMOSTASIS	17,598	4.157	0.025190
15	ATHEROSCLEROSIS SUPPLEMENTS	767	3.968	0.001220
16	ATHEROSCLEROSIS	24,587	3.919	0.036590
17	Journal of Atherosclerosis and Thrombosis	3,426	3.876	0.005190
18	AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY	26,114	3.864	0.020400

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
19	CURRENT HYPERTENSION REPORTS	3,143	3.856	0.005940
20	JOURNAL OF VASCULAR SURGERY	26,553	3.405	0.024980
21	Journal of Vascular Surgery-Venous and Lymphatic Disorders	1,115	3.137	0.002260
22	JOURNAL OF ENDOVASCULAR THERAPY	3,651	3.102	0.005110
23	JOURNAL OF VASCULAR AND IN-TERVENTIONAL RADIOLOGY	9,045	3.037	0.009790
24	SHOCK	7,919	2.960	0.010370
25	HYPERTENSION RESEARCH	5,169	2.941	0.005330
26	SEMINARS IN THROMBOSIS AND HEMOSTASIS	4,074	2.892	0.005260
27	THROMBOSIS RESEARCH	10,616	2.869	0.017410
28	MICROVASCULAR RESEARCH	4,028	2.730	0.003140
29	Journal of Clinical Hypertension	3,983	2.719	0.006980
30	Diabetes & Vascular Disease Research	1,520	2.707	0.003170
31	CEREBROVASCULAR DISEASES	5,699	2.698	0.005800
32	Current Vascular Pharmacology	1,786	2.672	0.002200
33	AMERICAN JOURNAL OF HYPERTENSION	8,915	2.669	0.010840
34	CURRENT OPINION IN NEPHROLOGY AND HYPERTENSION	3,281	2.539	0.004740
35	VASCULAR MEDICINE	1,770	2.509	0.002560
36	Thrombosis Journal	773	2.295	0.001890
37	JOURNAL OF HUMAN HYPERTENSION	4,372	2.260	0.004640
38	JOURNAL OF CARDIOTHORACIC AND VASCULAR ANESTHESIA	5,371	2.258	0.007310

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
39	ANGIOLOGY	3,063	2.255	0.003420
40	BLOOD PRESSURE	1,180	2.169	0.001920
41	MICROCIRCULATION	2,462	2.110	0.002700
42	Pregnancy Hypertension-An International Journal of Womens Cardiovascular Health	1,286	2.095	0.003810
43	INTERNATIONAL ANGIOLOGY	1,331	2.080	0.001320
44	JOURNAL OF THROMBOSIS AND THROMBOLYSIS	2,794	2.054	0.005740
45	PHLEBOLOGY	1,438	1.914	0.001850
46	KIDNEY & BLOOD PRESSURE RESEARCH	1,903	1.898	0.003130
47	Seminars in Vascular Surgery	720	1.889	0.000740
48	Vasa-European Journalof Vascular Medicine	1,008	1.831	0.001430
49	CLINICAL AND EXPERIMENTAL HYPERTENSION	1,887	1.789	0.002420
50	HYPERTENSION IN PREGNANCY	1,314	1.787	0.001450
50	Journal of Stroke & Cerebrovascular Diseases	6,838	1.787	0.016800
52	CLINICAL HEMORHEOLOGY AND MICROCIRCULATION	2,147	1.741	0.002180
53	JOURNAL OF VASCULAR RESEARCH	1,551	1.725	0.001150
54	HEART AND VESSELS	2,176	1.618	0.003670
55	JOURNAL OF THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM	918	1.417	0.001160
56	JOURNAL OF CARDIOVASCULAR SURGERY	1,825	1.415	0.002130

Publication 3: Li K, Zemmrich C, Bramlage P, Persson AB, Sacirovic M, Ritter O, Buschmann E, Buschmann I, Hillmeister P. Effect of ACEI and ARB treatment on nitric oxide-dependent endothelial function. Vasa. 2021 Nov;50(6):413-422. https://doi.org/10.1024/0301-1526/a000971

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publication List

Manuscripts

1. **Li K**, Zemmrich C, Bramlage P, Persson AB, Sacirovic M, Ritter O, Buschmann E, Buschmann I, Hillmeister P. Effect of ACEI and ARB treatment on nitric oxide-dependent endothelial function. Vasa. 2021 Nov;50(6):413-422. Impact factor (2020): 1.961.
2. Hillmeister P, Nagorka S, Gatzke N, Dülsner A, **Li K**, Dai M, Bondke Persson A, Lauxmann MA, Jaurigue J, Ritter O, Bramlage P, Buschmann E, Buschmann I. Angiotensin-converting enzyme inhibitors stimulate cerebral arteriogenesis. Acta Physiol (Oxf). 2022 Feb;234(2):e13732. Impact factor (2020): 6.311.
3. **Li K**, Kratzmann V, Dai M, Gatzke N, Rocic P, Bramlage P, Grisk O, Lubomirov LT, Hoffmeister M, Lauxmann MA, Ritter O, Buschmann E, Bader M, Persson AB, Buschmann I, Hillmeister P. Angiotensin receptor-neprilysin inhibitor improves coronary collateral perfusion. Front Cardiovasc Med. 2023 Feb 3;9:981333. Impact factor (2021): 5.846.

Poster Presentations

1. **Li K**, Hillmeister P, Buschmann I, Bader M. Kinin receptors in arteriogenesis: a new perspective for angiotensin-converting enzyme inhibitor therapies? Berlin PostDoc Day 2019, Berlin, June 6th, 2019.
2. **Li K**, Hillmeister P, Buschmann I. Introduction of cerebral arteriogenesis. Berlin Neuroscience Meeting 2019, Berlin, October 10th, 2019.
3. **Li K**. Role of angiotensin-converting enzyme inhibitor and kinin receptors in cerebral arteriogenesis. Neurizons 2020, Göttingen, May 28th, 2020.

Acknowledgments

I hereby express my sincere gratitude to Prof. Michael Bader, Prof. Ivo Buschmann, and Dr. Philipp Hillmeister. I am thankful to them for their scientific supervision.

I thank all my colleagues in the Max Delbrück Center for Molecular Medicine Berlin, and the German Center for Vascular Medicine Brandenburg-Berlin. Many thanks for their great support.

I also extend my appreciation to Mr. Ralf Ansorg from the International Graduate Program Medical Neurosciences, who patiently supported me for the doctoral application and administration.