SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Studying brain aging processes by neurovascular models and characterization of the cardiovascular effects of Omecamtiv mecarbil

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LAKI KALMAN DOCTORAL SCHOOL

DEBRECEN, 2023.

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The Examination takes place at the library of the Department of Cardiology and Cardiac Surgery, Faculty of Medicine, University of Debrecen, 12. 12. 2023., 11:00 a.m.

Head of the **Defense Committee**:	Prof. Dr. Pál Soltész, PhD, DSc
Reviewers:	Dr. Márta Sárközy, PhD
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Members of the Defense Committee:	Prof. Dr. Attila Cziráki, PhD
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The PhD Defense takes place at the Lecture Hall of Department of Obstetrics and Gynecology, Faculty of Medicine, University of Debrecen, 12. 12. 2023., 13:00

Introduction

Parallel to the development of medical science, it became possible to extend the human life span. Before the 1950s, most of the gains were made by reducing youth mortality, but in the second half of the 20th century this trend changed, and the increase in survival rates after the age of 65 helped progress (Oeppen & Vaupel, 2002). However, with increasing age, the prevalence of many diseases, including cerebrovascular and cardiovascular diseases, also increases, which poses new challenges to medicine (Arvanitakis & Bennett, 2019; Groenewegen et al., 2020).

Among the cerebrovascular diseases, dementia is one of the most common, which leads to a deterioration in the quality of life, and despite the fact that it affects millions worldwide, our treatment options are extremely limited. In order to make progress in the treatment of dementia, it is essential to understand the pathophysiology of the disease.

Numerous studies have been published on the possible causes, but the role of damage to the cerebral vasculature stands out among them. It is known that oxidative and nitrative stress, mitochondrial dysfunction, and damage to the antioxidant mechanisms affecting the cerebral vascular network can lead to vascular inflammation, thereby impairing vascular function, which can be linked to the pathomechanism of dementia (Cui et al., 2012; Pizzino et al., 2017; Tarantini et al., 2019; Ungvari et al., 2007). However, the pathological role of damage to vascular remodeling and age-related pathological changes in the cerebral venous system is less well known.

In addition to cerebrovascular diseases, the prevalence of cardiovascular diseases also increases with age, among which heart failure stands out, which according to 2020 data affected more than 60 million people worldwide. Despite the fact that many pharmacological and instrumental therapies have appeared to treat the disease in recent decades, the 10-year survival from diagnosis is still below 40% (Groenewegen et al., 2020).

In recent years, one of the main focuses of pharmacological therapy has turned towards the development of positive inotropic agents. Although traditional positive inotropic agents, such as dobutamine or milrinone, are still considered the cornerstone of acute heart failure care, many of their side effects are known. That is why active research has been started on the development of other drugs with positive inotropic effects, of which the Ca^{2+}-sensitizing Levosimendan and the direct myosin activator Omecamtiv Mecarbil (OM) should be highlighted (Teerlink, 2009).

Levosimendan is a so-called a positive inotropic agent acting through a "central" mechanism, which can increase the affinity of the troponin C (TnC) protein for Ca^{2+}, and by activating ATP-sensitive potassium channels, results in vasodilation and a consequent drop in blood pressure. Although it is a very effective drug, it is only available in an intravenous version and is not widely used due to its hypotonic effect and effect on serum potassium levels (Parissis et al., 2009).

The OM, on the other hand, can also be used per so-called positive inotrope acting through a "downstream" mechanism. In terms of its effect, it is a myosin ATPase, i.e. it accelerates the hydrolysis of ATP and thereby increases the number of strong actin-myosin cross-links, which results in a slower but more powerful cardiac muscle contraction in its kinetics (Teerlink, 2009). However, in parallel with the clinical trials conducted with OM, several publications were published that discussed the potential negative effects of the drug (Bakkehaug et al., 2015; Nagy et al., 2015; Nanasi et al., 2017). Based on these, it is crucial to understand the exact functioning of the applied therapies, which can improve their effectiveness and eliminate their harmful effects.

Aims

Although many studies have revealed the possible causes of dementia and the potential pathomechanisms of vascular cognitive impairment, a definitive therapy is still not available. At the same time, we have many options for drug therapy of heart failure, but despite the developments, the patient's long-term life prospects are still poor. In both cases, a more precise knowledge of the pathomechanism and the mechanism of action of the drugs is required. Accordingly, our goals were the following:

- We investigated the effect of high blood pressure on the remodeling of cerebral blood vessels in IGF-1 deficient animals as a model of aging.

- We studied the effect of increased cerebral venous pressure on neurological and cognitive function, as well as neurovascular coupling in an animal model.

- We tested the in vivo and in vitro effects of Omecamtiv Mecarbil in animal models.

Materials and methods

The role of IGF-1 in vascular remodeling

The animal model

For the experiment, we used male mice homozygous for the floxed 4th exon of the Igf1 gene (Igf1f/f; C57BL/6 background), in which circulating IGF-1 deficiency was induced by adeno-associated virus (AAV8) mediated expression of Cre recombinase in the liver at the age of 4 months. The control group consisted of C57BL/6 mice. High blood pressure was induced with subcutaneously placed osmotic minipumps containing angiotensin-II. A pump containing a saline solution was inserted into the normotensive group. All procedures were approved and followed by the University of Oklahoma HSC Institutional Animal Care and Use Committee.

Determination of the structural and mechanical characteristics of arteries

The dissected middle cerebral arteries (MCA) of the animals were attached to two glass micropipettes in an organ chamber, in oxygenated Ca^{2+}-free Krebs buffer, and the inflow and outflow pressures were controlled. The internal diameter and wall thickness were recorded after a stepwise increase in intraluminal pressure (between 5 and 160 mm Hg) using a video microscope system. VSMCs within the walls of MCAs were loaded with the fluorescent dye calcein AM to assess media thickness and VSMC hypertrophy. By measuring the above-mentioned morphometric data, we were able to calculate the circumferential strain, circumferential stress and Young's modulus of elasticity. Vascular remodeling was calculated by measuring the passive, pressure-dependent internal diameter change of MCAs, while VSMC hypertrophy was obtained by measuring the size of VSMCs.

Confocal/multiphoton microscopic imaging of arteries

Elastin content and cell number were determined using a confocal microscope. Cannulated arteries were fixed in 4% paraformaldehyde while pressurized at 70 mmHg for 1 hour. After permeabilization with Triton X-100, the preparations were incubated in 4′,6-diamidino-2-phenylindole (DAPI), Alexa Fluor 633 Hydrazide and Alexa Fluor 546 Phalloidin. Vessels were imaged with a Leica SP5 confocal/multiphoton microscope, images were processed, and the total volume occupied by VSMC nuclei, elastin, and actin was determined.

Determination of expression changes of genes related to vascular remodeling

Using the qPCR technique, we measured the mRNA expression of elastin, elastolytic and collagenolytic matrix metalloproteinases, genes encoding factors determining the structure of the elastin complex, genes responsible for extracellular matrix crosslinks, and genes responsible for coding growth factors regulating vascular remodeling.

Statistics

Differences between groups were determined by one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was significant. Data are expressed as mean ± SEM. Statistical analyzes were performed with Prism 5 software (GraphPad).

<u>Role of cerebral venous stasis and neuroinflammation in age-related vascular pathologies</u>

The animal model

We used 10-month-old adult male C57BL/6 mice for our experiments. To induce cerebral venous stasis in mice, bilateral jugular vein ligation was performed. The control group underwent a "sham" operation. Hypertension was induced as discussed above. All procedures were approved and followed by the University of Oklahoma HSC Institutional Animal Care and Use Committee.

Assessment of neurological function, behavior and cognitive function of animals

The neurological examination consisted of the assessment of spontaneous activity, limb symmetry, forelimb extension ability, climbing ability, body proprioception, whisker touch response, and gait coordination. In addition, we tested spatial and long-term memory in an 8-arm water maze, motor skill learning using a Rotarod device, and muscle strength of the front limbs using a grip meter.

Measurement of neurovascular coupling responses

To assess functional hyperemia (neurovascular coupling), anesthetized mice were immobilized and placed on a stereotaxic frame, the scalp and periosteum were pulled aside, and the skull was carefully thinned with a dental bur while cooled with liquid buffer. Whisker hair stimulation was used to determine functional hyperemia, and regional blood flow differences over the somatosensory cortex were recorded using laser-speckle contrast imaging equipment.

Measurement of blood-brain barrier damage and microglial activation, as well as proinflammatory gene expression in the hippocampus

We used immunofluorescent labeling and confocal microscopy to detect damage to the blood-brain barrier and to measure the activation of microglia. Damage to the blood-brain barrier was detected by identifying extravasated IgG, and

microglial activation was detected by labeling Iba1. The proinflammatory gene expression was investigated using the real-time qPCR technique.

Statistics

Differences between groups were determined by one-way ANOVA followed by Tukey's post hoc test. $p < 0.05$ was significant. Data are expressed as mean ± SEM. Statistical analyzes were performed with Prism 5 software (GraphPad).

In vivo and in vitro effects of Omecamtiv Mecarbil in an animal model

The animal model

The in vivo tests were performed on 13-15-week-old male Wistar-Kyoto rats weighing 300-330 g. Myocytes were isolated from mongrel dogs.

Hemodynamic measurements

Pentobarbital sodium anesthetized, intubated, ventilated rats were administered OM via the jugular vein. For the measurements, a 2-Fr pressure microcatheter was inserted into the right carotid artery and then lowered into the ascending aorta. The catheter was then advanced into the LV under pressure control. First, steady-state P-V relationships were recorded, and after 10 minutes of stabilization, the signals were continuously recorded at a sampling rate of 1000 samples/s. LV P-V relationships were determined at different preloads by transient occlusion of the inferior vena cava, in order to obtain load-independent parameters of LV systolic and diastolic function. After baseline parameters were recorded, DMSO (solvent) and three consecutive doses of OM (200, 400, and 600 μg/kg bw) were injected as intravenous boluses, resulting in a maximum cumulative dose of 1200 μg/kg bw.

Measurement of blood pressure

OM was administered to anesthetized rats via the jugular vein in increasing doses (200, 400 and 600 µg/kg body weight) and blood pressure was recorded invasively by cannulation of the right carotid artery. The first measurements were performed with the solvent, DMSO.

Echocardiography

After intoxication with ketamine and xylazine, the rats were placed on a heating pad, where the left jugular vein was cannulated so that increasing doses of OM boluses (200, 400 and 600 µg/kg body weight) could be administered. Afterwards, 2D and Doppler examinations were performed to determine diameters and systolic and diastolic function.

Mechanical measurements on permeabilized myocytes

For the measurements, cryopreserved (-80°C) human left ventricular free wall samples (from non-implanted donor hearts) were mechanically broken in isolation solution and then permeabilized using Triton X-100. Myocyte-sized preparations were fixed with silicone glue between a high-speed length gauge and a force gauge in ISO at 15 °C. The Ca^{2+}-activated force generation of cardiac muscle cells was induced by transferring the preparation from a relaxing solution to an activating solution. The pCa value of the relaxing solution was 9.0 (1 nM free Ca^{2+}), while the pCa value of the maximum activating solution was 4.75 (18 µM free Ca^{2+}). The maximum Ca^{2+}-activated force (Fmax) was determined at pCa 4.75. After each Ca^{2+}-dependent active force measurement, we measured the Ca^{2+}-independent passive force (Fpassive), which corresponds to cell relaxation of cardiomyocytes, by placing the preparation in the relaxing solution and reducing the cell length to 80% of normal for 8 seconds. During the force

measurements, LV samples of 4 different hearts (5-6 cardiac muscle cells per heart) were used. The effect of OM on the contraction of Triton-X-100 permeabilized human myocardium was tested in the presence of low (0.1 μM) and high (1 μM, 401 ng/ml) concentrations of OM.

Isolation of canine ventricular cardiomyocytes, then action potential, cell length and Ca^{2+}-transient measurements

Adult dogs of both sexes were anesthetized by intramuscular injection of 10 mg/kg ketamine hydrochloride and 1 mg/kg xylazine hydrochloride. Hearts were rapidly removed and placed in Tyrode's solution, and isolated cardiomyocytes were prepared by enzymatic dispersion. To record the action potential, viable ventricular cardiomyocytes with clear striations were placed in a 1 ml experimental chamber mounted on the stage of an inverted microscope. After sedimentation, the cardiac muscle cells were continuously superfused with 37 °C Tyrode solution at a rate of 1-2 ml/min. Cells were pierced with conventional borosilicate microelectrodes filled with 3 M KCl, with a peak resistance of 20–40 MΩ and connected to the input of a Multiclamp 700A amplifier. Action potentials (APs) were evoked through these intracellular electrodes using 2-ms-wide rectangular current pulses with an amplitude twice the diastolic threshold. APs were recorded at stimulation frequencies of 1 Hz, 2 Hz, 3.33 Hz, 4 Hz, and 5 Hz, in that order, in the absence and presence of 1 μM OM. Cell length (CL) was measured with a video-based edge-detector system simultaneously with AP recordings. For photometric detection of intracellular Ca^{2+} transients, cardiomyocytes were loaded with 5 μM Fura-2 AM in Tyrode's solution containing Pluronic F-127. Fluorescence was measured using an alternating, two-beam excitation fluorescence photometric device with an inverted microscope. The fluorescence signals of the Ca^{2+}-bound and Ca^{2+}-free Fura-2 dye were detected at an excitation wavelength of 340 nm (F340) and 380 nm (F380). The

emitted photons were detected at 510 nm with an R1527P photon multiplier tube. The F340/F380 fluorescence ratio was used to evaluate the intracellular Ca^{2+} transient (CaT).

Statistics

The results were expressed as mean ± SEM. Normally distributed data were first assessed using the Shapiro-Wilk test. In the case of a normal distribution, the series of data containing several groups were evaluated with analysis of variance (ANOVA) (ordinary in the absence of pairing, Greenhouse-Geisser correction in the case of repeated measurements), and multiple comparisons were evaluated with the Holm-Sidak test; while the data sets containing the two groups were evaluated with an ordinary, parametric T-test.

Non-parametric tests were used in case of deviation from the normal distribution or low numbers of observations ($n<6$). In these cases, we used the Friedman test for repeated measurements and the Kruskal-Wallis test for independent measurements. Dunn's test was used for multiple comparisons. The Wilcoxon paired signed rank test (repeated measurements) or the Kolgomorov-Smirnov test (unpaired values) were used to evaluate two groups. $p < 0.05$ was the criterion for significance.

Results

The role of IGF-1 in vascular remodeling

IGF-1 deficiency alters arterial morphology, vascular mechanics, and impairs structural adaptation of MCAs to hypertension

IGF-1 deficiency led to a significant decrease in wall thickness in normotensive mice. In addition, the structural adaptation of the MCAs to hypertension in control animals is clearly visible, which was manifested in a significant increase in wall thickness, a reduced internal diameter and a consequent increase in the wall/lumen ratio. Measurements on calcein-stained vascular smooth muscle cells (VSMC) showed that the increase in wall thickness was at least partially caused by medial VSMC hypertrophy. In the MCAs of hypertensive control mice, the stress-strain curve was shifted to the left compared to the curve obtained in the MCAs of normotensive control mice, indicating the stiffness of the cerebral arteries. IGF-1 deficiency itself was associated with a leftward shift of the stress-strain relationship of MCAs and increased circumferential strain. Infusion of Ang-II in IGF-1-deficient mice resulted in a further leftward shift of the stress-strain curve of MCAs, indicating the presence of increased vessel stiffness. We used confocal and multiphoton microscopy to further assess the vessel wall composition of MCAs. Based on these experiments, we were able to determine that the tendency of the elastin content in the walls of MCAs deficient in IGF-1 to decrease, which became more prominent after the induction of high blood pressure.

IGF-1 deficiency impairs hypertension-induced adaptive changes in the ECM-associated vascular gene expression profile

We found that in MCAs, IGF-1 deficiency inhibited the high blood pressure-induced changes in Eln mRNA expression, the assembly and remodeling of elastin fibers, the factors that regulate ECM cross-linking, the factors that regulate

vascular remodeling processes, and the expression of collagens, while had a significant effect on MMP expression. Among the factors involved in vascular redox regulation, we found that the lack of IGF-1 negatively affected the expression of the antioxidant transcription factor Nrf2 (Nfe2/2).

Role of cerebral venous stasis and neuroinflammation in age-related vascular pathologies

Jugular vein ligation results in impaired cognitive function in mice but does not affect neurovascular switching responses

During tests performed in the 8-arm water maze, we found that the combined error rate of the sham-operated mice was lower than that of the JVL-operated mice. To analyze working memory (short-term memory involved in immediate conscious perception), we examined re-entries to the incorrect arms (without the hidden platform). We found that working memory function was impaired in JVL-operated mice compared to sham-operated control mice. Analysis of non-cognitive parameters showed no differences in swimming speed and non-exploratory behavior (the total time mice spent not searching for the platform, such as hovering) between the two groups. Taken together, the aforementioned results suggest that cerebral venous stasis associated with JVL in mice impairs performance in the 8-arm water maze, which may be due to a decline in hippocampal spatial learning and memory, rather than changes in motor or motivational processes. Parallel to all this, the neurovascular switching responses were not affected by the bilateral JVL operation.

Effect of jugular vein ligation on neurological parameters, muscle strength and motor learning function

We found that JVL resulted in a significant decrease in the neurological score compared to the control group, indicating a clear neurological deficit. The muscle strength of the JVL-operated mice tended to decrease compared to that of the control animals. The learning of motor skills was assessed with a modified rotarod test. In terms of balance and endurance, there was no significant difference between the two groups.

Jugular vein ligation exacerbates hypertension-induced BBB damage in the hippocampus

IgG from extravasated plasma was used as a marker of increased hippocampal cerebrovascular permeability. Immunostaining of plasma-derived IgG revealed significant IgG deposits in the hippocampus of JVL-operated mice, which was further aggravated by hypertension. In the hippocampus of hypertensive sham-operated mice, the level of IgG release was significantly lower, while no IgG release was detectable in normotensive sham-operated mice.

<u>In vivo and in vitro effects of Omecamtiv Mecarbil in an animal model</u>

Omecamtiv mecarbil affects contraction, relaxation, Ca^{2+} sensitivity and stiffness of human cardiomyocytes permeabilized with Triton-X-100 in vitro

The calcium-dependent active force was significantly increased even at very low Ca^{2+} levels in the presence of 1 μM OM. For example, the force value in relaxing solution (pCa 9) was 1.1 ± 0.2 kN/m2 compared to 0 kN/m2 in the absence of OM. And the Ca^{2+}-sensitivity (pCa50) of myocyte force generation increased from 5.86 ± 0.02 to 6.42 ± 0.06 in the presence of 1 μM OM. The same dose of OM

increased Ca^{2+}-activated force production (Factive) several times at low Ca^{2+} concentration (at pCa 6.2, Factive from 1.7 ± 0.3 to 8.6 ± 1.9 kN/m2 grew). The rate of Ca^{2+}-dependent force generation (ktr) at 1 μM OM concentration decreased independently of Ca^{2+} concentration (from 0.97 ± 0.06 to 0.04 ± 0.01 1/s and from 0.28 ± 0.01 to $0{,}107 \pm 0{,}04$) at high and submaximal Ca^{2+} levels. In addition, the time required for half-maximal contraction and the kinetics of relaxation (trelax) were also slowed down at submaximal and high Ca^{2+} levels in the presence of 1 μM OM. Finally, the Ca^{2+}-independent (passive) stiffness (Fpassive) increased from 0.88±0.10 to 3.88±0.44 kN/m2 and from 1.03±0.12 to 3.25±0,4. It increased to 4 at submaximal and high Ca^{2+} levels in the presence of 1 μM OM.

Omecamtiv mecarbil induces positive inotropy in rats in vivo

Cumulative intravenous administration of OM at 200, 400, and 600 μg/kg body weight (BW) (with a maximum dose of 1200 μg/kg BW) improved cardiac systolic function as demonstrated by echocardiography. The ejection fraction (EF) increased from 73.3 ± 2.2 to 87.4 ± 3.6%, parallel to which the systolic ejection time (SET) increased from 75.2 ± 2.5 to $117.4 \pm 6{,}1$ ms, indicating the slowing down of the contraction kinetics. The improvement of the systolic function was also demonstrated by the LV P–V measurements, under the same conditions. The load-independent contractility indices also showed a clear improvement in systolic performance: both end-systolic P–V relationship (ESPVRq) and preload-recruitable stroke work (PRSW) increased.

Omecamtiv mecarbil causes diastolic dysfunction in rats in vivo

OM treatment was also associated with signs of diastolic dysfunction on echocardiography: the E:A ratio measured at the level of the mitral valve decreased from 2.02 ± 0.08 to 1.45 ± 0.03; Isovolumetric relaxation time (IVRT)

from 25.6 ± 1.6 ms to 52.7 ± 2.7 ms, and left atrial (LA) internal area from 29.4 ± 1.7 to 48.3 ± 2.00 mm^2 at the maximum applied OM concentration. Signs suggestive of diastolic dysfunction were also present with P–V analysis. The isovolumetric relaxation constant (Tau_w) increased from 9.2 ± 0.4 to 15.2 ± 0.7 ms, and the maximal rate of decrease in diastolic pressure decreased (dP/dt_{min}) from - 11,642 ± 0.4 mmHg/s to -8096 ± 614 mmHg/s.

Omecamtiv mecarbil induces hypotension in vivo at high doses in rats

OM significantly reduced both systolic (from 149 ± 6 to 49 ± 6 mmHg $P<0.05$) and diastolic (from 130 ± 4 to 37 ± 4 $P<0.05$) blood pressure values at a cumulative dose of 1200µg/kg. It is important to note that the drug had no effect on heart rate regardless of the development of hypotension (443 ± 18 and 445 ± 16 beats/min before and after treatment respectively).

Omecamtiv mecarbil induces transient electromechanical alternation in rats at high doses in vivo

OM induced transient, periodic, electromechanical alternations. They were characterized by an oscillation between normal and low or absent ejections from beat to beat. This pattern was observed in 23 of the 30 examined rats. Alternating contractile dysfunction manifests as partial or complete, and left ventricular diastolic filling distinguishes the two forms. Partial alternans was defined when normal systole was followed by partial filling, resulting in a decrease in left ventricular stroke volume. Complete alternans had virtually no left ventricular filling and consequently insufficient contraction after normal systole. The alternation pattern started with a high OM dose (mostly a cumulative dose of 1200 µg/kg body weight) after an increase in heart rate or after an extra beat. This alternation was also reflected in measurable parameters: the systolic ejection time

showed a marked alternation with a cumulative dose of OM 1200 µg/kg (from 111.9 ± 1.8 ms to 82.1 ± 3.0 ms).

Omecamtiv mecarbil alters intracellular Ca^{2+} handling in canine cardiomyocytes in vitro

Treatment with 1 µM OM increased the stiffness of unstimulated cells. The length of unstimulated cardiomyocytes in the presence of 1 µM OM decreased from 131 ± 14 to 112 ± 11 µm. Stimulation of isolated LV cardiomyocytes (faster than 3 Hz) resulted in beat-to-beat oscillations between normal and reduced contractions, similar to in vivo measurements. Alternation was considered real if there was ≥ 10% difference between successively recorded stimulated cell shortenings. This alternation was present in three of 14 cells at 4 Hz and in nine of 14 at 5 Hz. The alternation was then examined at 5 Hz by simultaneously recording changes in cell length, intracellular Ca^{2+} concentration, and membrane potential (AP). There was no difference in contraction (decrease in cell length) before OM treatment in successive contractions (14.20 ± 2.09 and 14.16 ± 2.06 µm), while a significant difference emerged after OM administration (16,6 ± 2,0 and 7,6 ± 0,9 µm). Simultaneously, the amplitude of Ca^{2+} transients (CaT amplitude) decreased from 0.69 ± 0.04 to 0.41 ± 0.08.

Discussion

During our experiments, we examined two aspects of cerebrovascular aging and based on our studies, we can draw several conclusions. First, the lack of IGF-1 seems to negatively affect the adaptive media hypertrophy caused by high blood pressure and the regulated ECM remodeling, reduces the elastin content and weakens the adaptive changes in ECM-related gene expression in a mouse model. Based on these, it seems that circulating IGF-1 plays a critical role in maintaining the structural integrity of cerebral arteries in both hypertensive and normotensive states. It is known that IGF-1 levels decrease with age, which may affect changes in VSMC phenotype and adaptive remodeling of the arterial wall, thus contributing to intracerebral microbleeds and consequent cognitive impairment in old age. Circulating IGF-1 levels decrease significantly with age in humans, and based on research, it appears that this might be the vascular aging process. Our results provide new evidence that IGF-1 deficiency contributes to remodeling and structural weakening of mouse cerebral arteries, mimicking many aspects of vascular aging.

To our knowledge, the manuscript we have published is the first study that has shown that IGF-1 deficiency alone causes age-like changes in the morphology and mechanical properties of cerebral arteries. First, our present results show that adaptive changes in cerebral arterial morphology and vascular mechanics induced by hypertension are impaired in IGF-1-deficient mice. Second, previous studies show that hypertension-induced hypertrophic remodeling in penetrating arterioles is impaired in IGF-1 deficiency (Toth et al., 2014). Third, our results show that IGF-1 deficiency adversely affects adaptive changes in vascular elastin content and the expression of ECM components, as well as factors that regulate ECM assembly, cross-linking, and remodeling. Fourth, previous studies show that autoregulation is deficient in hypertensive IGF-1-deficient mice, and arteria cerebri media from these animals have impaired myogenic tone (Toth et al.,

2014). Our results have important translational significance in the pathogenesis of many diseases that threaten the integrity of the arterial wall. An example of this is the development of intracerebral hemorrhages (ICH), which is also important because despite significant progress in the prevention of ischemic stroke, the incidence of ICH has not decreased in recent decades (Ungvari et al., 2017).

Secondly, regarding the effects of jugular venous pressure elevation, to our knowledge this is the first study to demonstrate that isolated cerebral venous stasis induced by bilateral jugular vein occlusion is associated with cognitive decline in mice. Elderly patients often develop jugular venous reflux, which leads to stagnation or reversal of internal flow in the jugular vein, promoting the transfer of increased central venous pressure to the cerebral venous circulation. The jugular venous valve, which is of critical importance in the prevention of jugular venous reflux, is often incompetent in the elderly, which promotes the development of cerebral venous pressure increase (Fulop et al., 2019). The mechanisms by which cerebral venous stasis/increased cerebral venous pressure promote cognitive impairment are likely multifaceted. Our studies provide direct evidence that cerebral venous stasis promotes BBB damage, which is associated with significant neuroinflammation (microglial activation and an increase in the local concentration of pro-inflammatory cytokines and chemokines). Proinflammatory cytokines, chemokines, proteases, and reactive oxygen radicals from activated microglia lead to neuronal dysfunction. It is important that, based on our experiments, the harmful effects of cerebral venous stasis on the integrity of the blood-brain barrier and inflammatory processes are aggravated by comorbid hypertension. This observation has important clinical significance.

Finally, based on our experiments with Omecamtiv Mecarbil, we reported two previously unrecognized features of the drug that overlap with its positive inotropic effect. One is the effect that severely impairs the diastolic function. The other is a periodic electromechanical alternation (at higher OM doses), in which

normal beats alternate with reduced efficiency myocardial contractions from beat to beat. The phenomenon is similar to pulsus alternans. Importantly, doses > 1200 ng/ml (about three times higher than in our in vitro experiments) in some studies led to excessive prolongation of systole, thus limiting coronary blood flow during diastole, leading to myocardial ischemia in some cases. The maximum intravenous OM dose used in our experiments corresponded to clinical applications (initial dose of 1000 μg/kg body weight in the first hour). In our in vitro experiments, the cellular effects were investigated at an OM concentration of 401 ng/mL (1 μM), which overlaps with previously published effective clinical serum concentrations of the drug. In the course of the present experiments, we confirmed our previous results, according to which a very robust Ca^{2+}-sensitizing effect can be achieved by using OM in a therapeutic dose. At the cellular level, this effect was associated with slowed contraction and relaxation kinetics and significantly increased passive stiffness. As a result, the drug also had a significant diastolic dysfunction-causing effect. Interestingly, some diastolic parameters (E:A ratio, IVRT and τw) were negatively affected even by the lowest dose of OM (200 μg/kg). OM treatment resulted in positive inotropy and diastolic dysfunction in isolated human cardiomyocytes, validating these cells for further experiments. It is interesting that in the presence of OM, even under passive (diastolic) conditions, these cells were shortened. This is consistent with the finding that OM increased the passive stiffness of permeabilized human cardiomyocytes in an apparently Ca^{2+} -independent manner, as well as slowed post-contraction relaxation. Our data suggest that the degree of increased Ca^{2+} sensitivity can be extreme: both diastolic shortening and increased passive stiffness of cardiac muscle cells occurred at very low (diastolic) Ca^{2+} concentrations. Both Ca^{2+}-dependent activation and dissociation appear to be much slower in the presence of OM. OM also increased the resting stiffness of cardiac muscle cells. This can be explained (1) by the Ca^{2+}-independent initiation of active contraction or (2) by the fact that the OM directly interacts with

contractile proteins other than myosin. These cellular data suggest an alternative mechanism behind the decrease in LV diameter: this contraction occurring at diastolic Ca^{2+} concentration may also be the result of significant Ca^{2+} sensitization. In addition, we confirmed that low concentrations of OM do not affect intracellular Ca^{2+} transients. In contrast, OM affected APD and T-wave morphology at higher concentrations. This last, previously unidentified property can be explained by (1) altered functioning of the ryanodine receptor (RyR) and (2) slowed dissociation of Ca^{2+} from the troponin complex. The second major novelty of our work is that OM induced dose- and apparently pulse-dependent, transient, electromechanical alternans in rats, which is similar to pulse alternans. This was present in 23 out of 30 rats at the high OM dose (1200 μg/kg). It mostly occurred at a slightly elevated heart rate or during periods of higher stimulation frequency in isolated dog LV cardiomyocytes (9 of 14 cells at a stimulation frequency of 5 Hz), suggesting that this property may be heart rate dependent. Examining the ECG, the reduced efficiency contraction is not associated with a change in QRS morphology or QT interval, however, the T-waves change from beat to beat during the alternans phenomenon. In patients with heart failure, T-wave alternation has mostly been described parallel to the progression of the disease. Based on research data, it is assumed that the appearance of alternating T-waves can increase the likelihood of developing life-threatening arrhythmias. The robust Ca^{2+}-sensitizing effect may also contribute to this effect.

Based on the above, we have identified two non-negligible potential side effects of OM, which can undermine its positive effects, and in clinical practice it is definitely recommended to act with special caution when using the drug.

The new research results included in the thesis

1. We were the first to demonstrate that IGF-1 deficiency alone causes aging-like changes in the morphology and mechanical properties of cerebral arteries. In addition, we showed that the IGF-1 deficiency negatively affects the changes in the arterial hypertensive data curve, which was also detectable at the gene expression level.

2. We proved that an increase in cerebral venous pressure leads to cognitive disorders in mice through damage to the blood-brain barrier and consequent neuroinflammation.

3. During the experiments conducted with Omecamtiv mecarbil, we established that even at a low dose (200 μg/kg body weight), the drug leads to deterioration of diastolic function, which was also an identifiable effect on isolated human cardiac muscle cells.

4. We also found that Omecamtiv Mecarbil in high doses (1200 μg/kg body weight) can lead to frequency-dependent electromechanical alternans. This effect can be identified not only in vivo, but also at the level of cardiomyocytes, where the length of successive action potentials appeared and the Ca^{2+} transient also changed from beat to beat. All these effects were shown in the form of T-wave alternation on the recorded ECGs.

Summary

The prevalence of many diseases shows an increase with aging. Dementia and heart failure are among these. Two diseases that affect a total of more than 100 million people today. In the case of dementia, despite intensive research, very limited results have been achieved both in the field of prevention and slowing down the progression. In contrast, heart failure therapy has developed explosively in recent decades, but the 10-year survival rate is still below 40% (Groenewegen et al., 2020). In both cases, understanding the pathophysiology and the effect of potential therapies can bring us closer to effective therapies.

In our work, our goal was, on the one hand, a more precise understanding of the pathophysiology of cognitive dysfunction. To this end, we examined two areas that are underrepresented in research: changes in adaptive vascular remodeling in aging, and the role of increased cerebral venous pressure. On the other hand, we aimed to better understand the in vivo and in vitro effects of Omecamtiv Mecarbil, a direct myosin activator developed for the treatment of heart failure with reduced ejection fraction.

In the course of our experiments, we made several findings using numerous in vivo and in vitro methods. On the one hand, we found that the IGF-1 deficiency that appears with aging causes impairment of the adaptive responses to high blood pressure in cerebral arteries. This effect can contribute to a deeper penetration of the arterial pressure wave, increase the vulnerability of blood vessels, which, based on further results by our research group, can lead to the development of microbleeds, local neuroinflammation and consequent cognitive dysfunction. On the other hand, we found that the increase in cerebral venous pressure causes damage to the blood-brain barrier in a mouse model, which leads to neuroinflammation and consequent cognitive impairment, also confirmed at the level of gene expression.

Finally, we proved that the direct myosin activator Omecamtiv Mecarbil can lead to diastolic dysfunction and transient electromechanical alternation in a dose-dependent manner. We were able to confirm these effects with in vivo and in vitro measurements on a rat model, as well as on human and dog heart muscle cells. Furthermore, we verified that transient electromechanical alternans is also associated with T-wave alternans, which can potentially lead to the development of ventricular arrhythmias. All these results may cast a negative light on the positive inotropic effect of OM.

Based on our research results, we can get closer to finding new attack points for the treatment of dementia, and to prevent the development of potentially fatal side effects by understanding the mechanism of action of Omecamtiv Mecarbil.

Keywords

Aging, Dementia, Vascular cognitive impairment, IGF-1 deficiency, Cerebral venous hypertension, Heart failure with reduced ejection fraction, Positive inotropic, Omecamtiv Mecarbil

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