Role of hemodynamic forces in the regulation of cerebral blood flow

Regulation of cerebrovascular resistance by flow-dependent mechanisms: Implication to normal and pathophysiological conditions

Ph.D. thesis

by

Peter Toth M.D.

Advisor:
Akos Koller M.D., Ph.D.

Program leader:
Samuel Komoly M.D., Ph.D.

Department of Pathophysiology and Gerontology

Doctoral School of Clinical Neuroscience

Medical School, University of Pecs, Pecs, Hungary

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ABBREVIATIONS

CBF: cerebral blood flow
TP receptor: thromboxane/prostaglandin endoperoxide receptor
CVR: cerebrovascular resistance
VCI: vascular cognitive impairment
α-SMA-GFP: α smooth muscle actin green fluorescent protein
Ang II: Angiotensin II
MCA: middle cerebral artery
HCA: human intracerebral artery
20-HETE: 20-hydroxy-5,8,11,14-eicosatetraenoic acid
HET0016: N-Hydroxy-Nhydroxytraenoic acidoic aciformamidine
TRPC6: transient receptor potential canonical type channel 6
SKF96365: 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole
BBB: blood-brain barrier
MCP-1: Monocyte chemoattractant protein-1
TNFα: tumor necrosis factor α
IP-10: Interferon gamma-induced protein 10 (also known as C-X-C motif chemokine 10 (CXCL10))
5-NT: 5-nitrotyrosine
Cyp4a12, Cyp4a10, Cyp4a14: cytochrome P 450 4 A family 12, 10, 14

Part I.
Discovering the role of flow-dependent mechanism in the regulation of cerebrovascular resistance. Contribution of hemodynamic forces to autoregulation of cerebral blood flow.

I.1. INTRODUCTION

Regulation of cerebral blood flow (CBF) is of utmost importance to supply the myriad functions of the brain. Many of the mechanisms operating in other organs and tissues are also contributing to the regulation of CBF, however all these mechanisms have to comply with limited space in the closed cranium [1]. Thus maintenance of a relatively constant cerebral blood flow despite of the variations in systemic blood pressure and flow, the so called "autoregulation" of CBF is extremely important and thus, has been always in the center of investigations.

Total cerebral blood flow has to be relatively constant in order to allow a stable and continuous supply of cerebral tissue and maintain intracranial volume and pressure constant. On the basis of Hagen-Poiseuille law it is assumed that CBF is related to the 4th power of vessel radius, thus an increase in the diameter of vessels elicits an *exponential* increase in blood flow. Therefore in the closed cranium, a general vasodilatation would lead to substantial increase in CBF and cerebral blood volume (CBV) and would lead to elevation of intracranial pressure (and vice versa)[2, 3] which would compress the brain and severely limits its function. Thus, tight control of CBF and CBV is essential for the brain. Indeed, in a wide range (from ~ 60 to 140 mmHg) of systemic arterial perfusion pressure CBF increases only slightly in a *linear* manner measured by different in vivo techniques.[4, 5] At this point it has to be noted, that although in mathematical models gain = 1 is used to indicate so called perfect autoregulation, [6, 7] as also depicted in Figure 1, it is likely that such perfect horizontal relationship does not exist in vivo and it would not be even beneficial to provide an appropriate blood supply of brain tissues. Rather, as Rosenblum suggested, it is likely that the slope increases linearly as pressure and flow increases.[4, 5, 8, 9] Nevertheless, the linear and not exponential (!) increase of CBF in the face of increasing blood pressure is achieved by cerebral autoregulation, although the underlying mechanisms have not yet clarified exactly.

Because changes in pressure are accompanied by changes in flow, in vivo responses of cerebral vessels to changes in hemodynamics are most likely a combination of pressure and flow-induced mechanisms. [10-13] Whereas, the role of changes in pressure has been well investigated in the cerebral circulation, the role of changes in flow eliciting vasomotor responses received much less attention. It is important to note however, that in previous in vivo studies of autoregulation of CBF and underlying cerebrovascular responses the effects of pressure and flow could not be separated and the effect of flow on the diameter of vessels was not even considered. [5, 14-24]

I. 1. 2. Intraluminal pressure-induced responses of cerebral vessels

Earlier findings and new interpretations

Until very recently, autoregulation of CBF has been primarily explained by the pressure-induced myogenic response: [25] the inherent property of vascular smooth muscle to dilate to decreases and to constrict to increases in intraluminal pressure. Since its first description by Bayliss, [26] early in the 20th century the myogenic response of different vessels (arterial, venous and lymphatic) has been widely investigated. [27-29] [6, 30] In these in vitro studies investigating the myogenic response only pressure was changed, flow was kept constant. [31-33] Therefore the observed diameter responses were due to changes in pressure alone and were not influenced by changes in flow. As mentioned above we reinvestigated these publications and found that in many of these studies cerebral vessels only maintained a constant diameter between 60-140 mmHg intraluminal pressure.[6, 34-36] This response is referred as the 2nd phase of in vitro arterial myogenic behavior proposed by Osol at al.[35] Interestingly

however, if one extrapolated these findings to in vivo conditions it would not achieve autoregulation of CBF. That is, because in the presence of constant diameter, increasing pressure would result in an increased blood flow velocity thus an increase in CBF. In contrast, as described above, in vivo measurements of CBF did show that CBF remained relatively constant while intraluminal pressure (and flow velocity) increased! These observations and facts prompted us to hypothesize the existence of a flow sensitive mechanism, which augments the gain of autoregulation close to 1 by eliciting additional constriction.

I.1.3. Flow-induced responses of cerebral vessels

As mentioned above, changes in pressure are accompanied by changes in flow,[10-13] and based on theoretical considerations flow-induced mechanisms may play a role in cerebral autoregulation. Interestingly, there have been only few studies investigating flow-induced responses of cerebral vessels, which varied between species, vessel types and methods used.[37-42 43, 44 45-47] Studies using a well-controlled methodological approach found dilation to flow in rat and mice in the vertebro-basilar circulatory area; constriction was found in cat and rat isolated cerebral arteries from the internal carotid circulatory area; biphasic responses were observed in rabbit and rat cerebral arterioles showing pressure and flow-rate dependency (dilated at lower and constricted at higher pressure and flow-rates).

I.2. HYPOTHESIS AND AIMS OF STUDIES

As described above flow-induced responses of cerebral vessels varied between species, vessel types and methods used.[37-42 43, 44 45-47] Importantly, no data are available regarding *human cerebral vessels* preventing the translation of knowledge from vertebrates to humans**.** In theory, flow-induced dilation (acting parallel to increase in pressure) would reduce the magnitude of myogenic constriction of cerebral vessels, which would reduce the gain of autoregulation of CBF, whereas if flow elicited constriction, it could contribute to a more efficient autoregulation of CBF. Importantly, the middle cerebral artery supplies those inner areas in which *arteries* have been shown to contribute substantially to vascular resistance controlling blood flow.

Thus, we hypothesized that increases in flow elicit constriction of isolated middle cerebral arteries of rats and intracerebral arteries of humans.

We aimed to assess the potential contribution of flow-induced response of cerebral arteries to the autoregulation of CBF and in a rat model elucidate the underlying molecular mechanisms.

I.3. MATERIALS AND METHODS

Isolation of human intracerebral arteries and rat middle cerebral arteries

All procedures were approved by the institutional animal care and use commeettes of University of Pecs, Medical School, Pecs, Hungary and New York Medical College, Valhalla NY, USA. Studies of human samples were carried out under the approvement of the Regional Ethic and Review Commeette of the University of Pecs.

Human samples

Human brain samples were provided by Dr. Tamas Doczi (Department of Neurosurgery, University of Pecs, Pecs, Hungary) from discarded tissues of patients undergoing neurosurgical treatment of epileptic disorder or cerebral tumors (n=6, age: 32±10 years).[36] The patients did not have any co-morbidity. Vessels for the study were selected to be removed from normal, non-enhancing areas that had to be removed due to operative technical reasons to be able to approach deep-seated tumors. Brain tissue from the fronto-temporal cortex was placed in 0-4 ºC physiological salt solution (PSS) composed of (in mmol/L) 110.0 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.0 KH_2PO_4, 5.5 glucose, and 24.0 $NaHCO_3$ equilibrated with a gas mixture of 20% O_2 and 5% CO_2, balanced with nitrogen at pH ~7.3.[48] Under an operating microscope, with microsurgical instruments

small human cerebral arteries (HCA, 200-300 μm active diameters) were isolated from cortical brain tissue.

Rat samples

Male Wistar-Kyoto rats (250-350 g) were anesthetized (intraperitoneal pentobarbital sodium) and decapitated. The brains were immediately removed and placed in PSS. Middle cerebral arteries (MCA) were isolated from both sides of brain of each animal (n=61).

Flow-, pressure-, and simultaneous flow and pressure - induced responses of isolated cerebral arteries

After isolation, cerebral arteries were transferred into a custom made pressure-flow chamber. First, changes in diameter of cerebral arteries were obtained to stepwise increases in flow elicited by pressure differences (ΔP; established by changing the inflow and outflow pressure to an equal degree, but opposite direction; ΔP = 5, 10, 20, 30, 40 corresponding to 3 to 320 μL/min intraluminal flow.[49] 2) Next, changes in diameter of cerebral arteries were measured to stepwise increase in intraluminal pressure (0-140 mmHg) in the absence of intraluminal flow by elevating simultaneously the inflow and outflow reservoir to the same level (10 minutes at each pressure step). 3) Then, changes in diameter were measured to stepwise simultaneous increase in pressure and flow. At the end of each experiment the passive diameters were measured at each intraluminal pressure step in the presence of Ca^{2+}-free PSS containing nifedipine 10^{-5} mol/L.

Theoretical calculations

We have estimated the change in CBF (in arbitrary units) by using the Hagen–Poiseuille equation ($Q = r^4\Delta P\pi/L8\ \eta$, where Q = flow, r = radius, ΔP = pressure difference, L = length, η : viscosity). We have also calculated a "gain factor (G)" indicating the strength or efficacy of the autoregulation of blood flow.[6] G = 1 indicates perfect autoregulation, whereas G<1 means inefficient autoregulation, when flow increases as a function of intraluminal pressure.

Administration of vasoactive agents and enzyme inhibitors

Flow-induced diameter changes of HCA and MCA were repeated in the presence of 20-HETE synthesis inhibitor HET 0016, cyclooxygenase inhibitor indomethacin, TXA_2 /PGH_2 receptor (TP) blocker SQ 29,548; free radical scavenger superoxide dismutase-SOD, and catalase-CAT, TXA_2-synthase inhibitor ozagrel. Afterward, 20-HETE was directly administered into the vessel chamber. In a series of experiments in the presence of Δ40 mmHg adenosine was added into the chamber.

Expression of CYP450 4A proteins in cerebral vessels

CYP450 4A protein expression was studied by western blot analysis (anti-cytochrome P450, 1:4000 dilution, #ab22615, Abcam, Cambridge MA).

Detection of Superoxide Level

Flow-induced superoxide production was assessed in MCA of rat by the dihydroethidium fluorescence method (EB).[50]

Statistical analysis

Statistical analysis was performed by two-way ANOVA followed by a Tukey's post hoc test or Student's t-test. P values less than 0.05 ($p<0.05$) were considered to be significant. Data are expressed as either micrometer or % of passive diameter (maximum diameter of a given vessel in Ca^{2+} free solution is taken as 100%) at corresponding intraluminal pressure and are presented as mean ± SEM.

I.4. RESULTS

Flow-induced responses of cerebral arteries and calculations of CBF

In the presence of constant pressure (80 mmHg) increases in flow elicited significant constrictions of vessels (human: from 74±4.9 to 63±5 %, rat: from 63.8±0.8 to 48.8±1.5 % of passive diameter at 80 mmHg, $p<0.05$). Diameter of MCAs incubated in the presence of flow Δ20mmHg increased when flow was decreased to Δ10 mmHg (to 111±1.7 % of diameter at flow Δ20 mmHg),

and decreased when flow was increased to Δ40 mmHg (to 84±1.5 % of diameter at flow Δ20 mmHg). Also, adenosine (10^{-5} mol/L) increased the diameter of MCAs perfused by flow Δ40 mmHg significantly above the baseline diameter (to 148±10 % of diameter at flow Δ20 mmHg).
We found that increases in intraluminal pressure decreased normalized diameter of MCA (from 84±3 to 53±4 %, n=6), whereas simultaneous increase of pressure+flow enhanced the only pressure-induced decrease in diameter (from 83.8±3 to 36±3 %, $p<0.05$). When only pressure was increased eCBF showed a linear increase from 1.4±0.1 to 23.3±7.6 in arbitrary units. In contrast, when pressure+flow increased simultaneously first the eCBF decreased significantly to 0.7±0.1 and then increased only to 5.4±1.4 in arbitrary unit. The gain of autoregulation (G) calculated using diameters induced by pressure alone was 0.8±0.1, whereas G was 0.99±0.1 when pressure+flow were increased simultaneously

Mechanism of flow-induced response of cerebral arteries

Incubation of the vessels with HET0016 (inhibitor of 20-HETE production by blocking Cyp4504A enzymes) abolished the decrease in diameter of both HCA and MCA elicited by increases in flow. Direct administration of CYP 450 metabolite 20-HETE (10^{-7} mol/L) decreased the diameter of MCA similarly to flow (at ΔP=40mmHg, flow:42±3, 20-HETE: 34±9.8 Δμm).
Dilations of MCA in response to ACh were not affected significantly by HET 0016 (before: 53±4.6 % after: 46±5.4 % of maximal dilation). Western blot analysis confirmed that cytochrome P450 4A enzymes are present in the MCA of rat.
Incubation of vessels with SOD/CAT significantly decreased the reduction in diameter of rat cerebral arteries elicited by increases in flow. EB fluorescent images of sections of MCA demonstrated an enhanced EB fluorescence in the vessels exposed to flow compared to control (absence of flow). Enhanced EB fluorescence was reduced to the control level by HET 0016 (10^{-5} m/L) (control: 0.05±0.02, flow: 0.18±0.04, flow+HET 0016: 0.07±0.02 integrated intensity/total area, respectively; $p<0.05$).
Incubation of the vessels with indomethacin or SQ 29,548 inhibited the constriction of MCA to increases in flow, whereas ozagrel did not have an effect.

I.5. DISCUSSION OF FINDINGS

Physiological significance of flow-induced responses of cerebral vessels. Developing a novel concept for the autoregulation of CBF.

The present studies established that increases in flow elicit constrictions in the isolated middle cerebral arteries of rats and isolated cerebral arteries of humans. These findings can have major impact on our understanding of the autoregulation of CBF, because previously only the pressure-induced myogenic response was used to explain autoregulation of CBF, which however, seems to be inefficient on its own (Figure 1).

In the cerebrum, in the internal carotid circulatory system resistance is profoundly determined by larger arteries.[5, 16, 18, 51] In line with this, larger arteries (i.e MCA) constrict to increases in flow, which enhances the pressure-induced tone of cerebral vessels leading to a more efficient autoregulation of CBF, and this way, flow-induced constriction of cerebral arteries plays an important role in regulating cerebral blood volume and intracranial pressure. Therefore, in addition to myogenic response, flow-induced constriction may also participate in the development of segmental resistance of the cerebral circulation, because both large arteries and arterioles respond to changes in flow with either constriction or dilation.[7, 37-39, 43, 46, 52, 53]

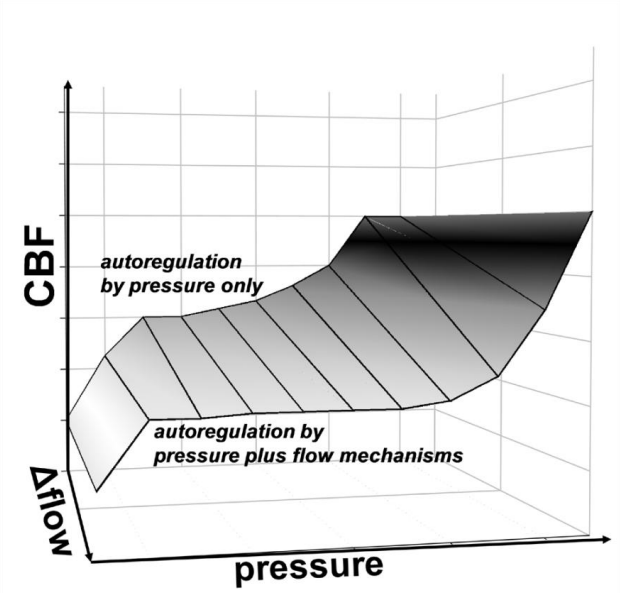

Figure 1. Proposed physiological role of flow-induced constriction of cerebral arteries in autoregulation of cerebral blood flow. *Combined effect of intraluminal pressure and intraluminal flow (Δ flow) achieves effective autoregulation of cerebral blood flow (CBF), while only pressure-induced diameter responses lead to increases in CBF, thus inefficient autoregulation.*

Importantly, the vasomotor tone "set" by the two hemodynamic forces can be modulated or overridden by other factors sensitive to the needs of neural tissues. That is blood flow still can be altered locally (for example due to metabolic factors) during increased demand. Such as, local neural needs can increase cerebral blood flow regionally via neural, glial and other regulatory mechanisms, which can also be propagated to upstream vessels.[54-56] This concept is in line with the suggestion of studies showing that metabolic dilation could overcome the constrictor effect of pressure or flow.[7, 44, 57, 58] Conversely, in the brain stem (vertebro-basilar system) arterioles are the major site of resistance,[18, 59] thus larger arteries, such as the basilar artery "can" dilate to flow participating in reactive hyperemia.[53]

Signaling mechanisms responsible for mediating flow-induced constriction of cerebral arteries

Harder and Gebremedhin at al. and others showed that AA is metabolized by cytochrome P450 ω-hydroxylases (CYP450 4A) into 20-hydroxyeicosatetraenoic acid (20-HETE)[60, 61] and it plays an important role in the regulation of cerebrovascular tone, by mediating agonists- and pressure-induced constrictions of vascular smooth muscle of cerebral vessels.[62, 63] We found that flow-induced constrictions of human cerebral arteries and MCA of rat were abolished by administration of HET 0016, an inhibitor of 20-HETE production. Consistently to these functional findings we found that CYP450 4A enzymes are present in the MCA of the rat, a finding similar to that of Gebremedhin and Dunn at al.[62, 64]

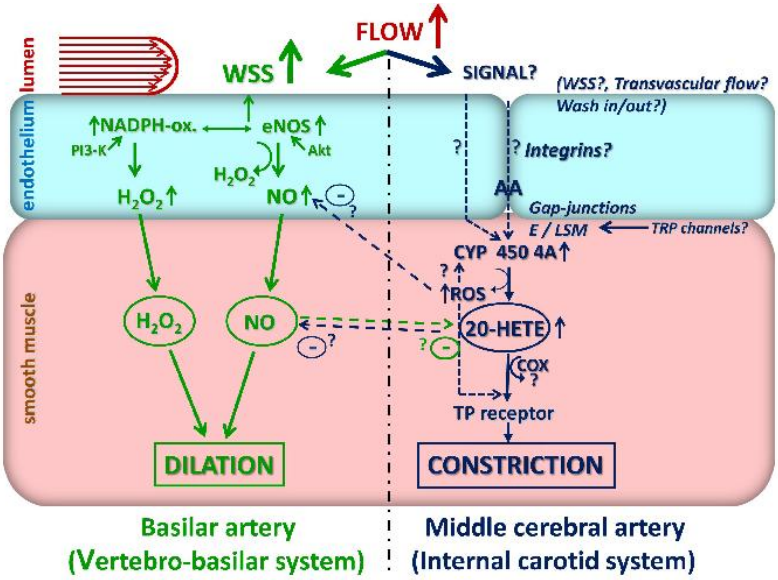

Figure 2. *Flow induces dilation, biphasic responses, or constriction of cerebral vessels depending on the regional and segmental localization of the vessels. We propose that in the internal carotid system larger arteries (such as the middle cerebral artery) constrict to increases in flow. The flow-induced constriction is mediated by 20-hydroxyeicosatetraenoic acid (20-HETE) (a metabolite of arachidonic acid (AA) produced by cytochrome P450 4A enzymes (CYP450 4A) acting via thromboxane A_2/prostaglandin H_2 (TP) receptors and requires COX activity. CYP450 4A also produces reactive oxygen species (ROS), which contribute to the constriction. Whereas, in the brain stem supplied by the vertebro-basilar system larger arteries, such as basilar artery, dilate to flow. Dilation is mediated by NADPH-oxidase (activated by phosphatydilinositol3-kinase (PI3-K) derived H_2O_2 and/or eNOS derived nitric oxide (NO). eNOS is activated in an Akt-dependent pathway.*

It is also known, that production and direct administration of 20-HETE by cytochrome P450 can produce ROS.[44, 65, 66] In the present studies we found that administration of ROS scavengers significantly reduced the flow-induced constriction of cerebral arteries. In addition, our findings showed increased EB fluorescence in MCA after exposing the vessels to flow, suggesting flow-induced increased ROS production. The enhanced production of ROS was reversed by inhibition of 20-HETE production, suggesting ROS is generated during synthesis of 20-HETE, which is elicited by increases in flow. These findings demonstrate that ROS are generated during flow-induced activation of CYP450 4A,[66] but because HET 0016 abolished flow-induced constriction, ROS unlikely have major direct vasomotor effect in this condition.

Our results that both inhibition of 20-HETE production and antagonizing TP receptor abolished flow-induced constriction suggest that 20-HETE acts on TP receptor. Consistently with this hypothesis previous studies[67, 68] by Schwartzman at al. proposed that 20-HETE caused constriction of arteries via TP receptor after 20-HETE was metabolized by COX into 20-endoperoxides (20-OH-PGH_2, 20-OH-PGG_2). This finding is supported by our finding that indomethacin also blocked flow-induced constrictions of MCA. The proposed molecular mechanisms mediating flow-induced constriction of human and rat cerebral arteries are summarized in Fig 2.

I.6. SUMMARY OF NOVEL FINDINGS OF PART I.

1) Increases in flow elicit constrictions in isolated human intracerebral arteries and in rat middle cerebral arteries;
2) simultaneous increases of pressure and flow elicit significantly greater constriction than pressure alone in isolated rat middle cerebral arteries;
3) pressure- and flow-induced constriction together can achieve a more efficient estimated autoregulation of CBF than pressure alone;
4) the underlying subcellular mechanism of flow-induced constriction of cerebral arteries involves increased production of ROS, increased activity of COX and CYP450 enzymes and consequently increased production of 20-HETE, which acts via TP receptors.

Part II.
Dysfunctional pressure- and flow-induced vasomotor mechanisms in hypertension and aging. Pathophysiological effects on the autoregulation of CBF.

II.1. INTRODUCTION

Epidemiological studies provide strong evidence that the deleterious cerebrovascular effects of hypertension are exacerbated in elderly patients, whereas young individuals appear to be more protected from cerebromicrovascular damage induced by hypertension.[69-71] Although the available human data suggest that advanced age and hypertension have synergistic effects, there are virtually no studies addressing the specific age-related mechanisms through which aging increases the vulnerability of the cerebromicrovascular system to hypertension leading to cerebrovascular diseases.[72]

Studies on young animals demonstrate that cerebral resistance arteries exhibit functional and structural adaptation to hypertension leading to increased vascular resistance, which provides and important protection of the distal portion of the cerebral microcirculation from pressure overload[73, 74]. Among these adaptive responses an increased pressure-induced myogenic constriction of cerebral resistance arteries is of great significance.[5, 6, 75] Previous studies demonstrated that in young hypertensive animals increased pressure sensitivity of the myogenic mechanism leads to an increased resistance at the level of small cerebral arteries, keeping pressure in the thin-walled, injury-prone arterioles and capillaries in the normal range with little change in tissue blood supply and oxygenation. As a result of this adaptive response, the range of cerebral blood flow autoregulation is extended to higher pressure values both in hypertensive experimental animals and hypertensive patients.[73, 74, 76] Studies in animal models of hypertension and stroke[77] suggest that *pathological loss of autoregulatory protection contribute to cerebromicrovascular injury.* Despite the paramount importance of the autoregulatory mechanisms in cerebromicrovascular protection, it is not well understood how aging affects the functional adaptation of the cerebral resistance arteries to maintain autoregulation of CBF in hypertension.

II.2. HYPOTHESIS AND AIMS OF STUDIES

The 2nd part of my work was designed to test the **hypothesis** that 1) in hypertension aging impairs functional adaptation of pressure- and flow-induced responses of cerebral vessels, 2) these are leading to impaired autoregulation of CBF, and 3) exacerbates hypertension-induced microvascular damage and neuroinflammation 4) promoting neural/learning dysfunction. I **aimed** to asses in young and aged hypertensive mice: 1) the changes of arterial myogenic and flow-induced constriction and 2) autoregulation of cerebral blood flow, 3) blood-brain barrier function, microvascular density, markers of neuroinflammation 4) and cognitive function.

II.3. MATERIALS AND METHODS

Animals

Young (3 month, n=80) and aged (24 month, n=80) male C57/BL6 mice were used. All procedures were approved by the Institutional Animal Use and Care Committee of the University of Oklahoma health Sciences Center.

Angiotensin II-induced hypertension

Young and aged mice received angiotensin II (Ang II, 1000 ng/min/kg) subcutaneously for 4 weeks. Systolic blood pressure was measured by the tail cuff method.

Behavioral studies

Mice were assessed for learning capacity using an elevated plus maze-based learning protocol.

Cerebrovascular autoregulation

In anesthetized, ventilated mice cortical blood flow was measured as a function of blood pressure between 40–160 mmHg by laser speckle flowmetry.

Assessment of pressure- and flow-induced responses in isolated middle cerebral arteries

In isolated middle cerebral arteries (MCA) myogenic response was assessed and repeated in the presence of the cytochrome P450 ω-hydroxylase inhibitor HET0016 (10^{-6} mol/L) and SKF96365 ($5x\ 10^{-6}$ mol/L, for 15 min), a potent blocker of TRPC channels. In isolated MCAs flow-induced constriction was also assessed.

Quantitative real-time RT-PCR

mRNA expression of *Cyp4a12, Cyp4a10, Cyp4a14* and *Trpc6* was analyzed in MCAs.

Assessment of the integrity of the blood- brain barrier

To quantify blood brain barrier (BBB) permeability we used the sodium fluorescein tracer assay. We also detected extravasated IgG by immunohystochemistry.

Western blotting

Immunoblotting studies for TRPC6 in MCA homogenates and for the tight junction proteins (ZO-1, occludin, and claudin-5) in hippocampal homogenates were performed.

Pericyte coverage

Pericyte coverage was assessed in brain slices of young and aged αSMA-GFP transgenic mice with or without angiotensin II-induced hypertension. Immunolabeling of endothelium was performed by using CD31.

Capillary density analysis

Capillary density (CD31 + capillaries) was quantified in different brain regions.

Neuroinflammation

Microglia activation was quantified in hippocampal sections by immunofluorescent labeling for CD68 and Iba-1. Neuroinflammatory cytokines/chemokines were determined by quantitative real-time PCR and protein levels of selected micoglia-derived pro-inflammatory factors (MCP-1, TNFα, IP-10) by fluorescent bead assay.

Determination of hippocampal protein 5-nitrotyrosine content

Oxidative/nitrosative stress was studied by 5-nitrotyrosine (5-NT; a marker for peroxynitrite action) in homogenates of hippocampi.

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) followed by Tukey post-hoc tests and Pearson's correlation analysis. A p value less than 0.05 was considered statistically significant. Data are expressed as mean±S.E.M..

II.4. RESULTS

Impaired cerebrovascular autoregulation in aged hypertensive mice

In young control mice CBF was independent of blood pressure in the range of 60–120 mmHg, which indicates that autoregulation was present and effective. No differences in

autoregulation were observed among young and aged normotensive mice. In young hypertensive mice there was a progressive expansion of the range of autoregulation, indicating an adaptive response, which was completely absent in aged hypertensive mice.

Aging impairs autoregulatory function of cerebral arteries: role of myogenic and flow-induced constriction

In MCAs of young control mice, increases in intravascular pressure increased myogenic constriction and myogenic tone was maintained at almost the same level up to ~120 mmHg, overlapping the autoregulatory range of CBF. At higher pressures myogenic tone tended to decrease and arteries tended to dilate. In MCAs from young hypertensive mice myogenic tone was maintained at almost the same level at up to ~160 mmHg. MCAs of aged mice developed a slightly decreased myogenic constriction and did not exhibit a similar hypertension-induced adaptive increase in myogenic constriction, which was observed in young mice.

Increases in intraluminal flow elicited vasoconstriction in MCAs of young mice and this response was significantly enhanced by hypertension. In contrast, there was no adaptive increase in flow-induced constriction in MCAs of aged hypertensive mice.

Role of 20-HETE and TRPC6 in functional maladaptation of aged cerebral arteries to hypertension

We found that in MCAs of young hypertensive mice increased myogenic tone was significantly inhibited by both HET0016 and SKF96365 eliminating the difference between the four groups, whereas neither HET0016 nor SKF96365 affect significantly the myogenic tone of MCAs of aged hypertensive mice. Hypertension was associated with up-regulated expression of the CYP 4A arachidonic acid ω-hydroxylases *Cyp4a12*, *Cyp4a10* and *Cyp4a14* and TRPC6 channels) in MCAs of young mice, whereas these adaptive responses were significantly impaired or missing in MCAs of aged hypertensive mice.

Aging exacerbates hypertension-induced BBB disruption

We found that aging exacerbates hypertension-induced fluorescein leakage in the hippocampi, cortex and white matter. Immunostaining for plasma-derived IgG revealed significant perivascular IgG deposits in the hippocampus of aged hypertensive mice. IgG leakage in the hippocampus of young hypertensive mice was significantly reduced and there was no detectable IgG leakage in young control mice.

Aging exacerbates hypertension-induced pericyte loss and microvascular rarefaction

In young mice hypertension resulted in a significant decline in the relative number of pericytes and capillary pericyte coverage (by ~29%). In aged mice hypertension-induced decreases in pericyte number and pericyte coverage (by ~41%) were exacerbated. Relative hypertension-induced decreases in capillary length density in CA1, CA3, and DG of the mouse hippocampus, retrosplenial cortex, primary somatosensory cortex and corpus callosum of aged mice were significantly greater than in young mice.

Aging exacerbates hypertension-induced inflammation and oxidative stress in the hippocampus

We found that aging is associated with a relative increase in the number of activated microglia in the hippocampi. Importantly, hypertension-induced microglia activation was exacerbated in the hippocampi of aged mice. Sustained activation of microglia was associated with an increased expression of several pro-inflammatory cytokines and chemokines in the hippocampi of aged hypertensive mice. These findings were corroborated by demonstration of increased protein expression of MCP-1, TNFα and IP-10, which are known to be secreted by activated microglia, in the hippocampi of aged hypertensive mice.

Aging exacerbated hypertension-induced increases in hippocampal 5-nitrotyrosine content, confirming that the effects of age and hypertension are synergistic.

Aging exacerbates hypertension-induced decline in hippocampal dependent learning

In young control mice, transfer latency on Day 2 was significantly decreased compared to Day 1, indicating an intact learning effect (learning index: 1). The learning indexes for young hypertensive mice (~0.7) and aged (~0.67) mice tended to decrease, compared to young control mice, although the differences did not reach statistical significance. For old hypertensive mice, transfer latency was similar on Days 1 and 2 (corresponding to a learning index: ~0).

II.5. DISCUSSION OF FINDINGS

In the cerebral circulation myogenic constriction of proximal branches of the cerebrovascular tree (i.e. MCA) is uniquely important for protection of the distal cerebral microcirculation.[16] In healthy young animals pressure-induced myogenic constriction of the cerebral arteries acts as a critical homeostatic mechanism that assures that increased arterial pressure does not penetrate the distal portion of the microcirculation, causing damage to the thin-walled arteriolar and capillary microvessels in the brain.[5, 75] In hypertensive young mice and rats[6, 78] the myogenic constriction of cerebral arteries is enhanced and the range of cerebrovascular autoregulation is extended, which represent functional adaptation of these vessels to higher systemic blood pressure, protecting the cerebral microcirculation.[5, 6, 73-75] We have found perhaps for the first time that cerebral arteries of aged mice do not exhibit a hypertension-induced adaptive increase in myogenic constriction observed in young mice.

As we have shown (Part I of the thesis) in addition to the myogenic response flow-induced constriction of cerebral arteries may also contribute to cerebrovascular autoregulatory function.[7, 79] We have demonstrated that in young hypertensive mice flow-induced arterial constriction is also enhanced, representing another component of functional arterial adaptation to high blood pressure. This adaptive response is also impaired in aged hypertensive mice. Taken together, hypertension in aging is associated with dysfunction of cerebrovascular autoregulatory mechanisms protecting the brain.

Our findings support the view that in young animals activation of a 20-HETE/TRPC6-dependent pathway underlies functional adaptation of cerebral arteries to hypertension and that this adaptive response is dysfunctional in aging. First, in young hypertensive mice 20-HETE mediation of myogenic constriction is up-regulated in the high pressure range, likely due to adaptive up-regulation of cytochrome P450 4A ω-hydroxylases. We have found that this, 20-HETE-dependent adaptive response is impaired in aged hypertensive mice. Previous studies demonstrate that activation of TRPC6 channels mediates 20-HETE-induced smooth muscle constriction[80] and contributes to myogenic constriction of cerebral arteries[81] and while in cerebral arteries of young mice hypertension up-regulates vascular TRPC6 expression and activity, this adaptive response is impaired in aged hypertensive mice. Because flow-induced constriction of cerebral arteries is predominantly mediated by 20-HETE,[7, 62] dysregulation of this pathway in aged hypertensive mice simultaneously impairs both the myogenic and the flow-induced components of cerebrovascular autoregulation.

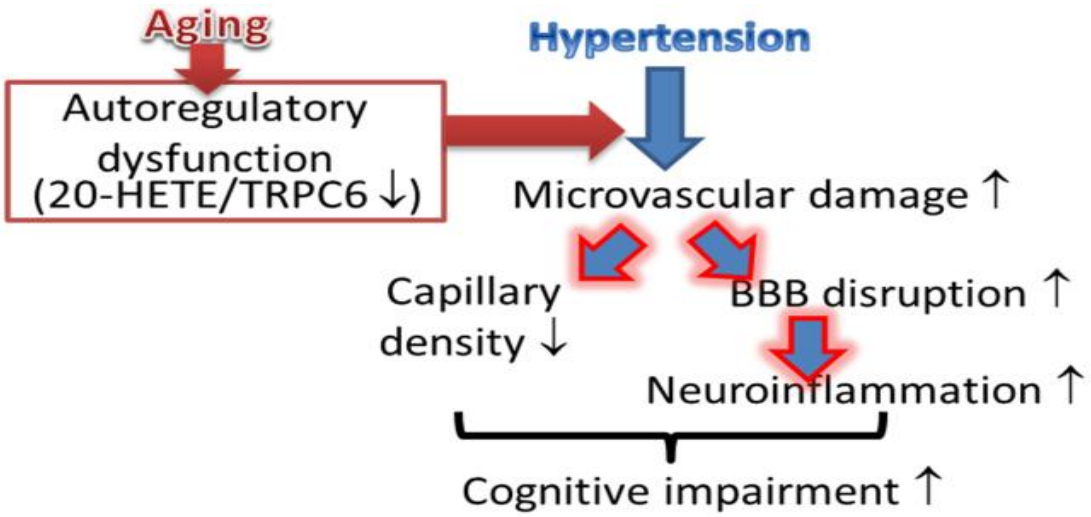

Figure 3. *Proposed scheme depicting the mechanisms by which age-related cerebrovascular autoregulatory dysfunction (due to lack of up-regulation of 20-HETE/TRPC6 pathway) exacerbates hypertension-induced microvascular damage, blood-brain barrier (BBB) disruption and neuroinflammation leading to learning/cognitive impairment.*

We found evidence that autoregulatory dysfunction in aged hypertensive mice leads to significant cerebromicrovascular damage. Lack of autoregulatory protection likely allows high blood pressure to penetrate the distal portion of the cerebral microcirculation, which leads to significant BBB disruption in the hippocampus and other brain regions in aged hypertensive mice. Pericytes are important cellular constituents of the BBB and they are sensitive to oxidative stress. Thus, it is likely that increased hypertension-induced loss of pericyte coverage contributes to BBB disruption in aged mice. Pericytes are sensitive to oxidative damage, thus it is possible that exacerbated hypertension-induced oxidative stress contributes to the increased pericyte loss in aged mice. Pericytes is also likely to contribute to microvascular rarefaction in brain of aged hypertensive mice. Increased BBB disruption in aged hypertensive mice is likely to impair neuronal function by multiple mechanisms involving the induction of neuroinflammation. We have found evidence that in aged hypertensive mice BBB disruption results in increased extravasation of IgG and an exacerbated neuroinflammatory response as shown by the increased number of activated microglia in the hippocampi. We also found that in the hippocampi of aged hypertensive mice there is an increased presence and expression of inflammatory mediators secreted by activated microglia. Microglia-derived pro-inflammatory cytokines, chemokines and proteases (i.e. MMPs) are thought to play a role in neuronal dysfunction and neurodegeneration in various pathophysiological conditions,[82] suggesting that exacerbation of neuroinflammation may importantly contribute to hypertension-induced neuronal dysfunction in aged mice. Activated microglia are also known to exhibit increased production of free radicals, thereby causing oxidative neuronal injury.[83] Our findings that aging exacerbates hypertension-induced oxidative/nitrosative stress in the hippocampus are consistent with this view.

To determine whether compromised BBB integrity, microvascular rarefaction, oxidative/nitrosative stress and chronic low-grade neuroinflammation were sufficient to induce neuronal dysfunction in aged hypertensive mice, we studied behavior. Importantly, hypertensive aged mice had the worst performance on behavioral tests of hippocampal function.

II.6. SUMMARY OF NOVEL FINDINGS OF PART II.

The novel findings of these studies are:
1) in cerebral arteries of young hypertensive mice there is an enhanced myogenic- and flow-induced constriction of cerebral arteries,
2) the up-regulation of 20-HETE/TRPC6 pathway is responsible for this adaptation.
3) By adapting to hypertensive condition cerebral arteries of young mice are able to and maintain an enhanced autoregulation of CBF,
4) in cerebral arteries of aged hypertensive mice there is an impaired myogenic- and flow-induced constriction of cerebral arteries,
5) the autoregulatory adaptation is lost in aged hypertensive animals,
6) in aged hypertensive mice there is exacerbated BBB disruption,
7) in aged hypertensive mice there is capillary rarefaction in cerebral tissue,
8) in aged hypertensive mice there is increased neuroinflammation, which likely
9) contributes to learning decline.

III. PEER-REVIEWED PUBLICATIONS OF THE AUTHOR (IF: 41.336)

The thesis is based on the following publications:

1. **Toth P**, Rozsa B, Springo Z, Doczi T, Koller A. Isolated human and rat cerebral arteries constrict to increases in flow. Role of 20-HETE and TXA2 receptors. J Cereb Blood Flow Metab. (10):2096-105. 2011 ***(IF:5.008)***
2. Koller A. and **Toth P**. Contribution of flow-dependent vasomotor mechanism to the autoregulation of cerebral blood flow. J Vasc Res. 49(5):375-89. 2012 ***(IF:2.65)***
3. **Toth P**, Tucsek Z, Sosnowska D, Gautam T, Mitschelen M, Tarantini S, Deak F, Koller A, Sonntag WE, Csiszar A, Ungvari Z. Age-related autoregulatory dysfunction and exacerbation of microvascular injury in mice. 2013 submitted

Other publications:

4. **Toth P**, Koller A, Pusch G, Bosnyak E, Szapary L, Komoly S, Marko L, Nagy J, Wittmann I. Microalbuminuria, indicated by total versus immunoreactive urinary albumins in acute ischemic stroke patients. J Stroke Cerebrovasc Dis. 20(6):510-516. 2011 ***(IF: 1.680)***
5. **Toth P**, Csiszar A, Sosnowska D, Tucsek Z, Cseplo P, Springo Z, Tarantini S, Sonntag WE, Ungvari Z, Koller A. Treatment with the cytochrome P450 ω-hydroxylase inhibitor HET0016 attenuates cerebrovascular inflammation, oxidative stress and improves vasomotor function in spontaneously hypertensive rats. Br J Pharmacol. 2012 *in press* ***(IF:4.409)***
6. Bailey-Downs LC, Mitschelen M, Sosnowska D, **Toth P**, Pinto JT, Ballabh P, Valcarcel-Ares MN, Farley J, Koller A, Henthorn JC, Bass C, Sonntag WE, Ungvari Z, Csiszar A. Liver-specific knockdown of IGF-1 decreases vascular oxidative stress resistance by impairing the Nrf2-dependent antioxidant response: A novel model of vascular aging. J Gerontol A Biol Sci Med Sci. 67(4):313-29. 2012 ***(IF:4.598)***
7. Bailey-Downs LC, Sosnowska D, **Toth P**, Mitschelen M, Gautam T, Henthorn J, Ballabh P, Koller A, Farley J, Sonntag WE, Csiszar A, Ungvari Z. Growth hormone and IGF-1 deficiency exacerbates high fat diet-induced endothelial impairment in obese Lewis dwarf rats: implications for vascular aging. J Gerontol A Biol Sci Med Sci. 67(6):553-64. 2012 ***(IF:4.598)***
8. Csiszar A, Sosnowska D, Tucsek Z, Gautam T, **Toth P**, Losonczy G, Colman RJ, Weindruch R, Anderson RM, Sonntag WE, Ungvari Z. Circulating factors induced by caloric restriction in the nonhuman primate Macaca Mulatta activate angiogenic processes in endothelial cells. J Gerontol A Biol Sci Med Sci. 2012 Aug 17. *In press* ***(IF: 4.598)***
9. Tucsek Z, Gautam T, Sonntag WE, **Toth P**, Saito H, Salomao R, Szabo Cs, Csiszar A, Ungvari Z. Aging exacerbates microvascular endothelial damage induced by circulating factors present in the serum of septic patients. J Gerontol A Biol Sci Med Sci. 2012 *in press* ***(IF:4.598)***
10. Bailey-Downs LC, Tucsek Z, **Toth P**, Sosnowska D, Gautam T, Sonntag WE, Csiszar A, Ungvari Z. Aging exacerbates obesity-induced oxidative stress and inflammation in perivascular adipose tissue in mice: a paracrine mechanism contributing to vascular redox dysregulation and inflammation. J. Gerontol A Biol Sci Med Sci. 2012 *in press* ***(IF:4.598)***
11. Ungvari Z, Tucsek Z, Sosnowska D, **Toth P**, Gautam T, Podlutsky A, Csiszar A, Losonczy G, Valcarcel-Ares NM, Sonntag WE, Csiszar A. Aging-induced dysregulation of Dicer1-dependent microRNA expression impairs angiogenic capacity of rat cerebromicrovascular endothelial cells. J. Gerontol A Biol Sci Med Sci. 2012 *in press* ***(IF:4.598)***

Abstracts in peer-reviewed journals

1. Degrell P, Molnar L, Pollak E, **Toth P**, Molnar GA, Nagy J, Wittmann I. Ultrastructural localization and pathophysiological importance of the IgA deposits of the mesangial matrix in IgA nephropathy. Act Phys Hun 93(2-3): 165-166. 2006.

2. Degrell P, Kellermayer M, Berta G, **Toth P**, Fincsur A, Gyomorei Cs, Molnar GA, Nagy J, Wittmann Exact localization of fibrinogen-fibrin using laser scanning confocal microscopy in various renal disease and its importance. Act Phys Hun 94(4): 341. 2007.
3. Toth P, Halmai R, Marko L, Bosnyak E, Toth M, Bagoly E, Szapary L, Wittmann I, Nagy J, Koller A, Komoly S. Manifestation of systemic vascular disease: microalbuminuria and renal dysfunction in acute stroke. J Vasc Res. 45 (suppl 2) 119; 2008.
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