THESIS FOR DEGREE OF DOCTOR OF PHYLOSOPHY

HEMODYNAMIC AND HEMOSTASIS INVESTIGATIONS IN CEREBROVASCULAR DISEASES

LÁSZLÓ OLÁH M.D.

TUTOR : DR. CSIBA LÁSZLÓ
PROFESSOR OF NEUROLOGY

UNIVERSITY OF DEBRECEN
FACULTY OF MEDICINE
DEPARTMENT OF NEUROLOGY

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I. Introduction

Cardiovascular diseases, cancer and stroke are the leading causes of death in both the Western European countries and Hungary. In addition, stroke is the most important cause of morbidity and long-term disability imposing an enormous economic burden. The incidence and mortality of stroke decreased in the developed countries during the last decades, however, the tendency is not so positive in Hungary. In order to decrease the incidence and mortality of stroke, prevention and treatment of cerebrovascular diseases have to be improved.

The most frequent form of stroke is the arterial focal cerebral ischemia, which could be caused by:

1) significant stenosis or occlusion of extracranial arteries (carotid and vertebral arteries), resulting in a decrease of cerebral perfusion pressure;
2) occlusion of intracranial arteries (anterior, middle, posterior cerebral arteries or their branches) by local thrombosis or artery-to-artery and cardiogen embolisations;
3) combination of these mechanisms.

I.1. Hemodynamic changes caused by significant stenosis of extracranial arteries

Atherosclerosis is the main cause of stenosis of extracranial arteries. Besides genetic factors, diabetes mellitus, hypertension, hyperlipidaemia and smoking play role in development of atherosclerosis. In case of severe stenosis (>70 % diameter reduction), the perfusion pressure deacreses distally to the stenosis. However, if the components of circle of Willis (mainly the communicating arteries) are well developed, the collateral flow through these arteries may compensate the decreased perfusion pressure. If it is not possible because of absence or small diameter of communicating arteries, the perfusion pressure decreases, leading to vasodilation of cerebral arterioles. This compensatory mechanism results in decrease of cerebrovascular resistance and thus increase of cerebral blood flow. If the vasodilation of cerebral arterioles reaches its maximum, the cerebral blood flow decreases parallel to the perfusion pressure. In this case the only compensatory mechanism is the increased oxygen extraction from the capillary blood in order to maintain the oxygen supply of the brain tissue. Further decrease of perfusion pressure, however, causes cerebral ischemia, leading to functional, or morphological deficit.

I.2. Hypercoagulability and its possible role in cerebral ischemia

Occlusion of intracranial arteries is mainly caused by local thrombosis or embolisation. Directly or indirectly, both mechanisms suppose coagulation of circulating blood in the arterial system. Main causes of coagulation of arterial blood are: A) damage of endothelium and/ or platelet activation, B) myocardial infarct or atrial fibrillation resulting in hypo- or akinesis and stasis, C) increased activity of components of coagulation cascade and/or decreased fibrinolytic activity. The use of recombinant tissue plasminogen activator and platelet inhibitors in ischemic stroke therapy points also to the importance of hemostasis factors.

In this dissertation, the main two mechanisms of ischemic stroke, namely the hemodynamic changes and hemostasis disturbances are discussed.

II. Aims of the thesis

The next questions were examined.

1) What is the frequency of carotid stenosis in patients with severe obliterating atherosclerosis of lower extremities? What is the incidence of atherosclerotic risk factors in this population?
2) What is the cerebrovascular reactivity and cerebrovascular reserve capacity in healthy subjects and in symptomatic or asymptomatic patients with severe unilateral carotid stenosis. What are changes of vasoreactivity in different stages of transient focal cerebral ischemia?
3) Are there hemostasis abnormalities in ischemic stroke? What are the aggregation properties of thrombocytes in cerebral ischemia? What is the frequency of increased coagulation and/or decreased fibrinolytic activity in young patients with focal cerebral ischemia?

III. Patients and methods

III.1. Experiences with carotid Doppler examinations in patients with severe obliterative atherosclerosis of lower extremities

Atherosclerosis is known to cause damage of not only one artery, but the whole arterial system. Development of cerebral ischemia is not rare in patients with severe obliterative atherosclerosis of lower extremities. Therefore, we examined the frequency of carotid stenosis in patients with severe arterial stenosis of lower extremities.

PATIENTS AND METHODS

Eighty-three patients (71 men, 12 women; mean age 62±10 years; range:36-81 years) with severe obliterative atherosclerosis of lower extremities were examined. Ninety-six subjects (72 men, 24 women; mean age: 63±11 years; range: 42-89 years) without neurological signs or claudication were selected as controls. Besides carotid Doppler examination, risk factors of atherosclerosis were also examined.

The severity of carotid stenosis was evaluated on B-mode images (diameter reduction at the level of the most severe stenosis of carotid system compared to the poststenotic part). Three subgroups were formed by severity of carotid stenosis: 1-30% stenosis (mild); 31-70% stenosis (moderate), 71-99% stenosis and occlusion (severe).

STATISTICAL ANALYSIS

The difference between the control and patient groups was evaluated by analysis of variance (ANOVA) and Fischer LSD post hoc test. A difference of $p<0.05$ was considered as statistically significant.

III.2. Examination of cerebral hemodynamics

III.2.1. Gender-related differences in cerebral hemodynamics

Cerebral hemodynamics have been examined in many stroke-related disorders. However, there have been only few studies investigating cerebrovascular vasoreactivity in healthy subjects, in addition the results were contradictory. Therefore, our aim was to investigate the cerebrovascular reactivity and cerebrovascular reserve capacity in healthy men

and women. Further subgroup analysis was performed between females before menopause and males of similar age, and between women after menopause and age-matched males.

PATIENTS AND METHODS

Twenty-nine women and twenty-seven age-matched men were investigated. Women were further divided into premenopausal and postmenopausal subgroups. Men were also divided into 2 (younger and older) subgroups by their age to make age-matched groups for the two female subgroups.

Transcranial Doppler measurements were performed by a 2 MHz probe (EME TC-2 64 B, EME Überlingen, Germany). The middle cerebral artery was insonated on both sides through the temporal window, and parameters of flow were recorded at a depth of 50 mm. Cerebrovascular reactivity and cerebrovascular reserve capacity were measured after intravenous administration of 1 g acetazolamide (Diamox, Lederle Parenterals, Puerto Rico, USA). Mean velocity in the middle cerebral artery was recorded before and at 5, 10, 15, and 20 minutes after acetazolamide administration. Cerebrovascular reactivity (CR) was assessed at each time point according to the following equation: $CR=100(vt-v0)/v0$, where vt and v0 are the mean velocity values measured at a depth of 50 mm in the MCA before (v0) and at time after acetazolamide administration (vt; t=5, 10, 15, 20 minutes). The rate of maximal velocity increase (i.e. cerebrovascular reserve capacity /CRC/) was calculated by the next equation: $CRC=100(vmax-v0)/v0$, where vmax is the highest of the four values of flow velocity measured after acetazolamide administration.

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) and unpaired t-test were used to compare the baseline mean velocity and the cerebrovascular reserve capacity between males and females. Repeated measure analysis of variance and Tukey post hoc test were used to detect differences in cerebrovascular reactivity between men and women. A difference of $p<0.05$ was considered as statistically significant.

III.2.2. Examination of cerebral hemodynamics in symptomatic and asymptomatic patients with severe unilateral carotid stenosis

Besides examination of cerebral hemodynamics of healthy subjects, cerebrovascular reactivity was examined in symptomatic and asymptomatic patients with severe unilateral

carotid stenosis as well. Our aim was to determine whether there is a difference in cerebrovascular reactivity between symptomatic and asymptomatic patients.

PATIENTS AND METHODS

Carotid arteries were examined by duplex ultrasound technique (Ultramark 4 Plus, ATL, Bothell, USA). Severity of carotid stenosis was determined by the North American Symptomatic Carotid Endarterectomy Trial criteria.

Patients were divided into 2 groups by their history, neurological examination and results of cerebral CT. Patients with transient or permanent signs of cerebral ischemia ipsilateral to the side of carotid stenosis were selected into the symptomatic group. Intracranial bleeding was excluded by cerebral CT. Patients were considered asymptomatic, if neither the detailed history nor the neurological examination revealed signs of cerebral ischemia. Cerebral CT was also negative in these patients.

Middle cerebral arteries were insonated on both sides. Absolute mean velocities were detected at a depth of 50 mm before and 10 and 15 minutes after intravenous administration of 1 g acetazolamide. Relative values after acetazolamide administration were also expressed in % of baseline mean velocity. Acetazolamide-TCD test was performed in 28 age-matched healthy subjects as well. Absolute and relative mean velocities of middle cerebral arteries were compared:

a) within subgroups, between the stenotic and the contralateral sides;
b) between the stenotic sides of asymptomatic and symptomatic subgroups;
c) between the patient subgroups and the controls.

STATISTICAL ANALYSIS

Statistical analysis was performed by Student t-test. In case of multiple comparison, Bonferroni correction was made. A difference of $p<0.05$ was considered as statistically significant.

III.2.3. CO_2 reactivity measured by perfusion-weighted MRI during transient focal cerebral ischemia in rats

Examination of cerebral vasoreactivity in acute phase of cerebral ischemia may be harmful in humans because of „steal“ phenomenon. Therefore, animal experiments were performed in order to investigate cerebrovascular reactivity at different phases of transient

focal cerebral ischemia. Further advantages of the animal experiments that the beginning and the duration of ischemia and reperfusion can be exactly determined.

Cerebral perfusion, severity of ischemic damage, and cerebrovascular reactivity were assessed with use of perfusion- and diffusion-weighted MR sequences during one hour middle cerebral artery occlusion (MCAO) and 4.5 hours of reperfusion. ATP levels of brain tissue were used as a measure of outcome. Our aim was to examine whether the cerebrovascular reactivity improves parallel with the recovery of the energy metabolism or it shows a prolonged disturbance even in the metabolically recovered tissue.

METHODS

Male Wistar rats (n=5; body weight: 300-350 g) were anesthetized with halothane in a 70%/30% mixture of N_2O/O_2. Animals were tracheotomized and mechanically ventilated. Arterial blood gases were measured repeatedly and kept within physiological limits by appropriate settings of the respirator. Focal ischemia was produced by intraluminal suture occlusion of the right middle cerebral artery using a remotely controlled occluding device. The distal end of the suture was thickened to 0.28-0.30 mm in diameter with silicone and was introduced into the right internal carotid artery via the proximal end of the isolated external carotid artery. The suture was connected to an extension catheter and passed through a guide sheath that was fixed to the neck of the animal. This arrangement permitted the manipulation of the thread position from outside the magnet to allow measurements during the pre-ischemic control phase, during MCAO and after retraction of the thread, without the need to reposition the animal. After one hour MCAO and 4.5 hours reperfusion the animals were frozen in liquid nitrogen.

NMR measurements were performed at 200 MHz using a BIOSPEC system (Bruker Medical, Ettlingen, Germany) with a 4.7-T magnet. One diffusion- and two perfusion-weighted images were performed at the pre-ischemic control phase, at the end of one hour MCAO, and 30, 90, 150, 210, and 270 minutes after reperfusion, at the level of the caudate-putamen. After the first perfusion-weighted image 6% CO_2 was added to the inhalation gas for 5 minutes. The second perfusion-weighted image was performed at the end of CO_2 inhalation. Arterial blood gases were measured during both perfusion-weighted measurements. CO_2 reactivity index was calculated by use of the perfusion signal intensity before and at the end of the CO_2 test and by the change of arterial pCO_2. CO_2 reactivity index was expressed in every pixel in %/mmHg (percent of change of perfusion signal intensity induced by 1 mmHg change of arterial pCO_2).

Apparent diffusion coefficient (ADC) was determined in every pixel at the level of the caudate-putamen. After ADC calculation, relative ADC maps were created by pixelwise division of the ADC, measured at the end of MCAO and at different phases of reperfusion, by the control, pre-ischemic ADC map. To investigate the CO_2 reactivity in areas with different degrees of ischemic damage, pixels on the relative ADC map at the end of MCAO were divided into 5 subgroups depending on their relative ADC (<70%, 70-79%, 80-89%, 90-99%, ≥100%). In these subgroups, the CO_2 reactivity values were determined at the pre-ischemic control phase, at the end of ischemia, and at different time points during reperfusion.

The ischemic tissue area at the end of MCAO was estimated by summing up all pixels with a relative ADC<80% of control, because this degree of relative ADC reduction has been described as correlating well with ATP depletion in the acute phase of permanent ischemia. The outcome at the end of the experiment was assessed by ATP images at the level of the caudate-putamen. Damaged tissue was defined as the brain area with ATP loss, while the remaining tissue with normal energy state was considered vital at the end of the experiment. Brain areas with relative ADC<80 % during ischemia, but with normal ATP levels at the end of the experiment were defined as recovered tissue, while the regions with relative ADC<80 % during ischemia and ATP loss at the end of the experiment were considered permanently damaged tissue. CO_2 reactivity and relative perfusion signal intensity (expressed in % of the contralateral homotopic area) were determined separately for the recovered and the permanently damaged tissues.

Brains were removed from the skull in a cold box at - 20 ^oC, and sliced at the same temperature into 20 μm thin sections, using a cryostat microtome. Coronal sections at the level of the caudate-putamen were processed for the regional distribution of ATP by evoking substrate-specific bioluminescence. Regional tissue pH was measured, using the umbelliferone fluorescence technique.

STATISTICAL ANALYSIS

The CO_2 reactivity values during ischemia and at different time points of reperfusion were compared to the pre-ischemic (control) period, using a paired t-test. Repeated measure analysis of variance and Scheffé post hoc test were used to detect differences in cerebrovascular CO_2 reactivity between the recovered and permanently damaged tissues at different phases of experiment. A difference with $p < 0.05$ was considered statistically significant.

III.3. Disturbances of hemostasis in cerebral ischemia

III.3.1. Examination of platelet aggregation before and after 100 mg acetil-salicylic acid (ASA) in patients with ischemic stroke

Platelet aggregation was examined in the acute phase of cerebral ischemia with use of multiparametric aggregation index (MAI) and spontaneous dysaggregation ratio (DR). Our aim was to examine whether low dose (100 mg/day) ASA normalizes the increased platelet aggregation after ischemic stroke.

PATIENTS AND METHODS

Fourty-three patients with cerebral ischemia (26 males and 17 females; mean age: 62.5±12.5 years) and 16 healthy subjects (9 men and 7 women; mean age: 44.1±16.8 years) were included in the study. Patients were divided into 2 groups based on their MAI: 17 patients (11 males and 6 females; mean age: 61.8±10.3 years) had normal (<2 l/μmol) MAI, while this value was elevated (>2 l/μmol) in 26 patients (15 men and 11 women; mean age: 62.9±14.0 years).

Patients were treated with a daily dose of 100 mg ASA. Platelet activity was measured in patients with increased initial MAI (n=26) not only before but also on the the 7^{th} and 28^{th} day of treatment. Serum levels of thromboxane-A2 (TXA2) and prostacycline (PGI2) metabolites (thromboxan-B2 /TXB2/ and 6-keto-prostaglandin-F1α /6KPGF1α/, respectively) were also determined before and on the 28^{th} day after treatment.

ADP and epinephrin were used to induce platelet aggregation for measurement of MAI. The MAI was assessed by the inductor concentrations at which primary and secondary aggregations occured and by the degree of aggregation induced by optimal inductor concentration. The DR was measured by the degree of spontaneous dysaggregation observed 15 minutes after platelet activation with 5μM ADP. Platelet aggregations were measured with Chrono Log Lumi 400 (Chrono Log Co., Havertown, USA) aggregometer. Serum levels of TXB2 and 6KPGF1α were determined with radioimmunoassay kit (IZINTA, Budapest).

STATISTICAL ANALYSIS

Paired t-test was used to compare the parameters before and after treatment. The difference between the control and patient groups was examined with unpaired t-test. Differences were considered statistically significant if the p value was < 0.05.

III.3.2. Examination of monocyte tissue factor in young patients with cerebral ischemia

Activated platelets may elevate the risk of cerebral ischemia by increased aggregation, but they may also induce tissue factor expression of monocytes, which is probably the most potent trigger of blood coagulation. Young patients with unknown cause of cerebral ischemia were studied in order to determine whether monocytes may contribute to thrombotic processes by the expression of the cell surface procoagulant tissue factor. In addition, the activation markers of coagulation and fibrinolysis were also investigated.

PATIENTS AND METHODS

Fourty-eight patients (23 men and 25 women; mean age: 39.8±9.3 years; age range: 17-50 years) with transient ischemic attack and ischemic stroke were studied. Patients above the age of 50, as well as those with cerebral hemorrhage, generalized atherosclerosis, migraine, diabetes mellitus, and cardiogen source of emboli were excluded. None of the patients were on aspirin, dicumarol, or received heparin at the time of the study. The control group consisted of 40 volunteers (21 men, 19 women; mean age: 36.9 years) who had never had any thromboembolic disease or cerebral ischemia.

Blood was drawn into vacutainer tubes containing sodium citrate. Part of the blood was centrifuged at 2000g for 20 minutes and aliqots of plasma samples were analyzed for D-dimer, thrombin-antithrombin (TAT) complex, F1+2 fragment, and C-reactive protein. Mononuclear cells were isolated from the remaining part of the blood, and the cell suspension was divided into two parts. Cells were either sonicated and aliquots were frozen for a one-stage clotting assay, or fixed in 1 % cold paraformaldehyde, then washed and stained for tissue factor. For each measurement, a minimum of 10^6 cells was used and stained with 10μg/ml final concentration of the monoclonal tissue factor antibody. Cells were washed thereafter, and incubated with 1:50 dilution of anti-mouse FITC. Cells, stained for surface immunofluorescence, were analyzed in a Becton Dickinson FacScan flow cytometer. Monocytes were identified on the basis of their forward and side scatter properties as well as by their CD 14 positivity. Immunofluorescence results were expressed as percent positive cells compared to an appropriately stained negative control. Mononuclear cell suspension, stimulated for 6 hours by lipopolysacharide, was used as positive control. These samples displayed 35-40% tissue factor positive monocytes, with no increase in lymphocyte fluorescence.

During the one-stage clotting assay, 100 μl of cell lysate, containing less than 5% neutrophils, were incubated with 100 μl of pooled normal plasma for 5 minutes and the

reaction was started by the addition of 100 µl 25 mM $CaCl_2$. Clotting times were recorded and the procoagulant activity results were compared to a serial dilution of recombinant human thromboplastin. A 10-fold dilution of the thromboplastin was arbitrarily taken as 100.000 mU. Based on the differential counts obtained for each sample, results were normalized as milli-units per 10^6 monocytes.

STATISTICAL ANALYSIS

Comparisons between groups for marker levels and surface immunofluorescence as well as tissue factor activity were carried out using the Mann-Whitney U test. Values of $p<0.05$ were reported as significant.

III.3.3. Natural coagulation inhibitor proteins and elements of fibrinolytic system in young patients with cerebral ischemia

The involvement of a prothrombotic state caused by abnormality of natural coagulation inhibitor protein (antithrombin-III, protein C, protein S) activities and/or by the decreased level of fibrinolytic activity, as a cause of cerebral ischemia of arterial type has been controversial. However, the association of a prothrombotic condition with secondary hypercoagulable states (smoking, pregnancy, puerperium, use of oral contraceptives, alcohol consumption, surgery, inflammation, malignancy) may elevate significantly the risk of cerebral ischemia. The purpose of the investigation was to study the role of abnormal activities of natural coagulation inhibitor proteins and disturbances of the fibrinolytic system in young patients with cerebral ischemia.

PATIENTS AND METHODS

Fifty three young adults between 15 and 49 years of age who had transient ischemic attack (TIA) or stroke were included in the study. Besides hemostasis screening test and chest x-ray, all patients underwent complete neurological and cardiological examinations, including brain CT or MRI, ultrasound examination or angiography of the carotid and vertebral arteries, electrocardiography (ECG) and two-dimensional echocardiography. Transoesophageal echocardiography was also performed in selected patients. Patients were divided into one of the following groups by the etiology of ischemic stroke: “atherothrombotic stroke”, “cardioembolic stroke”, “stroke with mixed etiology”, “stroke with other etiology”, and “stroke with unknown etiology”.

The measurement of the activities of AT-III, protein C, protein S, PAI-1, alpha-2-antiplasmin and plasminogen was carried out with use of commercially available assays (Diagnostica Stago, Asnières, France). Lipoprotein (a) /Lp(a)/ level was measured using an immunoturbidimetric assay. The normal ranges of the examined parameters were given by the manufacturers of these assays. The APC ratio was determined by the prolongation of clotting time in an activated partial thromboplastin time assay, and the normal value was given as > 2 (Diagnostica Stago, Asnières, France). The presence of Leiden and prothrombin gene mutations was also examined.

Quantitation of natural anticoagulant activities (AT-III, protein C, protein S) and activated protein C ratio was performed upon admission and 3 months later. PAI-1, alpha-2-antiplasmin, plasminogen activities, and /Lp(a)/ levels were also evaluated upon admission with the simultaneous measurement of C reactive protein. Patients with elevated C reactive protein (values above 10 mg/L) were excluded.

STATISTICAL ANALYSIS

The statistical analysis was performed using analysis of variance, 2x2 contingency tables, and by the chi-square test and Fisher's exact test. Differences were considered statistically significant if the p value was < 0.05.

IV. Results

IV.1. Experiences with carotid Doppler examinations in patients with severe obliterative atherosclerosis of lower extremities

Moderate and severe carotid stenosis were found in 37% and 19% of patients with severe obliterative atherosclerosis of lower extremities, respectively. On the contrary, the incidence of moderate carotid stenosis in the control group was only 2% and severe carotid stenosis was not found. The proportion of smokers (68%) and hypertensive patients (59%) was significantly higher ($p<0.05$) in the patient group compared with controls (smokers: 21%; hypertension: 25%). In addition, serum cholesterin, triglycerid, and LDL-C levels were significantly higher in patients than in controls.

IV.2. Examination of cerebral hemodynamics

IV.2.1. Gender-related differences in cerebral hemodynamics

No difference in age, blood pressure, heart rate, hematocrit, blood gases and weight was found between men and women. There were 18 women before and 11 after the menopause (premenopause and postmenopause subgroups). Men were also divided into 2 (younger and older) subgroups by their age to make age-matched groups for the two female subgroups.

The baseline mean flow velocity in the MCA was significantly higher in women (63±4 cm/s) compared with men (57±4 cm/s; $p<0.02$). After acetazolamide administration, significantly higher cerebrovascular reactivity was observed in females compared with males ($p < 0.001$), and the cerebrovascular reserve capacity was also higher in women (57.9±23.9%) than in men (43.7±17.8%; $p<0.001$). Subgroup analysis showed, that women before menopause responded with higher cerebrovascular reserve capacity than age-matched men ($p<0.01$), but no significant difference was found between females after menopause and men of similar age.

IV.2.2. Examination of cerebral hemodynamics in symptomatic and asymptomatic patients with severe unilateral carotid stenosis

Thirty-six patients with unilateral severe carotid stenosis or occlusion were examined (24 males, 12 females). Twelve patients had asymptomatic carotid stenosis. The remaining 24 patients belonged to the symptomatic group, 12 of them had severe carotid stenosis, the other 12 patients had internal carotid artery occlusion.

Significant difference in the absolute or relative mean velocity of middle cerebral artery was not detected either before or after acetazolamide administration between the stenotic and the contralateral sides in the asymptomatic group. However, in the symptomatic group, either 10 or 15 minutes after administration of 1 g acetazolamide, both the absolute and the relative mean velocities were lower ($p<0.01$) on the stenotic side than on the contralateral side. The absolute and relative velocities on the stenotic side in the symptomatic group were lower than those in the control subjects or on the stenotic side of asymptomatic patients ($p<0.01$), after acetazolamide administration. However, no difference was found between the velocities observed in healthy subjects and on the stenotic side of asymptomatic patients.

IV.2.3. CO_2 reactivity measured by perfusion-weighted MRI during transient focal cerebral ischemia in rats

Except for the short periods of the CO_2 reactivity tests, all general physiological parameters remained within the normal range during all phases of the experiment. Ventilation with 6 % CO_2 for 5 minutes increased arterial pCO_2 by 17 - 23 mmHg, and led to a significant decrease of arterial pH, and a slight but significant increase of systemic blood pressure. Successful occlusion of the middle cerebral artery and reperfusion of the ischemic area were proved with use of perfusion-weighted MRI. ADC in the ipsilateral MCA territory decreased significantly during 1 hour of MCAO, and improved after reperfusion.

The pre-ischemic CO_2 reactivity in the ipsilateral hemisphere was slightly lower (3.52 ± 0.88 % / mmHg) than in the contralateral hemisphere (4.05 ± 0.97 % / mmHg), but this difference was not statistically significant.

Severity of the ischemic damage was assessed by the end-ischemic relative ADC. During ischemia, an inverse CO_2 response was observed in the area with relative end-ischemic ADC below 90%. However, even in areas with normal or only slightly decreased ADC (i.e. relative end-ischemic ADC≥90 % of control), the CO_2 reactivity decreased dramatically to below 1 % / mmHg. It should be noted that these areas also showed a perfusion deficit, even though this did not lead to any significant ADC change.

After reperfusion, the CO_2 reactivity remained below 1 % / mmHg in the area which had suffered severe ischemic injury during MCAO (relative end-ischemic ADC < 80 %), indicating that vasomotor reactivity failed to recover within 4.5 hours of recirculation. However, the CO_2 response in the region with less severe ischemic damage (end-ischemic relative ADC≥80 % of control) showed gradual improvement, and by the end of the reperfusion was no longer significantly different from the response during the control period.

To differentiate between the tissues that were damaged at the end of ischemia but recovered during reperfusion and those that showed no recovery during recirculation, we used a combination of the end-ischemic relative ADC map and the ATP image at the end of the recirculation period. At first, the end-ischemic lesion was defined by the end-ischemic relative ADC < 80 % of control value. These pixels were then divided into two groups, depending on the ATP status at the end of the experiment: pixels with ATP loss (permanently damaged tissue) and pixels with normal ATP content (recovered tissue). A dramatic drop in CO_2 reactivity was observed in both regions during MCAO, which slightly increased during the first two hours of reperfusion. However, in the second half of the recirculation period, the

vasoreactivity declined in the permanently damaged tissue and reached approximately zero, while it continued to increase slowly in the recovered tissue. Although the difference between the CO_2 reactivities of these groups was significant at the end of the experiment (1.1±0.5%/mmHg in the recovered, and 0.2±0.5 %/mmHg in the permanently damaged tissue areas; $p<0.01$), the CO_2 response remained clearly below the control value ($p<0.01$) in the recovered tissue as well, indicating still impaired cerebrovascular reactivity after transient focal cerebral ischemia despite normalized ATP levels.

IV.3. Disturbances of hemostasis in cerebral ischemia

IV.3.1. Examination of platelet aggregation before and after 100 mg acetil-salicylic acid (ASA) in patients with ischemic stroke

Multiparametric aggregation index (MAI) was higher in patients with cerebral ischemia (3.8±0.5 l/μmol) than in controls (0.9±0.1 l/μmol). Measurement of MAI revealed increased platelet aggregation (MAI>2 l/μmol) in 60 % of patients. Platelet aggregation in this subgroup, measured by MAI or spontaneous dysaggregation ratio (DR), decreased significantly after administration of a daily dose of 100 mg acetil-salicylic acid (Table 1.). Serum level of thromboxan-B2 (TXB2) decreased significantly after administration of a daily dose of 100 mg acetil-salicylic acid for 4 weeks ($p<0.05$). However, similar change was not observed in concentration of 6-keto-prostaglandin F1α (6KPGF1α). (Table 1.)

Table 1. Laboratory data of patients (n=26) with increased platelet aggregation before acetil-salicylic acid treatment (mean ± standard error).

Parameters	Before treatment	7^{th} day of 100 mg ASA treatment	28^{th} day of 100 mg ASA treatment
MAI (1/μmol)	5.53±0.68	2.58±0.36 ***	2.05±0.32 ***
DR (%)	2.23±0.34	13.04±1.75 ***	13.35±1.66 ***
TXB2 (pg/ml)	1890±193	-	1462±197 *
6KPGF1α (pg/ml)	931±117	-	977±127 (NS)

Values, measured on 7^{th} and 28^{th} days of ASA treatment were compared with the initial (before treatment) values. ***$p<0.001$, *$p<0.05$, NS: not significant.

IV.3.2. Examination of monocyte tissue factor in young patients with cerebral ischemia

If blood was drawn for analysis within 48 hours after the onset of symptoms, patients were considered as acute (n=25), if it was drawn between days 4 and 14 they were considered as chronic (n=23). Results were compared to those obtained from an age- and sex-matched control group (n=40).

Tissue factor antigen levels on monocytes were significantly higher in both the acute ($p<0.001$) and chronic ($p<0.05$) phases of stroke as compared to controls (Table 2). Although tissue factor is fully expressed on the cell surface, there are marked differences and variation in specific functional activity due to the availability of charged phospholipids. Therefore, maximum activity can be ensured by measuring procoagulant activity in cell lysates. The one-stage clotting assay showed that tissue factor antigen displayed functional tissue factor activity. Results were higher in both the acute and chronic phase groups, however the elevation was significant only in acute phase patients ($p<0.05$; Table 2).

Table 2. Monocyte tissue factor antigen, monocyte tissue factor activity, and serum prothrombin fragment 1+2 (F1+2), thrombin-atithrombin complex (TAT), D-dimer levels are shown in acute and chronic phases of cerebral ischemia (means ± standard error).

	Control	Cerebral ischemia	
		acute	chronic
Tissue factor antigen (%)	3.2±0.7	10.7±1.8 **	9.1±1.7 *
Tissue factor activity (mU)	109±25	220±29 *	193±31
TAT (ng/ml)	3.1±0.3	23.9±8.3 ***	30.7±9.6 **
F1+2 (nmol/l)	1.41±0.20	5.53±1.24 ***	3.18±0.64 *
D-dimer (ng/ml)	254±33	301±59	488±80 *

(Tissue factor antigen is expressed as percent positive monocytes compared to a negative control. Tissue factor activity is expressed as milli-units per 10^6 monocytes. ***$p<0.005$, **$p<0.01$, *$p<0.05$, compared with controls.)

Patients displayed significantly elevated F1+2 fragment and TAT levels in both the acute and chronic phases of the disease ($p<0.005$ in the acute phase for both markers, and $p<0.05$ for F1+2 fragment, $p<0.01$ for TAT complex in chronic phase patients; Table 2). The activation of the fibrinolytic system was investigated by measuring D-dimer levels. It was

found that D-dimer levels were significantly increased in the chronic ($p<0.05$, Table 2.) but not in the acute phase of cerebral ischemia as compared to controls.

IV.3.3. Natural coagulation inhibitor proteins and elements of fibrinolytic system in young patients with cerebral ischemia

The mean age of patients (n=53; 21 men, 32 women) was 38.5±9.8 years. Fourty-six patients had ischemic stroke and 7 patients' neurological signs ceased within 24 hours, thus were considered as TIA.

Except for one patient who had lupus anticoagulant with a prolonged APTT, hemostasis screening tests (prothrombin time, thrombine time, activated partial thromboplastin time) were normal in all patients. The detailed screening tests revealed abnormally low activity of AT-III in 5, low protein C activity in 9, and low protein S activity in 13 of 53 cases at the first examination. APC ratio was lower than the cut-off value in 9 of 53 patients. Combined abnormalities were found in 10 patients, and there was at least one abnormally low value in 26 of 53 patients at the first sampling.

At the repeated examination (3 months later) AT-III activity was normal in all cases, the protein C activity remained abnormally low in 1 patient, while both the protein S activity and the APC ratio remained pathological in 3 of 49 patients. On repeated examination there was at least one abnormally low value in 7 patients, while combined abnormalities were not observed. The molecular biological investigations revealed one of the 3 patients with low APC ratio to be homozygous for factor V Leiden, while the other 2 patients did not display Leiden mutation. Three of 49 patients were heterozygous for the prothrombin 20210 G→A mutation.

Alteration of the fibrinolytic system was evaluated only upon admission. In order to minimize the acute phase effect, only patients with normal C reactive protein values were included. PAI-1 activity was elevated in 23 of 51 cases, Lp(a) level in 10 of 51 patients, alpha-2-antiplasmin activity in 4 of 50 cases, while plasminogen activity was decreased in only 2 of 48 patients. Combined abnormalities were found in 8 of 51 cases.

V. Summary

The role of hemodynamic changes and hemostasis abnormalities on development of cerebrovascular diseases was examined. Besides human studies, animal experiments were

also performed to investigate hemodynamic changes in the acute phase of transient focal cerebral ischemia.

Cumulative presence of atherosclerosis risk factors was found in patients with severe obliterative arterial disease of lower extremities. About 20% of patients also had severe carotid stenosis. Our data suggest that in case of a severe obliterative atherosclerosis of the lower extremities, the carotid arteries should also be investigated and the risk factors have to be reduced.

In order to evaluate the changes of cerebral hemodynamics in patients with cerebrovascular diseases, control values in healthy men and women have to be determined. Hemodynamic investigations in a healthy population revealed a significantly larger cerebrovascular reserve capacity in women before menopause than in age matched men. However, significant difference was not found between females after menopause and males of similar age. Our data suggest that sexual steroids have influence on cerebral hemodynamics.

The cerebrovascular reserve capacity in symptomatic patients with severe unilateral carotid stenosis was significantly smaller on the lesion side than on the contralateral side or than in control subjects. However, such a disturbance of cerebral hemodynamics was not found in asymptomatic patients with severe unilateral carotid stenosis. The most likely explanation of the normal vasoreactivity in asymptomatic patients is the presence of well formed communicating arteries, which may compensate the decreased perfusion pressure caused by severe carotid stenosis. The smaller reserve capacity in symptomatic patients is probably due to the insufficient collaterals.

Since thrombolysis is a proved therapy of ischemic stroke in selected patients, we investigated cerebral hemodynamics during the acute phase of transient focal cerebral ischaemia in animal experiments. Our data suggest that the improvement of CO_2 reactivity during the early phase of recirculation depends on the severity of ischemic damage caused by 1 hour middle cerebral artery occlusion. In case of severe ischaemic damage, a long lasting disturbance of CO_2 reactivity was observed during reperfusion even if the energy metabolism improved after restoration of blood flow.

Investigation of platelet aggregation with use of multiparametric aggregation index and spontaneous dysaggregation ratio revealed increased aggregation propereties in patients with cerebral ischaemia as compared to controls. Abnormal platelet aggregation was effectively inhibited by a daily dose of 100 mg acetyl-salicylic acid in most patients. Patients not responding to acetyl-salicylic acid therapy can be selected with use of the above

mentioned methods. In these cases changing to another platelet inhibitor (e.g. clopidogrel, ticlopidine) is recommended.

Platelet activation may contribute to monocyte tissue factor expression and thus may elevate the risk of cerebral ischemia. Higher tissue factor antigen level and higher procoagulant activity of monocytes were found in patients with cerebral ischemia than in controls. Our results suggest the importance of tissue factor expression on monocytes in ischemic stroke, becuase it may induce or enhance activation of blood coagulation.

In young patients with cerebral ischaemia (age < 50 years) a decreased activity of at least one natural coagulation inhibitor protein or low APC ratio was found in a surprisingly high proportion at admission. However, the number of patients with abnormal values decreased significantly at repeated measurement 3 months later. These data suggest that cerebral ischaemia may influence the results of measurements of hemostasis. However, it should be kept in mind that the increased coagulation, observed at admission, may contribute to the progression of cerebral ischaemia, independent of being cause or consequence of the diseaese.

Our results indicate the importance of both the neurosonologic and hemostasis investigations in cerebral ischemia or in risk factors of cerebrovascular diseases. In order to prevent the development of cerebral ischemia, patients with signs of other arterial diseases have to be urgently examined (carotid Doppler, echocardiography). Evaluation of degree of platelet aggregation is also suggested. Platelet aggregation should be decreased by administration of acetyl-salicylic acid or other platelet inhibitor. In case of decreased cerebrovascular reserve capacity caused by significant carotid stenosis, indication of carotid endarterectomy should be taken into consideration also in asymptomatic patients.

If cerebral ischaemia could not be prevented, activity of natural anticoagulant proteins and elements of the the fibrinolytic system should be screened in younger patients without significant carotid stenosis or cardiogen source of emboli. In case of hemodynamically significant carotid stenosis, platelet activity is probably also increased which may contribute to increased coagulation by increased production of thromboxan or expression of monocyte tissue factor. These disturbances may elevate the risk of thrombus formation and embolisation.

If thrombolysis could be induced within 3 hours in a patient with focal cerebral ischemia, the outcome depends mainly on the severity of ischemic damage. In case of severe damage, the probability of the recovery is poor. Severity of ischemic damage can be estimated

with use of combination of perfusion and diffusion-weighted MR techniques. It means that tissue volume with reversible and irreversible damage can be assessed, which helps to decide for or against thrombolytic therapy. MR investigations will probably have much more importance in the near future in diagnosis of cerebral ischemia and in follow up of therapeutical success.

Our results emphasize the complex role of hemodynamic and hemostasis investigations in prevention and diagnosis of cerebral ischaemia. In addition, hemodynamic or hemostasis parameters may be useful in evaluation of therapeutical effects.

V. List of publications

V.1. Full length articles. (Titles of articles related to the thesis are bolded.)

1. **Oláh L, Fülesdi B, Valikovics A, Csiba L, Olvasztó S, Bánfi Cs, Kozlovszky B. Alsóvégtagi verőérszűkület miatt operált betegek carotis-Doppler vizsgálatával szerzett tapasztalataink. [Experiences with carotid-Doppler examinations on patients with arterial bypass operation on the lower extremities]. Ideggy Szle 1993;46:322-325.**
2. Pallagi E, Oláh L. [Cluster headache]. A cluster fejfájás. Orv Hetil 1994;135:1515-9.
3. Valikovics A, Oláh L, Fülesdi B, Munkácsy Cs, Csiba L. A vazoreaktivitás vizsgálata egészséges személyek esetében transcranialis és carotis Dopplerrel. [Examination of vasoreactivity with transcranial and carotid Doppler in healthy subjects]. Ideggy Szle 1996;49:30-35.
4. **Oláh L, Misz M, Bereczki D, Fekete I, Bordánne JE, Takács EI. Kis dózisú acetilszalicilát hatásosan gátolja a thrombocyta-aggregációt ischaemiás stroke után. [Low doses of acetylsalicylic acid effectively inhibits thrombocyte aggregation after ischemic stroke]. Orv Hetil 1996;137:455-9.**
5. Valikovics A, Oláh L, Fülesdi B, Káposzta Z, Ficzere A, Bereczki D, Csiba L. Cerebrovascular reactivity measured by transcranial Doppler in migraine. Headache 1996;36:323-8.
6. Fülesdi B, Valikovics A, Orosz L, Oláh L, Limburg M, Dink L, Káposzta Z, Csiba L. A cerebrovascularis reaktivitás vizsgálata az arteria carotisok tünetmentes és tünetet okozó atheroscleroticus laesióiban szenvedő betegekben. [Assessment of cerebrovascular reactivity in patients with symptomatic and asymptomatic atherosclerotic carotid artery lesions]. Orv Hetil 1998;139:623-8.
7. **Misz M, Oláh L, Kappelmayer J, Blaskó G, Udvardy M, Fekete I, Csépány T, Ajzner É, Csiba L. Haemostasis eltérések ischaemiás strokeban. [Hemostatic abnormalities in ischemic stroke]. Orv Hetil 1998;139:2503-7.**
8. **Kappelmayer J, Berecki D, Misz M, Oláh L, Fekete I, Csiba L, Blaskó G. Monocytes express tissue factor in young patients with cerebral ischemia. Cerebrovasc Dis 1998;8:235-9.**

9. Grüne M, van Dorsten FA, Schwindt W, Oláh L, Hoehn M. Quantitative T*(2) and T'(2) maps during reversible focal cerebral ischemia in rats: separation of blood oxygenation from nonsusceptibility-based contributions. Magn Reson Med 1999;42:1027-32.
10. **Oláh L, Valikovics A, Bereczki D, Fülesdi B, Munkácsy Cs, Csiba L. Gender-related differences in acetazolamide-induced cerebral vasodilatory response: a transcranial Doppler study. J Neuroimaging 2000;10:151-6.**
11. **Oláh L, Franke C, Schwindt W, Hoehn M. CO(2) reactivity measured by perfusion MRI during transient focal cerebral ischemia in rats. Stroke 2000;31:2236-44.**
12. Franke C, van Dorsten FA, Oláh L, Schwindt W, Hoehn M. Arterial spin tagging perfusion imaging of rat brain. Dependency on magnetic field strength. Magnetic Resonance Imaging 2000;18:1109-1113.
13. Maeda K, Mies G, Oláh L, Hossmann KA. Quantitative measurement of local cerebral blood flow in the anesthetized mouse using intraperitoneal [14C]iodoantipyrine injection and final arterial heart blood sampling. J Cereb Blood Flow Metab 2000;20:10-14.
14. Paschen W, Oláh L, Mies G. Effect of transient focal ischemia of mouse brain on energy state and NAD levels: no evidence that NAD depletion plays a major role in secondary disturbances of energy metabolism. J Neurochem 2000;75:1675-80.
15. Oláh L, Wecker S, Hoehn M. Relationship of ADC changes and metabolic disturbances after 1 hour focal cerebral ischemia and at different reperfusion phases in rats. J Cereb Blood Flow Metab 2001;21:430-439.
16. Oláh L, Wecker S, Hoehn M. Secondary deterioration of apparent diffusion coefficient after 1-hour transient focal cerebral ischemia in rats. J Cereb Blood Flow Metab 2000;10:1474-1482.
17. **Oláh L, Misz M, Kappelmayer J, Ajzner É, Csépány T, Fekete I, Bereczki D, Blaskó G, Csiba L. Natural coagulation inhibitor proteins in young patients with cerebral ischemia. *Cerebrovasc Dis* 2001;12:291-297.**

V.2. Abstracts related to the thesis

1. Valikovics A, Oláh L, Fülesdi B, Bereczki D, Csiba L. Cerebrovascular reserve capacity measured by transcranial Doppler in migraine. Eur J Neurol 1995;2 (Suppl1):81.
2. Fülesdi B, Káposzta Z, Molnár C, Oláh L, Limburg M, Valikovics A, Csiba L. Evaluation of the cerebrovascular reserve capacity in asymptomatic and symptomatic carotid artery obstructive disease. Eur J Neurol 1995;2 (Suppl2):41.

3. Valikovics A, Magyar T, Hegedűs I, Kappelmayer J, Oláh L, Bereczki D, Csiba L. Microembolization in the middle cerebral artery of patients with high risk for embolic stroke. Eur J Neurol 1996;3 (Suppl5):47.
4. Csiba L, Valikovics A, Magyar T, Oláh L, Czuriga I. Transcranial Doppler investigations on hypertensive patients during physical exercise. Cerebrovasc Dis 1998;8 (Suppl3):18.
5. Franke C, Oláh L, Schwindt W, Hoehn M. CO_2 reactivity during transient focal cerebral ischemia: a perfusion-weighted MRI investigation in rat brain. Eight Scientific Meeting of International Society for Magnetic Resonance in Medicine Page 175; Number: 1283; 1-7 April, 2000 Denver USA (poster)

V.3. Other abstracts, posters, lectures

1. Mies G, Trapp T, Kilic E, Oláh L, Hata R, Hermann DM, Hossmann K-A (2001) Relationship between DNA fragmentation, energy state, and protein synthesis after transient focal cerebral ischemia in mice. In: Maturation Phenomenon in Cerebral Ischemia IV: Defensive Mechanisms versus Apoptosis Neuronal Recovery, and Protection in Cerebral Ischemia. Eds: Bazan N, Ito U, Kuroiwa T, Springer-Verlag, Berlin, Heidelberg, New York, (Oral presentation in New Orleans, USA, November 1999)
2. Oláh L, Wecker S, Hoehn M. Secondary deterioration of ADC after one hour transient focal cerebral ischemia in rats. A magnetic resonance imaging study. Eur J Neurosci 2000; 12 (Supp1):118. (Forum of European Neuroscience; 2000, Brighton; poster)
3. Daoud R, Mies G, Oláh L, Hossmann K-A, Stamm S. Intracellular translocation of transformer-2 beta protein following transient focal cerebral ischemia in mice. Eur J Neurosci 2000; 12 (Supp1):347. (Forum of European Neuroscience; 2000, Brighton; poster)
4. Wecker S, Oláh L, Hoehn M. Secondary deterioration of ADC in transient focal cerebral ischemia in rats. Eight Scientific Meeting of International Society for Magnetic Resonance in Medicine Page 176; Number: 1293; 1-7 April, 2000 Denver USA (poster)