Angiotensin-converting enzyme inhibitors stimulate cerebral arteriogenesis

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Abstract

Aim: Arteriogenesis constitutes the most efficient endogenous rescue mechanism in cases of cerebral ischaemia. The aim of this work was to investigate whether angiotensin-converting enzyme inhibitors (ACEi) stimulates, and angiotensin II receptor type 1 blockers (ARB) inhibits cerebral collateral growth by applying a three-vessel occlusion (3-VO) model in rat.

Methods: Cerebral collateral growth was measured post 3-VO (1) by assessing blood flow using the cerebrovascular reserve capacity (CVRC) technique, and (2) by assessing vessel diameters in the posterior cerebral artery (PCA) via the evaluation of latex angiographies. A stimulatory effect on arteriogenesis was investigated for ACEi administration ± bradykinin receptor 1 (B1R) and 2 (B2R) blockers, and an inhibitory effect was analyses for ARB administration. Results were validated by immunohistochemical analysis and mechanistic data were collected by human umbilical vein endothelial cell (HUVEC) viability or scratch assay and monocyte (THP-1) migration assay.

Results: An inhibitory effect of ARB on arteriogenesis could not be demonstrated. However, collateral growth measurements demonstrated a significantly increased CVRC and PCA diameters in the ACEi group. ACEi stimulates cell viability and migration, which could be partially reduced by additional administration of bradykinin receptor 1 inhibitor (B1Ri).

ACEi inhibits the degradation of pro-arteriogenic bradykinin derivatives, but combined ACEi + B1Ri + B1Ri (BRB) treatment did not reverse the stimulatory effect. Yet, co-administration of ACEi + BRB enhances arteriogenesis and cell migration.
1 | INTRODUCTION

Cardiovascular disease and stroke are recognized as the leading cause of mortality and morbidity worldwide. Stroke outcome is dependent upon pathological processes, such as ischaemic cerebral damage-induced chronic systolic dysfunction, and physiological process, such as arteriogenesis. Arteriogenesis is a type of flow-induced outward remodelling of pre-existing collateral arterial pathways into functional conductance arteries (biological bypass), and is recognized as the most effective endogenous rescue mechanism against cerebral ischaemia. Blood supply to the brain is primarily redistributed by an extensive collateral circulatory system within the circle of Willis. In cases of vascular occlusion, redistribution of cerebral flow via enlarged collateral arteries restores blood supply to the compromised ischaemic regions, and collateral status is associated with reduced infarct volumes.

In the context of cerebral ischaemia prevention, therapeutic arteriogenesis is considered a highly relevant clinically strategy, and pharmacological stimulation of collateral growth has been intensively researched. Angiotensin receptor blockers (ARBs) and angiotensin-converting enzyme inhibitors (ACEi) are pharmaceutical compounds commonly used as a first-line therapy in cardiovascular risk management and stroke prevention. ARBs and ACEi have both relevant roles for improving cerebral blood flow (CBF); however, the effectiveness of these drugs in lowering blood pressure cannot be considered the sole determinant of its effectiveness. ARBs and ACEi target the renin–angiotensin system (RAS), whereby renin is cleaved into angiotensin I (Ang I) and then converted into the vasoactive octapeptide angiotensin II (Ang II) by the zinc metallopeptidase Angiotensin I-converting enzyme/kininase II (ACE). Ang II then exerts its biological effects via AT1R initiating vasoconstriction. In the adult brain, structures related to the regulation of body fluid homoeostasis and blood pressure, in particular endothelial cells, smooth muscle cells and myocytes express AT1R. The role of AT1Rs in arteriogenesis remains unknown; however, it has been reported that AT1R play a major role in vascular remodelling processes such as atherosclerosis and cell proliferation. With this consideration, we hypothesize that blockade of the AT1R by ARBs should inhibit cerebral arteriogenesis.

ACEi are a second major group of drugs with great importance in cardiovascular medicine, affecting the RAS not only by inhibiting ACE activity but also by activating the kallikrein–kinin system (KKS) as well. In the KKS, the molecule kallikrein cleaves the substrate kinogen, liberating kinins. The effect of these kinin derivatives are mediated by two G-protein-coupled receptor subtypes bradykinin receptor 1 (B1R) and bradykinin receptor 2 (B2R). In regard to the KKS, our group has shown that this hormonal system is crucially important for modulating cerebral arteriogenesis. We previously showed that kininogen was selectively expressed in growing collaterals within the circle of Willis during cerebral arteriogenesis. Furthermore, we provided evidence that bradykinin receptor signalling regulates cerebral arteriogenesis, and treatment with B1R agonist leads to considerable stimulation of cerebral arteriogenesis. Here it is known that ACEi prolong and enhance bradykinin receptor signalling, which is proposed to be largely responsible for ACEi induced cardioprotective effects. ACEi stabilizes kinin, because otherwise kinin derivatives are rapidly degraded by ACE. Therefore, we hypothesized that cerebral arteriogenesis can be therapeutically enhanced beyond its natural time course by modulation with ACEi.

To test our hypotheses whether ACEi stimulates and ARB inhibits cerebral collateral growth, we utilized the three-vessel occlusion (3-VO) model of cerebral arteriogenesis in rats. The 3-VO model produces a non-lethal type of brain hypoperfusion followed by a gradual improvement of cerebral flow by collateral artery growth within the circle of Willis. Furthermore, we used cell viability and scratch assays, as well as transwell migration assays to obtain mechanistic data to important features of endothelial cell and monocyte-mediated arteriogenesis. To the best of our knowledge, this is the first experimental study investigating the modulating effects of ARB and ACEi on arteriogenesis in the brain.
2 | RESULTS

2.1 | 3-VO model—activation and modulation of cerebral arteriogenesis

For the investigation of a pharmacological stimulation of cerebral arteriogenesis by ACEi, a 7-day 3-VO study design was chosen, since a possible acceleration of collateral growth can be analysed on early time points (Figure S1A). For a pharmacological inhibition of cerebral arteriogenesis by ARB, a 21-day 3-VO study design was chosen. Here, natural arteriogenesis is already more advanced, and a possible inhibition can thus be represented by a reduction in collateral growth (Figure S1B).

The occlusion of three of the four arteries supplying the brain leads to a redirection of blood flow through the anastomotic circle of Willis and thus to an adaptive growth of the posterior cerebral artery (PCA) (Figure 1A-F). Cerebral arteriogenesis stimulation or inhibition was evaluated by measuring PCA vessel diameters post flow-induced remodelling using latex angiography under the condition of maximum vasodilation (Figure 1G-K).

2.2 | Vessel diameters

Seven days after surgery, vessel diameters of control-treated 3-VO animals showed significantly larger PCA diameters (ipsilateral 212 ± 10 μm, contralateral 205 ± 19 μm) compared with non-operated (untreated) rats (Figure 2A). Rats treated with ACEi showed significantly larger PCA diameters (ipsilateral 244 ± 18 μm, contralateral 232 ± 50 μm) compared with control-treated 3-VO animals 7 days after surgery. Rats treated with ACEi + BRB also showed larger PCA diameters (ipsilateral 274 ± 20 μm, contralateral 254 ± 23 μm) compared with untreated 3-VO animals. Furthermore, rats treated with ACEi + BRB had significantly larger PCA diameters compared with animals treated with ACEi alone.

With regard to the experiments in the ARB treatment group, results indicated 21 days after surgery that PCA diameters were significantly different in untreated 3-VO animals (ipsilateral 263 ± 30 μm, contralateral 253 ± 40 μm) compared with non-surgery animals (ipsilateral 155 ± 34 μm, contralateral 143 ± 19 μm). However, 21 days post-3-VO animals treated with AT1R antagonist showed no significant different PCA diameters (ipsilateral 265 ± 17 μm, contralateral 238 ± 44 μm) compared with untreated 21 days post 3-VO animals (ipsilateral 263 ± 30 μm, contralateral 253 ± 40 μm) (Figure 2B).

2.3 | Cerebrovascular reserve capacity

Cerebral arteriogenesis was evaluated by measuring cerebrovascular reserve capacity (CVRC), and therefore blood flow recovery, 7 and 21 days after 3-VO surgery. Post 3-VO surgery, perfusion drops quickly and recovers with time. To determine CVRC, the percentage change in cerebral blood flow before and 8 minutes after acetazolamide application was measured. Control-treated animals prior to 3-VO operation and non-operated (untreated) animals showed CVRC at 19% ± 5% (Figure 3A). Twenty-four hours after 3-VO operation, CVRC was almost eliminated at 1% ± 8%. Seven days after operation, CVRC remained significantly low in control-treated animals at 2% ± 7%. Conversely, animals treated with ACEi showed significantly higher CVRC (10% ± 9%) 7 days post 3-VO compared with control-treated animals 7 days post 3-VO. Animals treated with ACEi + BRB showed significantly higher CVRC (11% ± 5%) than control-treated animals, but not significantly different to animals treated with ACEi alone.

Three weeks after surgery, control-treated animals showed a CVRC (12% ± 15%) comparable with non-operated (untreated) animals (19% ± 5%) (Figure 3B). However, animals treated with ARB showed no different CVRC (AT1R antagonist 8% ± 5%) compared with control-treated animals 21 days after 3-VO surgery.

2.4 | Arterial growth morphology—histology

The stimulation of arteriogenesis by ACEi was validated by morphological assessment of cell proliferation in the collateral area of the PCA. Anti-Ki-67 primary antibody with Cy3-conjugated secondary antibody visualized proliferating cell nuclei in the interphase of the cell cycle. FITC-conjugated anti-α-smooth muscle actin antibody visualized smooth muscle cell layers in the PCA vessel wall, and Hoechst cell nuclei counterstain helped distinguish individual cells (Figure 4A-G). In the tunica intima of the PCA 7 days after 3-VO surgery, an average of 1.24 ± 0.67 proliferating cell nuclei were found in the control-treated group, and 1.11 ± 0.82 proliferating cell nuclei in the ACEi + BRB-treated group (Table 1). In contrast, the number of proliferating cell nuclei was significantly reduced in the ACEi-treated group (0.48 ± 0.65). In the tunica media, no significant differences in proliferating cell nuclei were observed between groups (control-treated 1.04 ± 0.80; ACE inhibitor-treated 0.41 ± 1.13 and the ACEi + BRB-treated 0.50 ± 0.66). In the perivascular area, however, the control-treated group showed significantly more
FIGURE 1  Visualization of the collateral anastomoses of the Circle of Willis, and schematic cross-section of the posterior cerebral artery: (A, D) before 3-VO, (B, E) activation-phase upon 3-VO, (C, F) remodelling phase with proliferating cells. I, ipsilateral; c, contralateral; VA, Aa. Vertebrales; BA, A. basilaris; SCA, A. cerebelli superior; PCA, A. cerebri posterior (posterior cerebral artery); PComA, A. communicans posterior; ICA, A. carotis interna; MCA, A. cerebri media; ACA, A. cerebri anterior. The transparent circle marks the analysed area (area of interest) of the posterior cerebral artery which was used under the binocular to determine the diameters by latex angiography. Representative images of latex angiography for the determination of vascular diameters, (G) = 3-VO control, (H) = 3-VO 21d + ARB, (J) = 3-VO 7d + ACEi, (K) = 3-VO 7d ACEi + BRB
proliferating cell nuclei (0.76 ± 0.55) compared with the ACEi-treated (0.17 ± 0.38) and ACEi + BRB-treated (0.13 ± 0.23) groups.

To further validate collateral growth, the number of smooth muscle cell layers in the vessel wall was quantified. Seven days after surgery the control-treated group
presented 2.62 ± 0.44 smooth muscle cell layers. Animals in the ACEi-treated and ACEi + BRB-treated groups presented significantly more smooth muscle cell layers at 3.23 ± 0.47 and 3.36 ± 0.45, respectively (Table 1).
FIGURE 4  Immunohistochemical examination. Ki-67 Cy3 staining for cell proliferation (red), anti α-smooth muscle actin-FITC staining (green), counterstained with a Hoechst cell nucleus labelling (blue). Representative image of the PCA in: (A) 7 days post 3-VO control rat; (B) ACEi 7 days post 3-VO rat; (C) ACEi + BRB 7 days post 3-VO. Morphological analysis of collateral vascular growth. Representative images with higher magnification. (D) 7 days post 3-VO, (E) ACEi 7 days post 3-VO, (F) ACEi + BRB 7 days post 3-VO. G, brain sagittal section overview displaying the PCA and the PcomA (A. communicans posterior). Histological quantification of collateral growth (numbers) is shown in Table 1.
In regard to HUVEC migration, and interaction we performed a wound healing (scratch) assay (Figure 5E-K). Cells were cultured for 24 hours in 10% FBS medium containing ACEi (E), ACEi + B1Ri (F), ACEi + B2Ri (G), ACEi + B1Ri + B2Ri (H) or serum only (J). Representative images were captured under the microscope (100 times magnification) and, in each well, four different scraped areas were randomly selected and analysed (Figure 5K). Cells treated by 1 μM ACEi showed significant higher migration ratio (ACEi group vs FBS group, ***P < .001). When cells were treated with 1 μM ACEi + B1Ri, the effect of facilitating migration by ACEi was significantly reduced (ACEi group vs ACEi + B1Ri group, **P < .01). When HUVECs were treated with concomitant administration of ACEi + B1Ri + B2Ri, this significantly promoted migration compared with the ACEi + B1Ri group and was now similarly increased compared with the group stimulated with ACEi alone.

### 2.5 Endothelial cell viability and migration—cell culture assay

To determine the toxicity of ACEi on human endothelial cells, we treated HUVECs with a series of doses of ACEi ranging from 0.1 to 20 μM in a time window between 24 and 96 hours (Figure 5A,B). Results show that when the concentration of ACEi was 1 μM (72 and 96 hours treatment), it promoted the cell viability of HUVECs significantly. However, when the concentrations of ACEi were at 10 and 20 μM, they significantly reduced the cell viability respectively. Based on these observations, we treated HUVECs with a well-tolerable concentration of 1 μM ACEi in the subsequent experiments in order to determine positive effects on cell viability. Here, HUVECs were cultured in 10% FBS RPMI-1640 medium containing ACEi, ACEi + B1Ri, ACEi + B2Ri or equal volumes of PBS for 24-96 hours. No statistical difference was observed between groups on 24 and 48 hours respectively. When cells were incubated for 72 (Figure 5C) and 96 (Figure 5D) hours, ACEi promoted the cells viability of HUVECs significantly. However, the capability of ACEi to promote cells viability cannot be weaken by B1Ri and B2Ri.

### 2.6 Monocyte transwell migration—cell culture assay

A cell culture migration assay was performed with THP-1 monocytic cells using transwell chambers (Figure 6). THP-1 cells were stimulated with ACEi, B1Ri and B2Ri and representative images were captured under the microscope (Figure 6A-E) and the number of cells was counted in four replicates. THP-1 cells show significantly increased migration after 3 hours of stimulation with ACEi compared with the control, in which THP-1 cells were incubated with serum only (Figure 6F, ACEi group vs FBS group, *P < .05). THP-1 cells showed the same migration behaviour after stimulation with ACEi + B2Ri compared with the cells stimulated with ACEi alone. When cells were treated by ACEi + B1Ri, the effect of facilitating migration by ACEi has been significantly reduced (ACEi group vs ACEi + B1Ri group, **P < .01). When cells were treated by ACEi + B1Ri + B2Ri, the number of migrated cells was increased more than cells treated with ACEi alone (ACEi group vs ACEi + B1Ri + B2Ri group, *P < .05).

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**Figure 5** A-B, The effects of different concentrations ACEi on cell viability in human umbilical vein endothelial cells (HUVECs). Cell viability was measured using CCK8 assay. Cell viability for (A) 72 hours and (B) 96 hours incubation time showed significant differences and a positive effect for cells treated with 1 μM ACEi. Cells viability assay with HUVECs treated for 72 hours (C) and 96 hours (D) incubation time with 1 μM ACEi (**other groups compared with this group, *P < .05, **P < .01, ***P < .001, abbreviation: ns, no statistical significance; PC, positive control; NC, negative control). E-K, Scratch assay for analysing HUVEC migration. Consistently shaped wounds were created. E-J, Representative images. Images were captured under the microscope (100 times magnification), and in each well, four different scraped areas were randomly selected and analysed. Cells were cultured for 24 hours in 10% FBS medium containing 1 μM ACEi (E), ACEi + B1Ri (F), ACEi + B2Ri (G), ACEi + B1Ri + B2Ri (H) or no drugs (J). Results quantification (K). (**all other groups were compared with ACEi-treated group, *P < .05, **P < .01, ***P < .001, abbreviation: ns, no statistical significance)**
We demonstrated for the first time the therapeutic effect of ACEi on cerebral collateral artery growth. Induction of cerebral arteriogenesis after 3-VO surgery can be stimulated with ACEi administration, significantly enhancing blood flow recovery after the experimentally induced occlusion of brain-supplying arteries. PCA vessel diameters also increased, confirming the stimulatory effect of ACEi administration for therapeutic cerebral arteriogenesis. This finding has great clinical relevance, as it has been proven that therapeutically increasing cerebral arteriogenesis significantly correlates with reduced stroke volume. In contrast, this work did not show that ARBs modulate cerebral arteriogenesis. The assumption that AT1R blockade via ARB administration is associated with the inhibition of collateral growth could not be confirmed here.

**FIGURE 6** Transwell migration assay shown that ACEi promotes THP-1 migration. A total of 60,000 cells were cultured on the upper chamber using FBS free RPMI-1640 medium containing 1 μM ACEi (A), ACEi + B1Ri (B), ACEi + B2Ri (C), ACE and B1Ri + B2Ri (D) or no drugs (E). 600 μL RPMI-1640 media containing 10% FBS was added into the lower chamber. Images were captured under the microscope (100 times magnification), and in each well, four different areas were randomly selected and analysed. Results quantification (F), (all other groups were compared with ACEi treated group, *P < .05, **P < .01. ns, no statistical significance)
We once again demonstrated the reproducibility of the 3-VO model in the rat (Figures 1-3). Experimentally induced occlusion of three of the four brain-supplying arteries after 3-VO surgery redirects blood flow from the remaining carotid artery via the collateral anastomoses of the circle of Willis, thereby placing severe stress on the PCA. Activation of adaptive collateral growth in the PCA occurs, resulting in restoration of blood supply and recovery of cerebral autoregulation as measured by CVRC. Collateral growth is especially evident in the ipsilateral PCA and is demonstrated by a significant increase in vascular diameter. Adaptive remodelling in the 3-VO model was further analysed in the context of additional cerebral arteriogenesis modulation by ACEi and ARB administration.

Both ARBs and ACEi lower hypertension and improve the vascular tone. In Hagen–Poisueille’s law, an increase in diameter means an improvement in blood flow to the power of 4. Hence, passive vasodilation by lowering blood pressure and the associated loss of constriction will promote blood flow. Greater flow may enhance outward remodelling of the distal arterial territory via shear stress-induced arteriogenesis. Hence, vasodilation should trigger active remodelling processes of collateral arteries by enhanced blood flow. We recently explored this hypothesis and investigated the role of the anti-hypertensive substances nitroglycerin (NO-Donor), metoprolol (β₁-adrenoreceptor blockers), verapamil (calcium channel blocks), ranolazine (piperazine derivatives) or candesartan (ARB) in a model of myocardial arteriogenesis.\textsuperscript{8} It was shown that ARBs and other antihypertensive agents, with the exception of nitroglycerin, have no beneficial effect on therapeutic arteriogenesis. In this context, we have never studied the effect of ACEi on arteriogenesis.

In the experimental setting presented here, ARB did neither improve positive outward remodelling nor did it increase CBF. Our experimental design was aimed at stimulating flow-induced arteriogenesis, which is regulated similarly in normotensive and hypertensive settings.

In summary, the effectiveness of these drugs in lowering blood pressure cannot be considered the sole determinant of its effectiveness in reducing mortality and morbidity. Mechanisms of action other than vasodilation by ARBs and ACEi in the treatment of cardiovascular disease remain to be explored in the context of arteriogenesis.\textsuperscript{22}

ARBs and ACEi have both relevant roles for improving cerebral CBF in hypertensive patients. Ito et al\textsuperscript{23} have shown in hypertensive rats that ARB and ACEi pre-treatment maintain CBF and protects against cerebral ischaemia. However, only the anti-proliferative properties of ARB have been shown to protect against a hypertension-induced pathological increase in vascular wall thickness and reduced vascular capacity. It was shown that ARB, but not ACEi administration, blocks cell proliferation because AT1R signalling induces the expression of various growth-promoting factors such as VEGF, platelet-derived growth factor and fibroblast growth factor.\textsuperscript{24,25} Hence, it is a reasonable assumption that inhibiting AT1R signalling would impair cerebral arteriogenesis. Secondly, multiple clinical trials in patients with various cardiovascular diseases have shown that blockade of the renin–angiotensin system by ARB reduces inflammation and oxidative stress.\textsuperscript{26–31} Inflammation, and even reactive oxygen species, is an important factor in arteriogenesis, also suggesting that ARBs inhibit collateral growth.\textsuperscript{9,32} However, our current work provided no evidence that blocking AT1R by ARB has an effect on inhibiting arteriogenesis. In summary, neither a stimulatory effect\textsuperscript{8} nor an inhibitory effect of AT1R blockers on arteriogenesis could be demonstrated. In regard to collateral growth, overall positive and negative properties of ARB appear to be in balance. Both ARBs and ACEi have relevant roles for improving CBF in patients. However, as discussed above, both might be relevant in the context of different underlying mechanisms.

In contrast to ARBs, ACEi show almost only properties that seem to be beneficial for arteriogenesis. Indeed, we show in this work a pro-arteriogenic effect after ACEi administration, demonstrated by increased blood flow recovery measured via rat brain CVRC 7 days after 3-VO surgery compared with the associated control-treated group (Figure 2). It is well known that ACEi plays a major role in activating the KKS and signalling via bradykinin receptors by increasing bradykinin through the degradation of its substrate ACE (kinase II).\textsuperscript{33} ACE is also known to modulate the RAS, in addition to KKS by converting angiotensin I to angiotensin II. However, ACE has a higher affinity for bradykinin than for angiotensin I, suggesting that ACEi is more effective for the KKS than for the RAS. Therefore, it is currently considered that the cardiovascular protective effect of ACEi is largely mediated by their role in downstream bradykinin signalling.\textsuperscript{34}

In a previous work, we showed that bradykinin receptors (B1R and B2R) act as positive modulators of arteriogenesis in mice and rats.\textsuperscript{18} Here, B1R showed a much stronger phenotype with respect to arteriogenesis, and B1R antagonist or agonist administration can effectively inhibit or stimulate cerebral arteriogenesis in the 3-VO model. Furthermore, numerous experiments including a THP-1 monocyte migration assay showed increased migration behaviour of the monocytes after stimulation by B1R agonists and suggested a prominent role of B1R-expressing leukocytes. Now, using a THP-1 monocyte migration assay we show in the present work that ACEi can improve the migration of monocytes (Figure 6). Appropriately, simultaneous stimulation of THP-1 cells with ACEi and B1Ri, but not with B2Ri, shows that monocyte migration is reduced.
This is consistent with our hypothesis and data published in 2011 that arteriogenesis is positively regulated mainly via the B1R. Presumably, ACEi will accumulate bradykinin, and this will stimulate monocyte migration via the B1R. Inhibition of the B1R signalling pathway will then also decrease monocyte migration.

Furthermore, we show for the first time that ACEi can also enhance HUVEC viability and HUVEC migration (Figure 5). Again, the migration of cells can be reduced by the administration of B1Ri, which also suggests a role of B1R signalling pathways in endothelial cell migration. It is well known that ACE and bradykinin receptors are localized in the plasma membrane of brain endothelial cells and in regard to monocytes we have recently shown the expression of ACE (and also bradykinin receptor 1 and 2) on peripheral blood mononuclear cells from 165 cardiovascular patients. Thus, we believe that in particular, the B1R signalling pathway significantly controls arteriogenesis by modulation of EC and monocyte migration, and here ACEi accumulated kinins act via a classical B1R agonist binding.

Finally, we showed that ACEi-treated animals exhibit a significant increase in ipsilateral PCA diameter 7 days post 3-VO compared with the control-treated animals (Figure 2). This result is consistent with increased PCA diameters after specific B1R agonist administration from our previous work. In this past work, we also used latex angiography and showed significantly increased PCA diameters, representing a state of active, positive outward remodelling after experimentally induced maximum vasodilation. Furthermore, immunohistological imaging analysis shows a significantly increased number of smooth muscle cell layers in the growing PCA of ACEi-treated animals (Figure 4, Table 1). Notably, fewer proliferating cell nuclei were seen in the ACEi-treated animals compared with the 3-VO control group, despite the high numbers of smooth muscle cell layers. This result is expected regarding the temporal development of collateral growth. Our group previously demonstrated that cell proliferation during cerebral collateral growth in the PCA was strongest 1-3 days post 3-VO. Seven to twenty-one days after experimentally induction of arteriogenesis, collateral growth enters the anti-proliferative maturation phase characterized by the expression of genes involved in extracellular matrix-associated remodelling. Therefore, the ACEi-treated animals presented fewer proliferating nuclei because cerebral arteriogenesis stimulation accelerated the kinetics of collateral growth bringing the maturation phase forward to day 7.

Previous work using a model for peripheral arteriogenesis demonstrated that ACEi administration can increase collateral-dependent blood flow and remodelling in the hind limb by promoting pro-angiogenic gene expression, supporting the potential utility of ACEi as a therapeutic agent in stimulating arteriogenesis. In a model for coronary arteriogenesis, Altin et al hypothesized that ACEi administration should have a negative effect on myocardial collateral development in patients with coronary occlusions. However, this work focussed on the role of ACEi on the RAS system, and hypothesized, that blocking angiotensin II signalling might impair collateral development. Indeed, ACEi is known to inhibit angiotensin II production and can therefore, together with other ARBs, prevent AT1R signalling. However, in the current work, the pro-angiogenic effect after ACEi administration can be explained by increased kinin activity followed by subsequent downstream signalling via bradykinin receptors, leading to the stimulation of cerebral arteriogenesis.

Our current work continues on from earlier publications exploring the relationship between ACEi, KKS and arteriogenesis, since ACEi-dependent stimulation of arteriogenesis most likely occurs via modulation of KKS. Indeed, we had previously demonstrated that kininogen is a highly effective biomarker for cerebral arteriogenesis in the 3-VO model, which has been shown to be highly expressed in the growing ipsilateral PCA. Kallikrein acts on kininogen to release vasoactive kinin peptides. These are the effectors of bradykinin receptor signalling, modulating arteriogenesis and playing an important role in cardiovascular homeostasis. After release, the various kinins activate the two different G-protein-coupled bradykinin receptors (B2R) and (B1R). B2R is constitutively expressed in the cardiovascular system, and is primary involved in the regulation of blood pressure as well as flow. B1R, on the other hand, is weakly expressed under physiological conditions but is activated in a variety of tissues during inflammation. While B2R undergoes rapid desensitization, B1R does not, but remains active, making it an interesting target for pharmacological modulation. Both bradykinin receptors may mediate their signalling in a nitric oxide (NO)-dependent manner via activation of different nitric oxide synthase isoforms. Presumably, the bradykinin receptors mediate their pro-angiogenic effect via NO, and the important role for nitric oxide for stimulating arteriogenesis was proven by Troidl et al. 

Hence, the second aim of this work was to investigate how administration of ACEi, which is linked to kinin-induced bradykinin receptor signalling, would interfere with bradykinin receptors in the context of collateral growth in the 3-VO model. We hypothesized that blocking both bradykinin receptors in vivo would impair the pro-angiogenic effect of simultaneous ACEi administration. Because although B1R signalling plays a much more important role in arteriogenesis, B2R also takes on minor modulatory effects. However, the ACEi induced
enhancement of collateral growth was not inhibited by pharmacological co-administration of B1Ri + B2Ri (BRB). Contrarily, administration of ACEi + BRB leads to significantly increased PCA diameters compared with ACEi alone. The synergetic, pro-arteriogenic effect of ACEi + BRB administration was also validated by assessing CVRC, which remained as high in the ACEi + BRB treatment group as it did in the group treated with ACEi alone. Likewise, muscle cell layer numbers also remained as high in the ACEi + BRB-treated group as it did in the ACEi-treatment group. Based on these findings, we provide further evidence for ACEi-modulated bradykinin receptor signalling and performed the monocyte migration assay with dual Bradykinin receptor (BRB) inhibition. Here, the number of migrated mononuclear cells was even more increased than with ACEi treatment alone. This result confirms our curious finding that dual inhibition by BRB with concomitant modulation by ACEi does not inhibit therapeutic arteriogenesis but, in fact, significantly further stimulates it. These results now show for the first time a completely new feature for bradykinin receptors and suggest a compensatory mechanism in case of failure of both bradykinin receptors system. We also showed that concomitant administration of ACEi + BRB now again significantly increased HUVEC migration compared with the ACEi + BR1i group. Although this stimulation was not stronger than that induced by ACEi alone, simultaneous inhibition of both bradykinin receptors again increased stimulation to a level comparable to the ACEi-stimulated group. The exact mode of this compensatory action remains elusive since we expected BRB administration would reduce the pro-arteriogenic effect of ACEi by preventing kinin signalling. Interestingly, there is growing evidence that ACEi affect B1R and B2R receptors signalling directly, independent of kinin signalling.44 ACEi were shown to be direct agonists of the B1R.45 Here, ACEi bind to a Zn-binding sequence on the second extracellular loop of B1R that differs from the orthosteric binding site of des-Arg kinin peptide ligands. Regarding the B2R, it was shown that ACEi can directly increase B2R functions as allosteric enhancers. ACEi are believed to induce a conformational change at ACE, causing heterodimerization at the plasma membrane of cells between ACE and B2Rs, thus inducing B2R signalling.44 Therefore, the effects of ACEi may go beyond inhibiting bradykinin degradation and may thus bind or interact with bradykinin receptors directly.44,45 The pleiotropic and compensatory therapeutic activation of bradykinin receptors by ACEi and how this synergizes with pharmacological inhibition requires further investigation at the molecular level.

In summary, there is long-standing evidence that ACEi have a protective effect for primary and secondary prevention of stroke through other modes of action beyond hypertension regulation. In this context, this work could not provide evidence that ARB has a modulatory effect on arteriogenesis. Instead, we demonstrate for the first time that ACEi stimulates cerebral arteriogenesis, which is considered the most efficient rescue mechanism against cerebral ischaemia. We consider that the pro-arteriogenic effects after ACEi administration are more likely a result of inhibiting bradykinin degradation, rather than preventing AT1R signalling to inhibit angiotensin II production. Our results also indicate an additional stimulatory effect of ACEi presumably through direct bradykinin receptor interaction. We show that ACEi stimulation of EC and monocytes enhances cell viability and migration and that B1R antagonists can inhibit the improved cell migration. Furthermore, we demonstrate a new and special mode of action for bradykinin receptors. In case of a double failure of B1R and B2R, a so far completely unknown and strong compensation mechanism occurs, which can restore the properties (rescue) and even seems to fulfil them better.

The results presented regarding the stimulation of cerebral arteriogenesis by ACEi have clinical relevance. Yet, in active-control comparisons of studies on patients with hypertension, primary stroke risk reduction within ACEi-treated groups was similar to groups treated with diuretics, beta-blockers and calcium channel blockers. Therefore, it is premature to conclude that ACEi therapy might be superior for primary or secondary prevention of stroke.46 However, further research should investigate the molecular mechanisms in which ACEi drugs stimulate cerebral arteriogenesis, and how this relates to bradykinin receptor signalling. In this way, novel clinical therapies can be developed that may selectively stimulate cerebral collateral artery growth.

### 4 MATERIAL AND METHODS

Animal experiments were carried out in accordance with the German Law for the Protection of Animals, the National Institute of Health Guidelines for Care and Use of Laboratory Animals, and in compliance with the ARRIVE guidelines for how to report animal experiments. Procurement of animals, the husbandry and the experiments conform to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985). The approval was granted by the Berlin ethics committee (LaGeSo) under trial number G 0071/09. All the material submitted is conform with good publishing practice in physiology according to the Acta Physiologica guidelines.47
4.1 | Non-ischaemic brain hypoperfusion 3-vessel occlusion (3-VO) model

The 3-VO model was used for pharmacological modulation by ACEi and ARBs in male Sprague–Dawley rats (weight 330 grams, age 11-12 weeks). The 3-VO model was first described in the form applied here by Busch et al & Schneeloch et al. In brief, the 3-VO model targets the activation of the circle of Willis, a collateral vascular system which restores cerebral blood supply through a biological bypass. Under normal conditions, inflow into the collateral vascular system in the brain occurs primarily via four vessels, the two vertebral arteries (dextra et sinistra) and the left and right A. carotis interna. In the 3-VO model, three of the four arteries supplying the brain, both vertebral arteries and one carotid artery, are experimentally ligated.

The present project once again confirmed the reproducibility of this 3-VO model in the rat. It demonstrates its suitability to analyse the vascular remodelling processes during cerebral arteriogenesis. In this work, therefore, the use of Sham groups was abandoned, as it was shown here in previous publications that a Sham surgery has no effect. Although animal experiments are essential in research, there is agreement that they should be kept to a necessary minimum. The guiding principle is the ethical principle of the ‘3Rs’: Replacement (avoid), Reduction (reduce or limit) and Refinement (improve).

4.2 | Analgesia

Anaesthesia during 3-VO surgery was performed by administering a combination anaesthesia of ketamine (50 mg/kg body weight [bw]) and xylazine (4 mg/kg bw) injected intraperitoneally. Ketamine served both as a narcotic as well as an analgesic. After administration, the rats were individually kept in a darkened cage in a quiet environment for 15 minutes. Surgery was maintained under anaesthesia with isoflurane inhalation through a breathing mask (mixture of 100% oxygen as carrier gas with 1.5%-2% isoflurane by volume). During preparation, an additional 1 mL (5 mg/kg bw) of ropivacaine was locally administered subcutaneously for local anaesthesia.

4.3 | Measurement of CVRC

To functionally quantify cerebral arteriogenesis, flow changes were assessed by quantifying the cerebrovascular reactivity. To quantify cerebrovascular reactivity, CVRC is measured to determine the highest theoretical increase in flow under maximum vasodilation. CVRC is presented as a percentage of vessel diameter increase after a vasodilatory stimulus compared with the initial situation and measured using laser Doppler flowmetry (Perimed, Sweden) as described by Duelsner et al. Cerebrovascular reactivity is considered a haemodynamic parameter that is a measure for the increase in perfusion following vasodilatory stimuli, such as increased CO₂ partial pressure (pCO₂) and reduced blood pH. Rats were anesthetized 7 or 21 days after 3-VO, and veins were catheterized for administering the carboanhydrase inhibitor acetazolamide (Diamox®, Sanofi Aventis, 30 mg/kg bw). Acetazolamide administration leads to a reduced blood concentration of hydrogen carbonate, and concomitant rise in blood pCO₂. Consequently, blood pH decreases resulting in maximum vasodilation.

4.4 | Latex angiography

Collateral growth was evaluated morphologically by means of latex angiography. Here, the vascular system is perfused with black-stained latex milk. After latex perfusion, the extracted brains were examined with the help of a stereomicroscope.

For ink injection, a catheter was placed in the left common carotid artery in the cranial direction. Subsequently, 2 mL of saline solution and 1 mL of papaverine (50 to 65 mg/kg bw) were successively administered through the catheter. The resultant maximum vasodilation quickly led to a hypotension that was lethal for the animal. The catheter was then connected to a perfusion pump operating at a constant pressure of 150 mm Hg. The cerebral vasculature was perfused with 2 mL of vasodilation solution heated to 37°C, followed by perfusion with black coloured latex milk. In order to allow the latex to harden, the animal body was placed on ice for approximately 15 minutes.

In the rat circle of Willis, both the ipsi- and contralateral posterior cerebral arteries were evaluated. The diameters of each vessel were measured at four different fixed points. From these four measurements, an average vessel diameter was determined. Reliable remodelling processes and evaluation of posterior cerebral arteries in 3-VO model studies enable cross-project comparisons. Based on altered flow physiology after 3-VO operation, growth processes are more prominent on the ipsilateral posterior cerebral artery in comparison to the contralateral side.

4.5 | Drug administration

Each of the ARB group animals received one daily dose of candesartan (Atacand, AstraZeneca, Germany, 2 mg/kg bw) dissolved in 0.5 mL 0.5% methylcellulose solution per
os. Each animal of the ACEi group received one daily dose of ramipril (CT Arzneimittel, Germany, 0.2 mg/kg bw) dissolved in 0.5 mL drinking water per os. Thus, medication for all treated animals was administered similarly via drinking water. Similarly, each non-3-VO animal and the control treated 3-VO animal (untreated) was administered 0.5 mL of drinking water by os once daily. The respective dosages of candesartan cilexetil (ARB) and ramipril (ACEi) were selected according to the recommendations of intake for an adult human. The dosage was converted to the rat taking into account the metabolic body weight.

In follow-up tests, animals were treated with both ACEi and bradykinin receptor blocker 1 and 2 (BRBs). Here, the B2R inhibitor Icatibant (Firazyr) was administered subcutaneously once daily at a dose of 0.5 mg/kg bw. Due to its higher sensitivity, the B1R inhibitor des-Arg9-[Leu8]-bradykinin (Biomol GmbH) was continuously administered via osmotic mini-pumps for a daily dose of 50 nmol/kg bw.

4.6 | Brain histology

The brains were preserved in formalin solution before histological preparation. For this purpose, the animal’s anaesthesia was deepened with 4% isoflurane, the thorax was opened and the right atrium was incised to allow vascular pressure relief. The previously placed catheters were also removed from the femoral artery and vein. Formalin (20 mL of 4%) administered into the left ventricle led directly to the death of the animal. The brain was removed and preserved in 10 mL of 4% formalin solution until further processing. Immunohistochemical imaging of the brain sections was utilized: anti-Ki-67 primary antibody (mouse mAb, clone MIB-5, Dako, Denmark) with Cy3-conjugated secondary antibody (goat anti-mouse, Jackson ImmunoResearch Laboratories) for visualizing cell proliferation. FITC-conjugated anti-α-smooth muscle actin (Sigma-Aldrich) for visualizing smooth muscle layers and Hoechst counterstaining for cell nucleus visualization. A total of three slides per animal were then examined corresponding to the beginning, middle and the end of each section series.

4.7 | Cell lines and cell culture

Human monocytic THP-1 cells were purchased from CLS (Cell Lines Service GmbH) and cultured in RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin solution (Thermo Fisher Scientific). HUVEC were purchased from Sigma and cultured in RPMI-1640 medium supplemented with 10% FBS, 1% endothelial cell growth supplement (Sigma) and 1% penicillin/streptomycin solution at 37°C in a humid atmosphere with 5% CO₂.

4.8 | Cell viability assay

To assess the effect of different concentrations of enalapril stimulants on HUVEC viability, HUVEC were seeded in 96-well plate with 4000 cells/well. After overnight attachment, the cells were treated in 10% serum RPMI-1640 medium containing varying concentrations of enalapril, des-Arg9-[Leu8]-bradykinin (bradykinin receptor 1 inhibitors, B1Ri), and HOE 140 (bradykinin receptor 2 inhibitors, B2Ri).

Cell viability was detected using a Cell Counting Kit-8 (Abcam) according to the manufacturer’s instructions. Briefly, the CCK-8 solution was added to the complete medium after 24 to 96 hours of compound stimulation. Cells were incubated for 2 hours at 37°C in a humid atmosphere with 5% CO₂. The absorbance was measured at 460 nm using a Microplate Reader (Bio-Rad).

4.9 | Wound healing assay

HUVEC were seeded on six-well plates with a density of 5 × 10⁵ per well and grown to 100% confluence. Consistently shaped wounds were made using a sterile 200 μL pipette tip across bottom surfaces of each well, creating cell-free areas. Cultures were gently washed with PBS to remove loose cells. Cells were cultured in 10% FBS medium containing enalapril, enalapril+0.5 mg/mL B1Ri, enalapril+0.5 mg/mL B2Ri or no compounds for 24 hours, fixed in 70% methanol for 30 minutes at room temperature. Cell images were captured under an optical microscope. Four different scraped areas of each well were randomly selected, and scratch wound healing rates were counted using Image J software.

4.10 | Transwell monocyte migration assay

Migration assay was performed using Transwell chambers (8 μm in pore size, Corning). THP-1 were diluted to 3 × 10⁵ cells/mL using FBS free RPMI-1640 medium with enalapril, enalapril+0.5 mg/mL B1Ri, enalapril+0.5 mg/mL B2Ri, enalapril+0.5 mg/mL B1Ri + 0.5 mg/mL B2Ri, or PBS. A total of 600 μL RPMI-1640 media containing 10% FBS were added into the lower chamber and 200 μL cell suspension was added into the upper chamber. The whole Transwell chambers were incubated at 37°C for...
3 hours. Cells in upper chamber were tenderly removed using cotton swabs, whereas lower cells were fixed with 4% paraformaldehyde for 20 minutes and stained with crystal violet for 30 minutes. Micrographs of four fields were randomly obtained under an inverted microscope, and then cells were counted using Image J.

### 4.11 Statistic

Data were tested for Normal Distribution by one-sample Kolmogorov–Smirnov tests with a predefined threshold of \( P \geq 10\%\). Our choice of a 10% threshold for the KS-Test was motivated by the rather low power to detect deviation from a Gaussian distribution in small samples. Accordingly, we decided that even a tendency for systematic deviation should be treated as an indicator for non-normality. For comparing means we utilized ANOVA with the post-hoc Fisher’s Least Significant Difference (LSD) test controlled with respect to the research, authorship and/or publication of this article.

All results are presented as mean and standard deviation (SD). Data were analysed with PASW (SPSS 25) and visualized using Graph Prism 9.

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### CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

### AUTHOR CONTRIBUTIONS

PH, SN, NG and AD contributed to concept, design, acquisition of data, analysis and interpretation of data, drafting the article and revision, approved the version to be published. KL, JJ, PB, MD, ML and ABP performed design, acquisition of data, analysis and interpretation of data, revision of data, approved the version to be published. EB, PB, OR and IB concept, design, analysis, interpretation of data, drafting the article and revision and approved the version to be published.

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