THE ROLE OF THE BLOOD-BRAIN BARRIER IN THE PATHOGENESIS OF NEONATAL AND ISCHAEMIC BRAIN INJURIES: INVESTIGATIONS USING ANIMAL MODELS

Ph.D. thesis

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II. Szabó CA, Deli MA, **Németh L**, Krizbai I, Kovács J, Ábrahám CS, Joó F: Histamine-induced vasogenic brain oedema formation in newborn pigs: a role for endothelial acid phosphatase?

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III. **Németh L**, Deli MA, Falus A, Szabó CA, Ábrahám CS: Cerebral ischaemia-reperfusion induced vasogen brain oedema formation in rats: effect of an intracellular histamin receptor antagonist.

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IV. Megyeri P, **Németh L**, Pabst KM, Pabst MJ, Deli MA, Ábrahám CS: 4-(2-aminoethyl)benzenesulfonyl fluoride attenuates tumor necrosis factor-α-induced blood-brain barrier opening

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V. **Németh L**, Szabó CA, Deli MA, Kovács J, Krizbai IA, Ábrahám CS: Cerebral microvascular acid phosphatase isoenzymes may contribute to the histamine-induced changes in the blood-brain barrier permeability.

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II. Megyeri P, Deli M, Joó F, **Németh L**, Kovács J, Torpier G, Pabst MJ, Ábrahám CS: A serine protease inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride, prevents tumor necrosis factor-induced vasogenic brain edema formation in newborn pigs.

Pediatric Research **39:** 298A, 1996

III. **Németh L**, Kovács J, Joó F, Füzesi K, Pintér S, Megyeri P, Deli MA, Ábrahám CS: Histamine is an important mediator of hypoxic-ischemic brain edema formation in newborn pigs.

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IV. Kovács J, **Németh L**, Szabó CA, Deli MA, Krizbai I, Joó F, Ábrahám CS: Endothelial acid phosphatase may participate on the histamine-induced vasogenic brain edema formation in newborn pigs.

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V. Szabó CA, Deli MA, **Németh L**, Krizbai I, Kovács J, Ábrahám CS, Joó F: Histamine-induced vasogenic brain oedema formation in newborn pigs: a role for endothelial acid phosphatase?

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VI. Deli MA, **Németh L**, Falus A, Kovács J, András I, Szabó CA, Krizbai IA, Eszlári E, Kovács T, Ábrahám CS: Effect of an intracellular histamine receptor antagonist on the blood-brain barrier permeability of rats.

Journal of Neurochemistry **69:** S151, 1997

VII. Szabó CA, Krizbai IA, Deli MA, András I, Joó F, **Németh L**, Kovács J, Ábrahám CS: Changes in the activity of acid phosphatase isoforms in isolated cortical microvessels and in cortical tissue during global cerebral ischaemia in rats.

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SUMMARY

The integrity of the blood-brain barrier (BBB) formed by cerebral microvascular endothelial cells is crucial to the maintenance of normal brain activity. Cerebral insults are relatively common in the perinatal period and may result in brain damage and permanent neurodevelopmental deficits. There is an increased claim to ensure a better chance to the neonatal and paediatric patient groups by the prevention of these complications.

The purpose of our study was to investigate the changes in the BBB permeability during pathological conditions. The following animal experiments were undertaken to reveal several pathophysiological mechanisms leading to the damage of the BBB.

(1) In newborn pigs, intracarotid administration of exogenous histamine resulted in a dose-dependent increase in the BBB permeability and in an induction of the acid phosphatase enzyme activity in cortical microvessels.

(2) Tumour necrosis factor-α injection provoked a time- and dose-dependent BBB opening in the neonatal porcine brain. Prae-treatment with a water-soluble serine protease inhibitor could attenuate the cytokine-induced BBB damage.

(3) In adult rats, 4-vessel occlusion and reperfusion caused a significant increase in the BBB permeability for albumin. Administration of an intracellular binding site antagonist failed to prevent this ischaemia-reperfusion-induced damage. Moreover, blockade of the intracellular effect of histamine could also induce an opening of the BBB in healthy animals.

(4) Bilateral carotid occlusion resulted in significant forebrain ischaemia, and a time-dependent increase in cerebral water content and BBB permeability in the rat. An induction of acid phosphatase enzyme in cortical capillaries might contribute to the development of brain oedema.

The results presented here may give a better insight into the pathomechanism of the BBB changes during brain injuries and may help in establishing new therapeutical strategies with specific cellular targets in the near future.

1. INTRODUCTION

In the central nervous system (CNS) of vertebrates intravascular and interstitial spaces are separated by a highly specialised endothelial lining, which is the morphological basis of the blood-brain barrier (BBB). The cerebral microvascular endothelial cells, functioning in co-operation with the neighbouring cellular elements, are responsible for the unique penetration barrier, which restricts the passage of nutrients, water-soluble compounds, hormones, immunologically relevant molecules and drugs to the brain. The integrity of the endothelial cells and the cerebral vasculature are crucial to the maintenance of the normal brain activity. The modulation of the BBB regulatory function during pathological conditions results in an imbalance in the cerebral homeostasis. The CNS responds to several injuries in a limited number of ways that may cause a late, persistent disorder of the brain.

1.1. Brain injuries in paediatrics

In neonates, the incidence of the severe brain injuries is between one and four cases per 1,000 live births but at later ages may be different, since some affected children die. On the other hand, in the paediatric critical care practice a wide repertoire of acute brain injuries, caused by a variety of toxic-metabolic, ischaemic, and traumatic disorders, is commonly seen providing a basis for the potential life-threatening problems. The primary pathology may arise from within or outside the CNS (Table 1). In the paediatric critical care practice a consistent variety of encephalopathic clinical patterns is present but head and brain trauma is the leading cause of death in children; those who die of their trauma usually do so as a result of brain injury (Conroy and Kraus, 1988). The clinical neurological features observed relate to the injury caused by the primary insult at the time of trauma, e.g. bleeding, contusion and neuronal shearing, or by the secondary brain injury resulting from subsequent complicating events and processes, which can be attributed to intracranial or systemic factors. Epidemiologically, accidental or non-accidental head injury is probably the major cause of the brain injuries in childhood. However, non-traumatic damages resulting from hypoxic-ischaemic insult remain the most important causes of chronic morbidity. Hypoxic-ischaemic brain injury is the single most important neurological problem and accounts for more of the non-progressive neurological deficits in childhood than any other type of brain injury (Volpe, 1983). Mental retardation, epilepsy and the various forms of cerebral palsy are its main sequels. The brain may be deprived of oxygen by two main pathogenetic mechanisms:

hypoxaemia (reduced amount of oxygen in the blood supply) or ischaemia (reduced amount of blood perfusing the brain). Hypoxaemia is associated with acceleration in the uptake of glucose,

Table 1. **Causes of Acute Brain Injuries in Childhood**

1 Trauma
- *Accidental*
- *Non-accidental*

2 Hypoxic-Ischaemic Injury
- *Cardiorespiratory arrest*
- *Near-miss sudden infant death syndrome*
- *Near drowning*
- *Smoke inhalation*
- *Shock syndromes*

3 Intracranial Infection
- *Meningitis*
- *Encephalitis*
- *Post-infectious*

4 Mass lesion
- *Haematoma*
- *Abscess*
- *Tumour*

5 Fluid Electrolyte and Acid-Base disorders
- *Hypernatraemia*
- *Hyponatraemia*
- *Water intoxication*
- *Acidosis*
- *Alkalosis*

6 Acute Ventricular Obstruction

7 Seizure Disorders

8 Complication of Malignancy

9 Systemic Infection
- *Sepsis syndrome*
- *Septic encephalopathy*

10 Poisoning

11 Vascular
- *Arterio-venosus malformation*
- *Hypertensive encephalopathy*
- *Embolism*
- *Migraine*
- *Venous thrombosis*
- *Arteritis*
- *Homocysteinuria*

12 Endocrine Dysfunction
- *Hypoglycaemia*
- *Diabetes mellitus*
- *Diabetes Insipidus*

13 Respiratory Failure

14 Renal Failure

15 Hepatic Failure

16 Reye's Syndrome

17 Inherited Metabolic Disorder
- *Lactic Acidosis*
- *Urea cycle disorder*
- *Aminoacidopathies*

18 Hypothermia/Hyperthermia

19 Iatrogenic
- *Overcorrection of acidosis*
- *Overhydration*
- *Drug-overdosage*

Tasker and Cole 1997;

rate of glycolysis and production of lactate, and with diminution in the concentration of various intermediates of the tricarboxylic acid cycle and in the production of adenosine triphosphate (ATP) and phosphocreatine (P-creatine). Conversion of pyruvate to lactate, in the absence of oxygen and with an impaired mitochondrial electron transport system, results in raised levels of lactate which at first cause local vasodilatation and an increased supply of substrate. Any associated hypercapnia may also contribute to this vasodilatation. At higher levels of lactate more serious tissue acidosis occurs and leads to inhibition of glycolysis at the phosphofructokinase step, local oedema and loss of vascular autoregulation (Duffy 1972). Ischaemia has very similar biochemical effects on the CNS to those of hypoxaemia. Glycolysis is increased but uptake of

glucose can not be augmented since the blood supply is impaired. The concentrations of glucose, ATP and P-creatine in the brain fall. The increased lactate resulting from accelerated glycolysis cannot be removed from the tissue and an added complication is seen with ischaemic but not with hypoxaemic injury (Ames 1968). This refers to small vessel lesions, which prevent the reflow of blood into the ischaemic zones when perfusion pressure is restored. The reduced energy availability gives rise to impaired cell membrane and endothelial integrity, resulting in cytotoxic and vasogenic oedema with rise in intracranial pressure. The energy failure allows the influx of neurotransmitters into the cells, notably calcium, oxygen-free radicals, and excitatory neurotransmitters (Vannucci 1990).

Mortality and morbidity is in the third highest level in encephalopathy seen in septic or intracranial infection patients (meningitis, abscess, cerebritis) (Young 1992). The aetiology of these states may include metabolic disturbances, cerebral microcirculatory dysfunction and cerebral oedema. An increase in cerebral capillary endothelial permeability, as a result of main pathogenetic processes, will result in the exudation of proteinaceous, higher osmotic pressure fluid into the cerebral white matter (intra and extracellular space). The increased brain volume will raise the intracranial pressure and may cause alterations in cerebral function and fluid dynamics.

1.2. The blood-brain barrier: developmental and functional considerations

1.2.1. Blood-brain barrier differentiation

During the foetal development, vascularization of the CNS involves angiogenesis. In this process capillary sprouts originating from the leptomeningeal capillary plexus invade the early embryonic neuroectoderm. The "impermeability" of the cerebral capillaries is induced by the neural microenvironment consisting of neuronal and glial elements. In vertebrates, astrocytes provide a continuous ensheathment for brain capillaries, which is an important factor in the induction and maintenance of the BBB phenotype. From the first day of intraneural vascularisation onwards some morphological properties of the BBB are already present in the embryonic rodent cortex (Bauer et al., 1993). However, the barrier is gradually built up during embryonic development, some markers appear even postnatally. The immigrating endothelial cells lose their fenestration's and exhibit junctional complexes, one of the hallmarks of the BBB. While the number of these

interendothelial junctional complexes remains constant in intraneural capillaries at the following stage, the frequency of pinocytotic vesicles decreases significantly which is the other most important anatomical feature of the mature barrier. Glucose transporter 1 expression also appears very early in the foetal intraneural capillaries (Bauer et al., 1995). Parallelly, most parts of the CNS and spinal cord become impermeable to trypan blue and horseradish peroxidase during intrauterine development in rodents. Multidrug-resistance P-glycoprotein is also an early marker of the BBB in the developing human foetal brain (Schumacher and Mollgard, 1997). Mature brain capillaries express, beside the special anatomical features, transport systems for nutrients (glucose, amino acids, transferrin receptor), a well-developed functional barrier (monoamine oxidase enzyme, P-glycoprotein), and polarity evidenced by differential distribution of enzymes (e.g. Na^+-K^+-ATPase abluminally, alkaline phosphatase luminally etc.), glycoconjugates and anionic sites (Joó, 1996). A few special brain regions, such as the choroid plexus, the neurohypophysis, and the circumventricular organs (e.g. median eminence, organum vasculosum of the lamina terminalis, subfornical organ, and area postrema) positioned adjacent to the cerebral ventricular system, contain fenestrated capillaries, and therefore lack specific BBB properties even in adults.

1.2.2. Selective vulnerability in the early postnatal period

The human brain forms over an unusually long period compared to other organs, and there is also great postnatal activity in the development of receptors and transmitter systems. Concomitantly, permeability to small, lipid-insoluble molecules is greater in developing brain and the cerebrospinal fluid (CSF) contains high concentrations of proteins. More specific transport mechanisms, such as those involved in transfer of ions and amino acids, develop sequentially as the brain grows (Saunders 1999). While most of the basic structure is laid down before birth, not all of the members of barrier system (BBB, blood-CSF barrier, CSF-brain barrier) responsible for the separation of CNS are fully developed until the middle of the first year of life. Though tight junctions excluding proteins from extracellular space are present from very early in foetal development both at the BBB and blood-CSF barrier, proteins can be transferred from blood to CSF even after birth. Proteins can pass through the immature epithelial cells by an intracellular mechanism of tubulo-endoplasmic reticulum, and then from CSF to brain through the strap junctions in the developing neuroependyma. These age-specific differences may create potential temporal windows of selective vulnerability to damage in newborns.

1.2.3. BBB transport mechanisms, lysosomes

The BBB is formed by cerebral microvascular endothelial cells having specific morphological (presence of tight intercellular junctions, paucity of pinocytotic vesicles, lack of endothelial fenestrations) and functional (barrier properties, polarity, carrier functions) characteristics (Joó, 1996). Brain endothelial cells are functioning in close co-operation with the neighbouring astrocytes, neurons, pericytes, and microglial cells, and maintaining the homeostasis of the brain. A solute can permeate from blood to brain through the BBB either transcellularly or paracellularly, both pathways being regulated by sophisticated machineries (Broadwell and Banks, 1993; Anderson and Van Italie, 1995). Transendothelial permeation of a macromolecule

Table 2. **Acid phosphatase enzyme (AcP; EC 3.1.3.2.) family in the central nervous system**

high-molecular-weight (HMW) AcP (tartrate-sensitive AcP; fluoride-sensitive AcP)
mw > 100 kDa
lysosomal localisation
inhibited by tartrate and fluoride
effect: non-specific phosphomonoester hydrolase

Zn^{2+}-induced AcP (Zn^{2+}-dependent tyrosine phosphatase; Mg^{2+}-dependent myo-inositol monophosphatase; galactose-1 phosphatase)
mw = 62 kDa (31 kDa dimer)
dependent on Zn^{2+}; inhibited by vanadate
effect (at pH 5.5): hydrolase; phosphotyrosyl protein phosphatase

Low-molecular-weight (LMW) AcP (tartrate-resistant AcP; fluoride-resistant AcP)
mw = 18 kDa
cytosolic localisation
resistant to tartrate, fluoride, and vanadate
effect: hydrolase; phosphotyrosyl protein phosphatase

[phosphotyrosine]protein phosphatase
mw = 23 kDa
partly resistant to tartrate, fluoride; inhibited by vanadate
effect: phosphotyrosyl protein phosphatase; hydrolase

3-phosphohistidine/6-phospholysine phosphatase
mw = 150 kDa (holoenzyme: 94 kDa; subunit: 50 kDa)
partly resistant to fluoride; inhibited by tartrate, and vanadate
effect: hydrolase, phosphoamidase

Mg^{2+}-dependent p-nitrophenyl phosphate phosphatase
mw = 84 kDa
dependent on Mg^{2+}; resistant to tartrate; inhibited by fluoride, Zn^{2+},vanadate, and Ca^{2+}
effect: phosphotyrosyl protein phosphatase; hydrolase

Phosphoprotein phosphatase type 1
Mw = 56 kDa
dependent on Mn^{2+}; inhibited by Zn^{2+}
effect: phosphoprotein phosphatase

References: Attwood et al., 1988; Caselli et al.,1993; Chernoff J et al.,1985; Okada et al.,1986; Ohmori et al., 1994; Parthasarathy et al., 1987; Shimohama et al., 1993; Vincent and Averill, 1990; Weber et al.,1987; Singh et al.1990

by adsorptive endocytosis including endocytosis, transcellular passage and exocytosis, is suggested to involve the Golgi complex, endosomes, and transport vesicles. Paracellular permeability is thought to be regulated by the complex interaction of different tight junction proteins. Endogenous compounds, pharmaceuticals, or diseases may initiate a series of molecular events in the cerebral endothelium which later can have an effect on the regulation of the BBB permeability by either one or both of these pathways.

Though cerebral endothelial cells have relatively few lysosomes, a role for these cell organelles in the regulation of macromolecular transport through the BBB is proposed. Specific lysosomal enzymes, such as acid phosphatase (AcP), trimetaphosphatase, phosphoprotein phosphatase, β-galactosidase, and aryl sulphatase have been identified in cerebral endothelium (Audus and Raub, 1993), and increased AcP activity was supposed to be involved in the enhancement of transendothelial transport (Lossinsky et al., 1981; Tagami et al., 1983). AcP enzyme (orthophosphoric monoesther hydrolase, EC 3.1.3.2) has multiple molecular isoforms in the brain (Table 2), which differ from each other in their subcellular localisation, molecular weight, sensitivity for inhibitors and substrate requirement. The effect of AcP isoenzymes on the regulation of the BBB, however, has not been completely revealed yet.

1.2.4. Blood-brain barrier research in Szeged

In the field of the BBB research, the late Prof. Ferenc Joó was a well-known and highly respected scientist (Joó and Csillik, 1966; Joó, 1968; Joó, 1972). By the development of isolation technique of cerebral microvessels, he did pioneer work in the in vitro BBB research (Joó and Karnushina, 1973), and became a founding father of this rapidly growing subspecialty. He also gave an impetus to the in vitro investigations on cultured cerebral microvascular endothelial cells in Szeged (Pákáski et al., 1990; Kása et al., 1991; Deli et al., 1993; Krizbai et al., 1995). His "laboratory without walls" provided an unique opportunity for a number of clinicians interested in scientific research. As a result of these fruitful collaborations, excellent studies were published about the role of BBB permeability changes in the pathogenesis of subarachnoid haemorrhages (Dóczi et al., 1984; Dóczi et al., 1986) of neonatal bilateral pneumothorax (Temesvári et al., 1984a; Temesvári et al., 1984b; Dux et al., 1987); and of kainic acid-induced seizures (Sztriha et al., 1985, Sztriha et al., 1986). In our Department of Paediatrics, research on the BBB continued during the last decade, and a model of neonatal meningitis was also developed (Megyeri et al., 1992; Temesvári et al., 1993; Temesvári et al., 1995).

I regard as a great privilege that I had the possibility to discuss my research plans and to collaborate with the late Prof. Ferenc Joó and his group. I think I could not reach my achievements without his support and help.

1.3. Vasoactive and inflammatory mediators in the pathogenesis of cerebral damages

1.3.1. Mediators and brain injury

Clinical studies revealed that a large number of mediators are released during CNS injury caused by a wide variety of stimuli, such as brain trauma, tumour, cerebral ischaemia with or without reperfusion, infection, inflammation, neurodegenerative diseases etc. In the last decades, pathogenetic role for these diverse endogenous compounds, e.g. bradykinin, histamine, serotonin, arachidonic acid, prostaglandins, leukotriens, platelet-activating factor, free oxygen radicals, nitric oxide, was proved (see for review: Wahl et al. 1988; Joó, 1996). The role of leukocytes and some inflammatory mediators (cytokines, chemokines, and adhesion molecules) is also an emerging topic in the research of cerebral ischemia and reperfusion (see for review: del Zoppo, 1994; Pantoni et al., 1998). These inflammatory mediators, and in particularly the tumour necrosis factor-α (TNF-α), has long been in the focus of the research on the CNS infections, inflammations, and trauma (Beutler and Grau, 1993; Feuerstein et al., 1994).

1.3.2. Histamine

It has long been known that histamine plays a role in the brain both under physiological and pathological conditions. In the CNS, histamine can be released from three different cellular compartments, such as histaminergic neurons, perivascular mast cells, and cerebral microvessels (Edvinsson et al., 1993). On the other hand, histamine can act as a neurotransmitter (Schwartz et al., 1991), as well as a regulator of cerebral blood flow (Edvinsson et al., 1993), and BBB permeability (Joó, 1996). Different stimuli, such as cryogen injury (Orr, 1988), brain trauma (Mohanty et al., 1989), focal cerebral ischaemia (Adachi et al., 1992), perinatal asphyxia (Kovács et al., 1995), could increase the intracerebral histamine content. Previous studies revealed that both H_2-receptor-dependent, adenylate cyclase-mediated and H_1-receptor dependent, phosphoinositol-mediated mechanisms could contribute to the histamine-induced changes in the BBB permeability (see for review: Edvinsson et al., 1993; Joó, 1996). Recently, Mayhan (1996) published evidences that histamine could induce the BBB-permeability by a nitric oxide-mediated

activation of guanylate cyclase. Though indirect data, such as beneficial effect of antihistamines (Dux et al., 1987) and cerebral release of histamine (Kovács et al., 1995) during perinatal asphyxia, suggested a role for histamine in neonatal brain oedema formation, the direct effect of histamine on neonates was not previously known.

Cerebral endothelium possess both histamine type 1 (H_1)- and type 2 (H_2)-receptors (Joó, 1996, Karlstedt et al., 1999), but there are no data available about the presence of histamine type 3 (H_3) receptors or intracellular histamine binding sites (Hic). Though isolated cerebral microvessels were shown to have high histamine content, and low activity of histamine-synthesising L-histidine decarboxylase, (HDC) and histamine-metabolising (histamine-N-methyltransferase) enzymes (Karnushina et al., 1980; Robinson-White and Beaven, 1982), Karlstedt and colleagues (1999) failed to prove the histamine-producing capacity of cerebral endothelial cells. Huszti et al. (1995) described a specific high affinity uptake and release system for histamine in primary cultures of cerebral endothelial cells, while a non-saturable histamine transport was demonstrated in immortalised rat brain endothelial (RBE4) cells (Karlstedt et al., 1999). In RBE4 cells, internalised histamine was distributed not only in cytoplasm, but in the nucleus also, which might support a role to be elucidated for the Hic in the regulation of the BBB function. On the other hand, not even the possible contribution of histamine, as an intracellular messenger, to the CNS activity has been completely understood, yet. Controversial data are available about the cerebral effect of the Hic antagonist *N,N*-diethyl-2-(4-(phenyl-methyl)phenoxy)ethanamine (DPPE): it potentiated the chemically-induced convulsions in mice (Sturman et al., 1994), it suppressed *N*-methyl-D-aspartate currents in hippocampus slices (Sharonova et al., 1996), and it reduced the cerebral infarction due to focal ischaemia in mice (Cramer and Toorop, 1998). Our working hypothesis was that Hic antagonist DPPE, similarly to the H_2-antagonists and some H_1-blockers (Edvinsson et al., 1993; Tósaki et al., 1994; Joó, 1996) could prevent the cerebral ischaemia-reperfusion-induced vasogenic oedema formation. Later we decided to check the effect of DDPE in healthy adult rats, too.

1.3.3. Tumour necrosis factor-α

TNF-α plays a crucial role in the development of acquired immunodeficiency syndrome, malaria, meningococcal disease, parasitic infections and sepsis (Beutler and Grau, 1993; Feuerstein et al., 1994) and it proved to be an important mediator of brain injury during cerebral ischemia-reperfusion (del Zoppo, 1994) and neurodegenerative diseases (Sharief and Thompson, 1992).

The BBB plays an active role in TNF-α-induced cerebral damage. Stimulated cells located both at luminal and abluminal sides can produce TNF-α which induces capillary endothelial cell proinflammatory responses (e.g. leukocyte-endothelial adhesion, neutrophil transmigration), and increased procoagulant activity by the activation of some serine proteases (Beutler and Grau, 1993; del Zoppo, 1994; Feuerstein et al., 1994). On the other hand, TNF-α has a specific bidirectional transport system through which it passes the BBB (Gutierrez et al., 1993).

Previous studies indicated that both intravascular and intracisternal TNF-α administration resulted in vasogenic brain oedema formation in newborn pigs (Megyeri et al., 1992; Ábrahám et al., 1996). It is assumed that activated leukocytes contribute to the acute BBB opening, because no similar changes in permeability were found in an in vitro reconstituted model of this barrier (cerebral endothelial cells co-cultured with astrocytes) (Deli et al., 1995c). The vasoconstrictor effect of intracisternal TNF-α (Megyeri et al., 1992; Tureen, 1995) suggests the involvement of cerebrovascular smooth muscle cells, too. It is known that oxygen free radicals produced by endothelial cells, brain cells or white blood cells play a role in the mediation of the TNF-α-induced brain injuries (del Zoppo, 1994; Feuerstein et al., 1994; Tureen, 1995). High superoxide production of human monocytes and neutrophil granulocytes evoked by TNF-α was blocked in vitro by the addition of 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), a non-toxic, water-soluble, irreversible inhibitor of serine proteases (Megyeri et al., 1995; Remold-O'Donnell and Parent, 1995).

1.4. Animal models of cerebral ischaemia

1.4.1. Cerebral ischaemia and reperfusion related injury

Alterations in the cerebral blood flow (CBF) induced by the changes of the oxygen level in the blood are of primary importance in understanding the genesis of ischaemia-reperfusion related injuries. Numerous authors suggest that the loss of vascular autoregulation coupled with hypotension reduced CBF to the point of producing tissue necrosis and subsequent cerebral oedema. With reoxygenation cerebral perfusion rebounds. The role of postischemic impairment of microvascular perfusion and "luxury perfusion" in the genesis of tissue damage in the asphyxiated, resuscitated human neonate is still unclear. The release of cytotoxins, notably free radicals, during reperfusion could contribute to hypoxic-ischemic brain damage. Increased amounts of inhibitory neuromodulators, such as endorphins, adenosine, and gamma-amino butiric acid, are believed to cause persistent depression of neuronal activity.

1.4.2. Rat models of cerebral ischaemia and reperfusion

Experimental animal models can help us in understanding the changes occurring during cerebral ischaemic damage in patients. In the rat, similarly to the human anatomical situation, but in contrast with that of the gerbil, there is a Willis circle, so a simple bilateral carotid occlusion is not enough to produce a total forebrain ischaemia. Therefore, in a series of models, other factors, such as systemic hypotension, hypercapnia, or hypoxia, were also added to the procedure of carotid clamping (Kagström et al., 1983). Definitive ligation of bilateral common carotid arteries could also result in reproducible brain injuries in sensitive rat strains, such as in Long Evans rats (Lespinasse et al., 1983), or in female Sprague-Dawley CFY rats (Tósaki et al., 1985). These experimental models are suitable for the investigation of the effect of global, subtotal ischaemia, while animal models of focal cerebral ischaemia mimics the changes during thromboembolic ischaemic attacks (Olsson et al., 1971; Yoshimoto et al., 1978). The consequence of neonatal hypoxic-ischaemic brain injury can be evaluated in a model where 7-day-old rats were subjected to unilateral common carotid artery ligation and hypoxia (Altman et al., 1984). The 4-vessel occlusion model of brain ischaemia (both vertebral arteries and both common carotid arteries are closed) described by Pulsinelli and Brierley (1979) is appropriate to reveal the changes occurring in resuscitation and reperfusion in clinical practice. The 3-vessel occlusion model was developed by a slight modification (electrocoagulation of basilar artery instead of both vertebral arteries) of the previous technique (Kameyama et al., 1985).

2. AIMS OF THE STUDY

The present series of animal experiments were undertaken to reveal some pathophysiological mechanisms leading to the damage of the blood-brain barrier and cerebral oedema formation. We looked for answers to the following questions:

2.1. The effect of histamine on the BBB in newborn pigs

2.1.1. Can exogenous histamine increase the permeability of the BBB in newborn pigs?

2.1.2. Are there any regional differences in the histamine-induced BBB permeability changes?

2.1.3. Does intracarotid histamine administration regulate the activity of acid phosphatase

isoenzymes in cortical microvessels and brain tissue?

2.2. The effect of TNF-α on the BBB in newborn pigs

2.2.1. What kind of regional and temporal BBB permeability changes can be induced by intracarotid TNF-α administration in newborn pigs?

2.2.2. Can serine protease inhibitor AEBSF prevent the TNF-α-induced BBB disruption?

2.3. BBB permeability changes after cerebral ischaemia-reperfusion induced by the 4-vessel occlusion in male Wistar rats

2.3.1. What kind of regional and temporal BBB permeability changes develop after cerebral ischaemia-reperfusion in rats?

2.3.2. Can Hic antagonist DPPE prevent the cerebral ischaemia-reperfusion-induced BBB damage?

2.3.3 Does Hic antagonist DPPE have any in vivo effect of the BBB permeability?

2.4. BBB permeability changes after forebrain ischaemia induced by the 2-vessel occlusion in female Sprague-Dawley CFY rats

2.4.1. Can bilateral carotid occlusion effectively reduce the CBF in the parietal cortex of female Sprague-Dawley rats?

2.4.2. What kind of temporal changes in cortical water content and BBB permeability changes develop in carotid-ligated rats?

2.4.3. Does bilateral carotid occlusion regulate the activity of acid phosphatase isoenzymes in cortical microvessels and brain tissue?

3. MATERIALS AND METHODS

3.1. Animal models

3.1.1. Ethical issues

The experimental procedures followed the National Institute of Health (Bethesda, MD, U.S.A.) Guidelines for the care and use of laboratory animals and were approved by the local Ethical Committee on Animal Investigation (ÁTB 53).

3.1.2. Histamine and TNF-α in newborn pigs

Newborn pigs of either sex (age: 4-8 h; weight: 1.030-1.610 kg) were included. After pentobarbital (30 mg/kg) anaesthesia, one of the umbilical arteries was catheterised, cardiovascular, blood gas and acid-base parameters were monitored (Ábrahám *et al.*, 1996). The left internal carotid artery of the animals was catheterised through the external branch (**Fig. 1**), and the vasoactive mediator diluted in 0.5 ml isotonic saline was given in slow intraarterial injection. Then the catheter was removed and the external carotid artery was ligated. Animals receiving isotonic saline served as control.

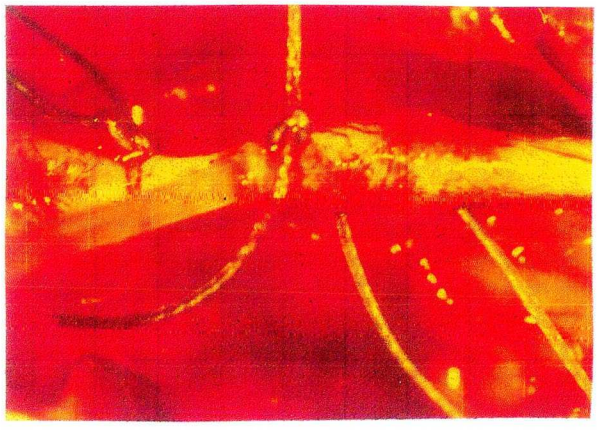

Figure 1. Microphotograph of the implanted catheter. The external branch of carotid artery is ligated (left), the internal branch and the common carotid artery (right) are looped only, to keep the free blood flow

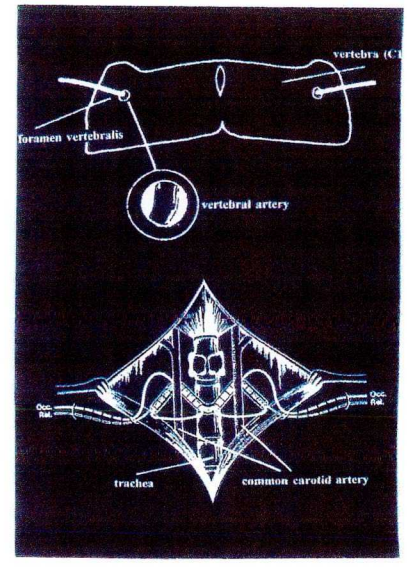

Figure 2. Schematic drawing of the vertebral arteries with the foramens (upper); and the device with thin wires inside placed around the two common carotid arteries (lower draft)

In the histamine study, exogenous histamine was administered intracarotidly in the following doses: 0 mol, 10^{-6} mol, $5x10^{-6}$ mol, 10^{-5} mol, $5x10^{-5}$ mol, 10^{-4} mol (n=12 in each group). Newborn pigs were sacrificed 1 h after the challenge. BBB permeability changes were determined in the frontal, parietal, and occipital cortex, hippocampus, and periventricular white matter of 6 animals. On the other hand, brain tissue samples for enzyme activity determination were also taken from the same brain regions of the remaining 6 animals. Cortical microvessels were also isolated from the brain tissue of the same animals (Tontsch and Bauer, 1989)

In cytokine study, recombinant human TNF-α was infused into the left internal carotid artery in the following doses: 0 IU; 1,000 IU; 10,000 IU; 100,000 IU. The BBB permeability changes were determined in 5 brain regions (parietal cortex, hippocampus, striatum, periventricular white matter and cerebellum) 1, 2, 4, 8, 16 h after the challenge (n=5 in each group). In the drug study with AEBSF, the animals (n=6 in each group) were prae-treated intravenously with 0, 2.4, 4.8, 9.6 and 19.2 mg/kg AEBSF (mw: 239.5) diluted in 1 ml isotonic saline1 h prior to the intracarotid infusion of 10,000 IU TNF-α.

3.1.3. Cerebral ischaemia-reperfusion: the 4-vessel occlusion model in male Wistar rats

Cerebral ischaemia was induced according to the 4-vessel occlusion model of Pulsinelli and Brierley (1979) (**Fig. 2**). Male Wistar rats were anaesthetised by pentobarbital (30 mg/kg) during the surgical intervention. The animal was turned prone and a sagittal approach was performed on the cranial part of the neck. The first cervical vertebra (C1) was approached via a midline muscle-splitting incision. The alar foramina were identified on both sides and the neurovascular branch was coagulated with a bipolar diathermic forceps. During this part of the procedure we used a Zeiss operating microscope. The wound was closed with 4/0 Vicryl. Then, the animal was turned on the back and a median incision was done on the neck. Common carotid arteries on both sides were isolated and an occluding device described by Tomida *et al.* (1987) was implanted around the vessels (**Fig. 3A**). This device is a W-shaped tube, where the two lower angles of the W are flexible fixed. The instrument has two wires inside the lumen. One of them around the arteries is similar to the number 8 (occluding wire), while the other one is a simple line in the tubes (releasing wire). When we pull the thinner, 8-shaped wire, it closes the two parts of the device and occludes both common carotid arteries (**Fig. 3B**). The occlusion can be released with the help of the thicker wire and the reperfusion of vessels can begin (**Fig. 3C**). We placed these wires subcutaneously and pulled through the skin. The wires were fixed behind the animal's head until the beginning of the experiment. On the next day, the animals were subjected to total cerebral ischaemia by the occlusion of carotid arteries for 20 min under superficial ether narcosis. After the procedure, the device was released and a reperfusion period with different duration (1, 2, 4, 8, and 16 h; n=6 in each group) was allowed to the rats. After the releasing of the occluding device a group of ischaemic animals were given intravenous injection of 5 mg/kg DPPE diluted in 0.5 ml isotonic saline and the BBB permeability changes were determined 2, 4 and 8 h after the injection (n=6 in each group).

To reveal the effect of the Hic binding site antagonist on the BBB permeability in healthy animals, DPPE (5 and 20 mg/kg) was also given intravenously to control male Wistar rats. The extravasation of the BBB tracers was measured 2, 4 and 8 h after the intravenous injection (n=6 in each group).

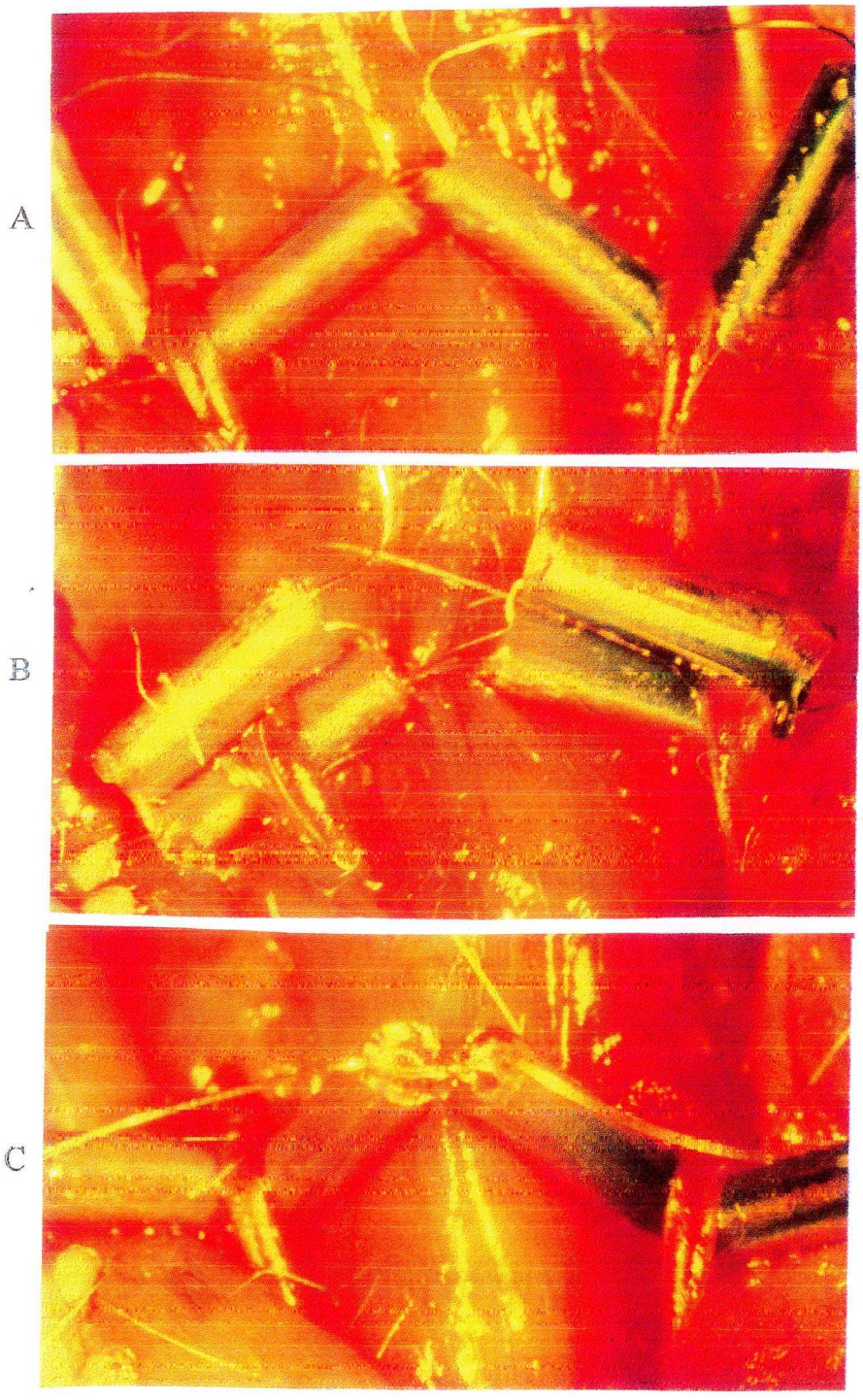

Figure 3. Microphotograph of the occluding device: **(A)** implanted around the two common carotid arteries; **(B)** occlusion indicated by closing of tubes with the controlling wires inside; **(C)** position after the release procedure with the wires, during the reperfusion

3.1.4. Forebrain ischaemia: 2-vessel occlusion in female Sprague-Dawley CFY rats

A closed colony of randomly bred Sprague-Dawley CFY rats (200-250 g) originally produced by the Laboratory Animals Institute (Gödöllõ, Hungary) was used. Female animals of this strain proved to be very sensitive to forebrain ischaemia evoked by bilateral common carotid artery occlusion (Tósaki *et al.*, 1985; Ábrahám *et al.*, 1992). Under light ether anesthesia, both common carotid arteries were exposed through a middle incision in the neck, then the vessels were carefully separated and tied with silk. The skin was sutured, and the rats were allowed to recover from anesthesia.

After carotid occlusion, clinical data and regional CBF in parietal cortex were monitored in 6 ischaemic animals for 2 h. Sham-operated rats (n=6) served as control. In a subgroup of ischaemic animals, rats were sacrificed 1, 2, 4, 8, 16 h after the beginning of the cerebral ischaemia (n=6 at each time point), and samples from parietal cortex were taken for the determination of percentage water content, and the activity of acid phosphatase isoenzymes. Cortical microvessels were also isolated from the brain tissue of the same animals. In another subgroup, BBB permeability changes in parietal cortex were also determined 1, 2, 4, 8, 16 h after the beginning of the cerebral ischaemia (n=6 at each time point).

3.2. Measurement of clinical parameters

3.2.1. Hearts rate and mean arterial blood pressure

In newborn pigs, one of the umbilical arteries was catheterised, while in female Sprague-Dawley CFY rats a cannula was inserted into the left femoral artery under pentobarbital anaesthesia (30 mg/kg intraperitoneally). Changes in heart rate and mean arterial blood pressure were determined by a Statham P230 transducer (Statham Instruments Inc., Los Angeles, CA, U.S.A.).

3.2.2. Arterial acid-base and blood gas parameters

Arterial blood chemistry parameters (pH, standard bicarbonate, partial CO_2 tension, partial O_2 tension) were determined by the standard Astrup method using an ABL-330 equipment (Radiometer, Copenhagen, Denmark).

3.2.3. Regional cerebral blood flow in parietal cortex (in 3.1.4. model)

Measurement of cerebral blood flow (CBF) was performed using the hydrogen-clearance

technique (Pásztor *et al.*, 1973). Two permanent platinum-irridium alloy electrodes (diameter= 100 μm) were implanted in the parietal cortex (one into the left, the other into the right hemisphere, 2 mm lateral from bregma) of female Sprague-Dawley CFY rats (n=6) anesthetized by pentobarbital injected in a dose of 35 mg/kg intraperitoneally. The electrodes were in contact with the upper 1 mm of the brain and positively polarized at +200mV with reference to a silver-silver chloride reference electrode placed subcutaneously in the back of animals. Two fixation screws were inserted into the skull beside the electrodes, and the total arrangement was embedded in dental cement as a cylindrical "crown". After a recovery period of 3 days the rats were re-anaesthetized and CBF was measured by polarography. The animals inhaled hydrogen-air mixture, mixed by a Dräger rotameter (Dräger Medizintechnik GmBH, Lubeck, Germany), partial H_2 pressure: 6.67 kPa (= 50 mmHg), for 10-50 seconds. The hydrogen clearance of the parietal cortex was registered by a Tesla BM 483 picoammeter (Tesla, Prague, Czech Republic) and a Kutesz type 175 recorder (Kutesz, Budapest, Hungary). CBF was determined before, and 3, 20, 40, 60, 90, 120 min after bilateral carotid occlusion. CBF was calculated by the following mathematical formula:

$$CBF = 1 \times 2t^{-1} \times \ln \{(I_{80} - I_{40}) \times (I_{40} - I_x)^{-1}\},$$

where I_{80}= 80% of the maximal current intensity (CI); I_{40}= 40% of the maximal CI; t= time, during which CI decreased by I_{80} to I_{40}, I_x= CI 2t time after I_{80}.

3.3. Blood-brain barrier permeability measurements

All through the animal experiments, BBB permeability was measured according to the method of Ábrahám *et al.*, (1996). We determined the extravasation of the following BBB tracers (both from Sigma, St. Louis, MO, U.S.A.): sodium fluorescein (mw: 376, Stokes radius: 0.55 nm) and Evan's blue-labelled albumin (mw: 67,000, Stokes radius 3.5 nm). Animals were given a solution of both dyes in isotonic saline (2%, 5 ml/kg) 30 min before the planned end of the experiments. After this perfusion period, the animals were sacrificed, blood samples were taken and the intravascular tracers were removed by a perfusion with 200 ml/kg isotonic saline. Then brain tissue samples were collected. Sera and brain tissue samples were homogenised in 3.0 ml of cold 7.5 % trichloroacetic acid and centrifuged at 10,000 x *g* for 10 min. From the supernatants, the absorbency of Evan's blue was measured at 620 nm, while the emission of sodium fluorescein at 525 nm, after excitation at 440 nm by a Hitachi F2000 fluorimeter (Tokyo, Japan). The concentrations of tracers in samples were determined using freshly prepared dilution series each

time. Extravasation was expressed as brain tissue concentration divided by final serum concentration: μg tracer/mg brain tissue x (μg tracer/μl serum).

3.4. Water content in the brain tissue

In the 2-vessel occlusion model, for determination of brain water content, rats were decapitated, tissue samples from parietal cortex were taken and their wet weights were immediately measured. The brain samples were then dried at 110°C for 48 hours to obtain their dry weights. Water content was expressed as a percent of the total wet tissue weight.

3.5. Biochemical determination of acid phosphatase enzyme activity in cerebral microvessels and brain tissue

AcP enzyme activity was measured by the rate of hydrolysis of *p*-nitrophenylphosphate both in homogenised brain tissue and in isolated cortical microvessels (Shimohama *et al.*, 1993; Szabó *et al.*, 1996). Samples in triplicates were incubated in 96-well microtiter plates in 160 μl solution containing 0.1 M acetate buffer (pH=5.5) and 2.5 mM *p*-nitrophenylphosphate at 37 °C for 1 h. In separate triplicated samples, enzyme activity was influenced by adding one of the following materials: L-(+)tartrate (10 mM), sodium fluoride (5 mM), or $ZnSO_4$ (2.5 mM) in order to determine the activity of different isoenzymes. After incubation, 45 μl of 1 M NaOH was added to the samples to stop the reaction and the absorbency was read at 405 nm by a Multiscan Biochromatic type 348 ELISA reader (Labsystems, Helsinki, Finland). Enzyme activities were determined from a calibration curve using increasing concentrations of purified AcP (AcP Lintrol, Sigma, St. Louis, MO, U.S.A.). Protein content of the samples was determined according to the method of Bradford (1976), and each enzyme activity was expressed as mU/mg protein.

3.6. Statistical analysis

All data presented are means ± S.E.M., the number of animals in each group is indicated in the corresponding legend. The values were compared between different experimental groups using the appropriate one of the following statistical methods in each case: one way analysis of variance, Kruskal-Wallis one way analysis of variance on ranks, Student's t-test, Mann-Whitney rank sum test. Changes were considered statistically significant at $P < 0.05$.

4. RESULTS

4.1. Histamine in newborn pigs

4.1.1. Clinical data

Histamine administration resulted in no significant change in the blood gas and acid parameters of the animals during the 1 hour long experiment (data not shown). However, a temporary cardiac arrhythmia was seen in 2 of 12 newborn pigs receiving 10^{-4} mol histamine in slow intraarterial injection of 0.5 ml isotonic saline within 5 min. The other animals were unaffected and no significant change in the mean arterial blood pressure and heart rate compared to the values measured in control pigs was recorded (data not shown).

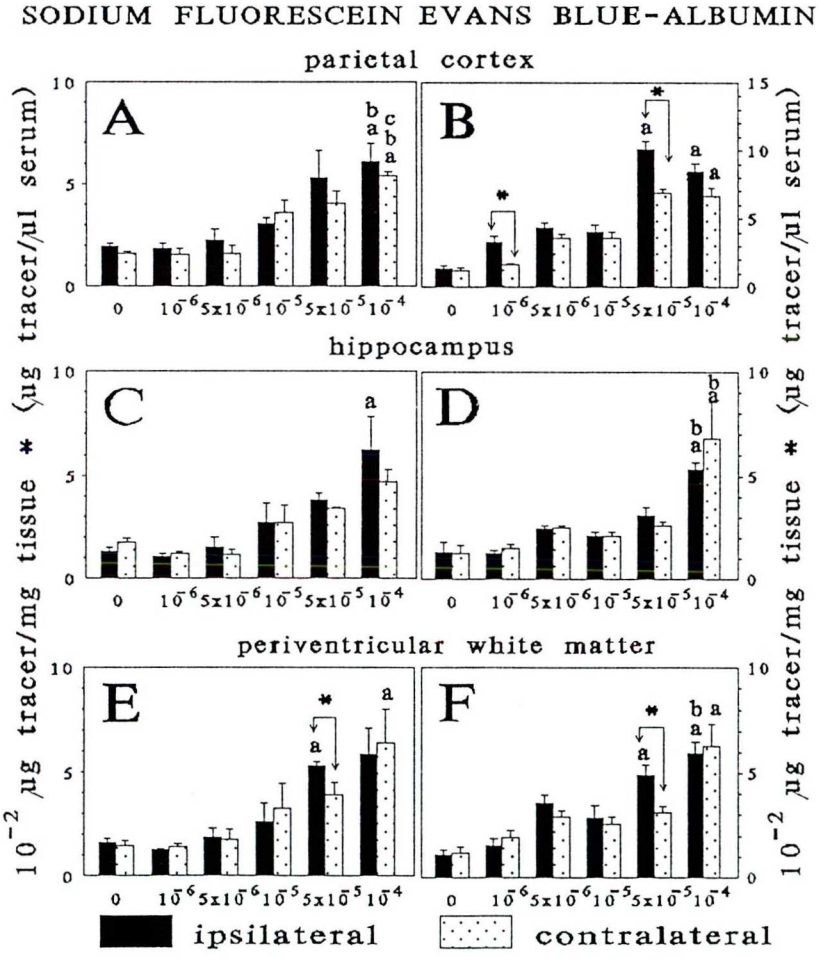

Figure 4. BBB permeability changes in parietal cortex (A,B), hippocampus (C,D), and periventricular white matter (E,F) of newborn pigs 1 h after the intracarotid administration of histamine. The permeability markers were: sodium fluorescein (A,C,E) and Evan's blue-albumin (B,D,F). Extravasations were expressed as 10^{-2} µg dye/ mg brain tissue x (µg dye/µl serum)$^{-1}$ for both dyes. Symbols indicate significant differences ($P < 0.05$) compared to the following treatments: a: 0 mol, b: 10^{-6} mol; c: $5x10^{-6}$ mol histamine; while * shows significant difference between ipsi- and contralateral sides in the same animal group

4.1.2. BBB permeability

Histamine increased the BBB permeability in each brain region (**Fig. 4**), but only higher doses resulted in significant ($P < 0.05$) changes: 10^{-4} mol for sodium fluorescein, and $5x10^{-5}$ and 10^{-4} mol for albumin transport. The highest dose of histamine caused a 3- to 6-fold increase in the permeability of tracers in each brain region compared to that in control group.

4.1.3. Acid phosphatase isoenzymes in cerebral microvessels and brain tissue

Intracarotid histamine administration resulted in a dose-dependent increase in total acid phosphatase activity in isolated microvessels, but not in brain tissue samples. Linear correlation was found between the dose of histamine and acid phosphatase enzyme activity in microvessels:

enzyme activity = 81.3 + 1,21 x 10^6 x [histamine dosis] (n=24, R=0.602, p < 0.01)

Each dose of histamine, but 10^{-6} mol, significantly ($P < 0.05$) increased the tartrate-resistant acid phosphatase activity in ipsilateral cortical microvessels, and a similar tendency was also seen in the contralateral side (**Fig. 5A**). However, tartrate-sensitive acid phosphatase activity was

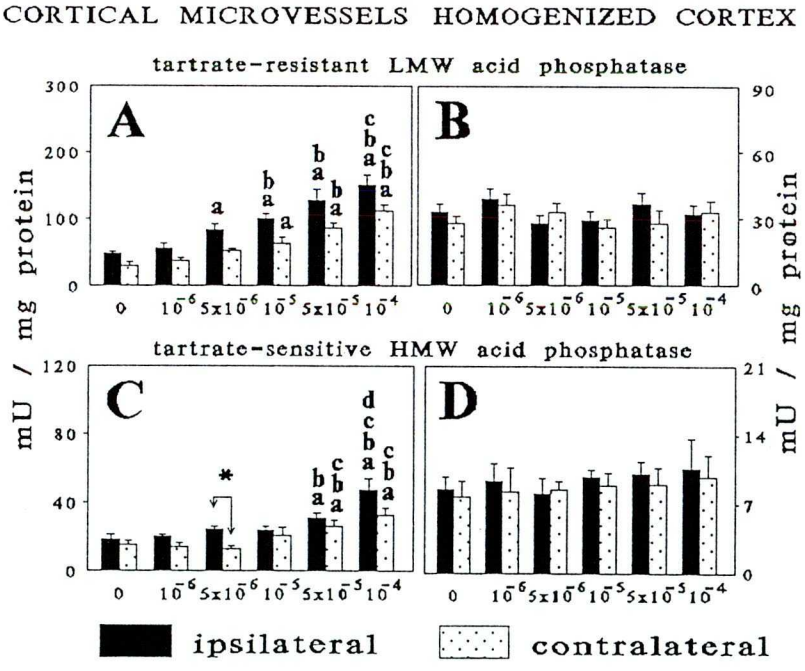

Figure 5. The activity of acid phosphatase (AcP) isoenzymes in isolated cortical microvessels (A,C) and homogenised parietal cortex (B,D) of newborn pigs 1 h after the intracarotid administration of histamine. Changes in the tartrate-resistant LMW (A,B) and tartrate-sensitive HMW (C,D) AcP isoforms were expressed as mU/mg protein. Symbols indicate significant differences ($P < 0.05$) compared to the following treatments: a, 0 mol, b, 10^{-6} mol; c, $5x10^{-6}$; d, 10^{-5} mol histamine; while * means significant difference between ipsi- and contralateral sides in the same animal group.

significantly increased only after the injection of $5x10^{-5}$ and 10^{-4} mol histamine (**Fig. 5C**). There was no significant histamine-induced change in the enzyme activity in homogenised brain tissue samples (**Fig. 5B-D**).

4.2. TNF-α in newborn pigs

4.2.1. Clinical data

During the experimental period vital cardiovascular parameters did not change significantly after administration of TNF-α and AEBSF compared to those measured in control animals (data not shown).

4.2.2. BBB permeability: dose- and time-dependent changes in different brain regions

In newborn pigs, rhTNF-α administration (in the doses of 1,000 IU; 10,000 IU; and 100,000 IU) resulted in significant ($P < 0.05$) increases in BBB permeability for sodium fluorescein both in ipsi- and contralateral parietal cortex (**Fig. 6A**), hippocampus (**Fig. B**), striatum (**Fig. C**), periventricular white matter (**Fig. D**), and cerebellum (**Fig. E**). The BBB opening for the small molecular weight tracer started as soon as 1 h after the cytokine infusion, and a time- and dose-dependent pattern was seen (**Fig. 6**). A similar elevation in BBB permeability was measured for Evan's blue labelled albumin in 5 brain regions examined, however, the significant ($P < 0.05$) changes started later, about 2 h after the TNF-α treatment (**Fig. 7**).

4.2.3. Protective role of AEBSF in TNF-α-induced BBB-damage

Injection of 10,000 IU rhTNF-α resulted in BBB opening both for sodium fluorescein and albumin in porcine brain (**Fig. 8**). AEBSF prae-treatment, in the doses of 4.8-19.6 mg/kg, significantly ($P < 0.05$) inhibited the TNF-α induced increase in sodium fluorescein permeability in all regions (**Fig. 8 A-C-E-G-I**). The effect was dose-dependent and the highest dose could prevent the BBB opening for this intravascular tracer. However, the effect of AEBSF on the increased albumin permeability was less expressed: only the dose of 19.6 mg/kg AEBSF could significantly ($P < 0.05$) decrease the extravasation in parietal cortex of both sides (**Fig. 8B**), as well as in ipsilateral hippocampus (**Fig. 8D**). Comparing BBB permeability measured in the same region of 2 hemispheres, statistically significant ($P < 0.05$) differences were only seen in 5 cases for sodium fluorescein (**Fig. 8 A-C-G-I**) and in 1 case for Evan's blue-albumin (**Fig. 8H**).

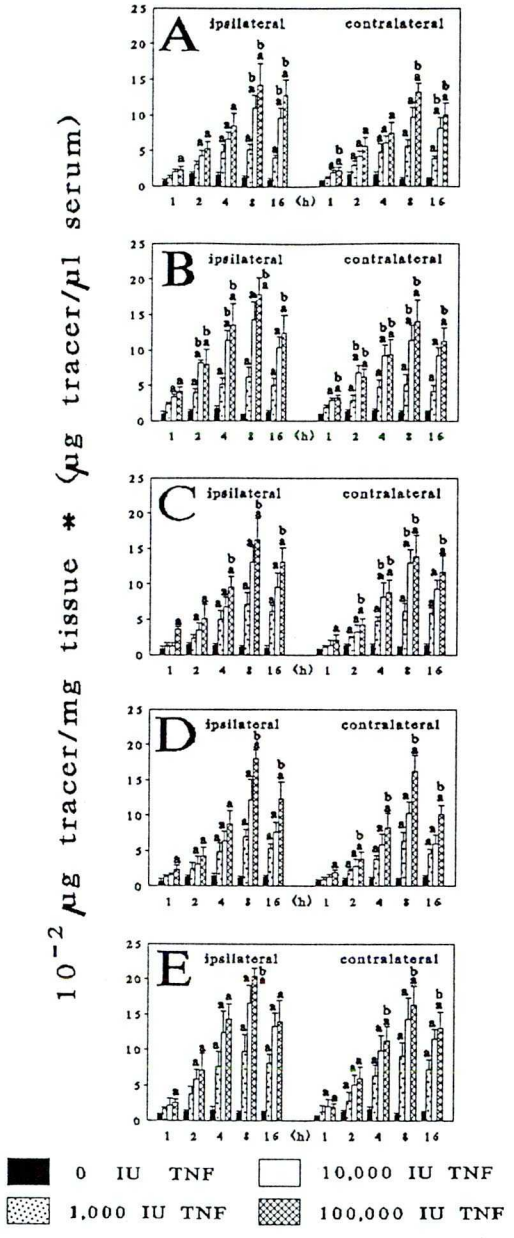

Figure 6. BBB permeability for sodium fluorescein in parietal cortex (A), hippocampus (B), striatum (C), periventricular white matter (D), and cerebellum (E) of newborn pigs 1, 2, 4, 8, and 16 h after the intracarotid administration of TNF-α. The doses of cytokines were 0 IU (closed bars), 1,000 IU (dotted bars), 10,000 IU (open bars), and 100,000 IU (horizontal line bars) TNF-α, respectively. Extravasations were expressed as 10^{-2} µg dye/ mg brain tissue x (µg dye/µl serum)$^{-1}$. Letters indicate significant differences ($P < 0.05$) compared to values measured concomitantly in groups receiving the following doses of TNF-α: a, 0 IU; b, 1,000 IU; c, 10,000 IU, respectively.

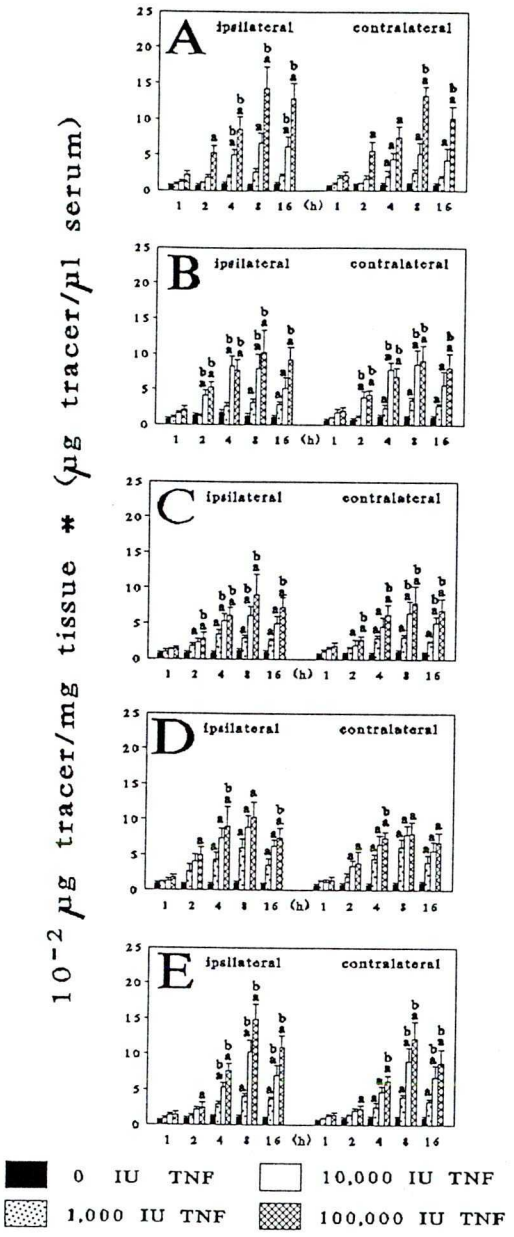

Figure 7. BBB permeability for Evan's blue-labelled albumin in parietal cortex (A), hippocampus (B), striatum (C), periventricular white matter (D), and cerebellum (E) of newborn pigs 1, 2, 4, 8, and 16 h after the intracarotid administration of TNF-α. The doses of cytokines were 0 IU (closed bars), 1,000 IU (dotted bars), 10,000 IU (open bars), and 100,000 IU (horizontal line bars) TNF-α, respectively. Extravasations were expressed as 10^{-2} µg dye/ mg brain tissue x (µg dye/µl serum)$^{-1}$. Letters indicate significant differences ($P < 0.05$) compared to values measured concomitantly in groups receiving the following doses of TNF-α: a, 0 IU; b, 1,000 IU; c, 10,000 IU, respectively.

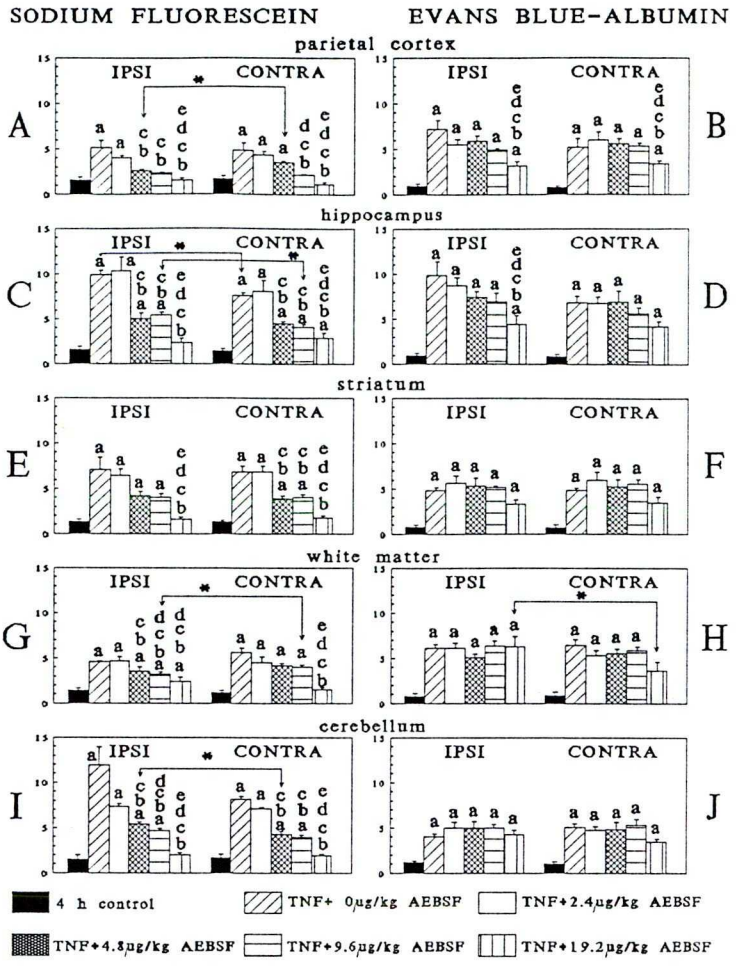

Figure 8. The effect of 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) pretreatment on the BBB permeability 4 h after intracarotid challenge with 10,000 IU TNF-α. Filled bars: control animals, while the other columns represent the 5 TNF-α-treated groups according to the doses of AEBSF applied: 0 mg/kg (hatched bars), 2.4 mg/kg (open bars), 4.8 mg/kg (cross-hatched bars), 9.6 mg/kg (horizontal line bars), and 19.2 mg/kg (vertical line bars), respectively. Permeability was determined in parietal cortex (A,B), hippocampus (C,D), striatum (E,F), periventricular white matter (G,H), and cerebellum (I,J) of newborn pigs. The permeability markers were: sodium fluorescein (A,C,E,G,I) and Evan's blue-albumin (B,D,F,H,J). Extravasations were expressed as 10^{-2} μg dye/ mg brain tissue x (μg dye/μl serum)$^{-1}$ for both dyes. Letters a-e indicate significant differences ($P < 0.05$) compared to values measured a, in control group; as well as in TNF-α-treated groups receiving the following doses of AEBSF b, 0 mg/kg; c, 2.4 mg/kg; d, 4.8 mg/kg; and e, 9.6 mg/kg, respectively. Symbol * shows significant difference between permeability of ipsi- and contralateral sides of the same brain region.

4.3. The 4-vessel occlusion rat model

4.3.1. BBB permeability: time-dependent changes in different brain regions

Cerebral ischaemia-reperfusion resulted in a significant ($P < 0.05$), time-dependent increase in BBB permeability for albumin in each region examined (**Fig. 9 B-D-F-G**). While sodium fluorescein transport was elevated only at 2-h-reperfusion in the striatum compared to the value measured in untreated control animals (**Fig. 9E**).

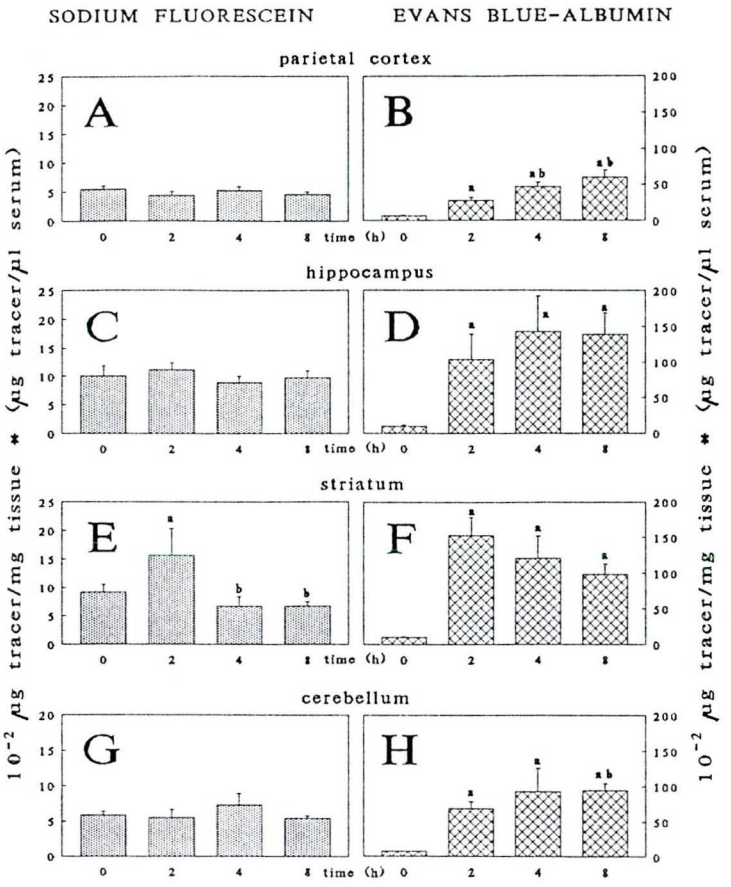

Figure 9. The effect of cerebral ischaemia and reperfusion on the BBB permeability 2, 4, and 8 h after the end of ischaemia. Permeability for sodium fluorescein (A,C,E,G) and Evan's blue-albumin (B,D,F,H) was determined in parietal cortex (A,B), hippocampus (C,D), striatum (E,F), and cerebellum (G,H) of newborn pigs. Extravasations were expressed as 10^{-2} µg dye/ mg brain tissue x (µg dye/µl serum)$^{-1}$ for both dyes. Letters indicate significant differences ($P < 0.05$) compared to values measured at the following time-points in the same brain region: a, 0 h; b, 2 h, respectively.

4.3.2. Effects of Hic-receptor antagonist DPPE on BBB permeability in control and 4-vessel-occluded rats

Sodium fluorescein permeability was significantly ($P < 0.05$) increased compared to the values measured in untreated control group 2 h after the DPPE treatment (in intravenous dose of with 5 mg/kg) in hippocampus, striatum, and cerebellum (**Fig. 10 C-E-G**), but this extravasation decreased later, and it could significantly decrease below the control value at 8 h in parietal cortex. There was a time-dependent increase in Evan's blue-albumin extravasation in all brain regions measured (**Fig. 10 B-D-F-H**). The peak in albumin flux occurred 2 h after the injection, with a 5- to 12-fold increase in permeability. A separate group of animals were given a dose 20 mg/kg DPPE, which resulted in similar, but more expressed disturbance in the BBB permeability (data not shown).

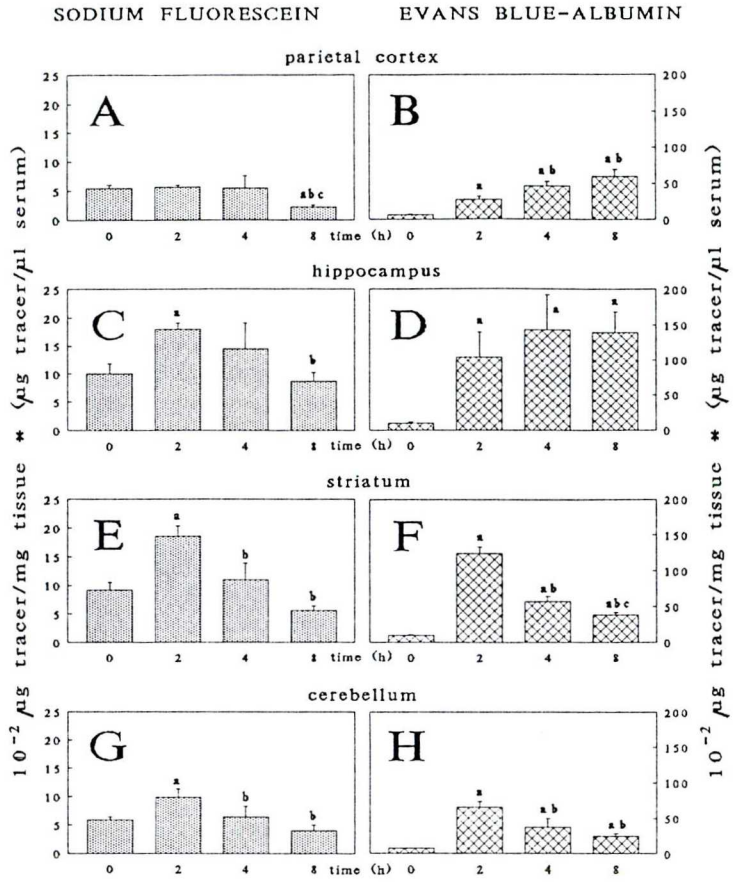

Figure 10. The effect of the treatment of Hic antagonist DPPE on the BBB permeability 2, 4, and 8 h after the intravenous injection. Permeability for sodium fluorescein (A,C,E,G) and Evan's blue-albumin (B,D,F,H) was determined in parietal cortex (A,B), hippocampus (C,D), striatum (E,F), and cerebellum (G,H) of newborn pigs. Extravasations were expressed as 10^{-2} µg dye/ mg brain tissue x (µg dye/µl serum)$^{-1}$ for both dyes. Letters indicate significant differences ($P < 0.05$) compared to values measured at the following time-points in the same brain region: a, 0 h; b, 2 h, c, 4 h, respectively.

A similar significant increase ($P < 0.05$) in sodium fluorescein extravasation could be seen at 2 h, but not at 4 and 8 h, in 3 brain regions of those DPPE-treated rats which also underwent ischaemia-reperfusion (**Fig. 11 C-E-G**). Though the elevations in BBB permeability for albumin were more prolonged in the brain of these animals (**Fig. 11 B-D-F-H**) than that in healthy DPPE-treated group, at 2 h these permeability values in each region were significantly lower in DPPE-treated postischaemic rats than those in the groups of DPPE-treated healthy animals, or postischaemic rats without DPPE administration.

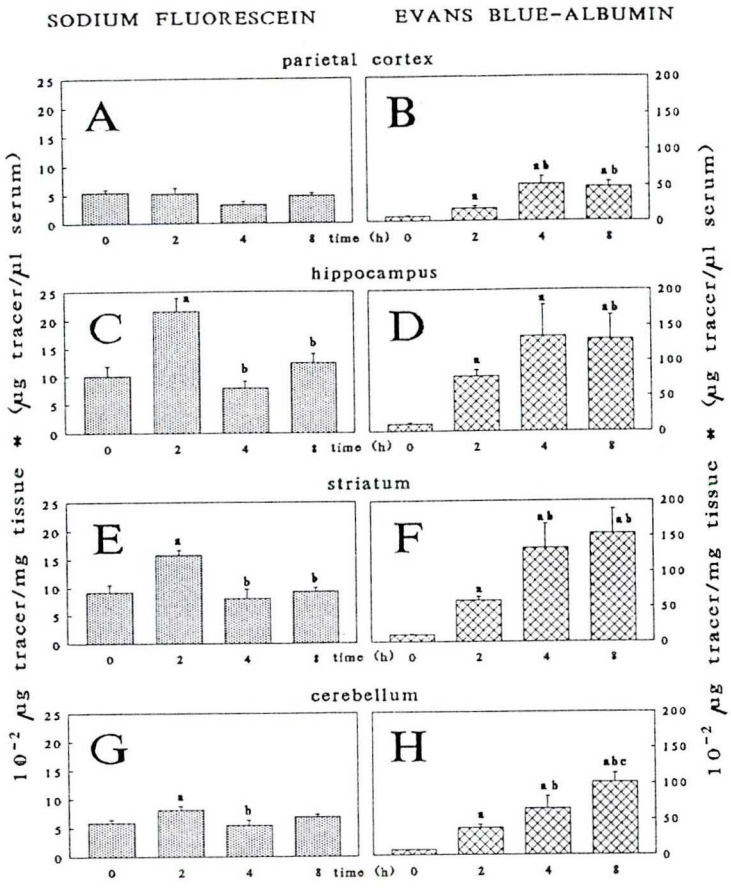

Figure 11. The combined effect of cerebral ischaemia-reperfusion and DPPE treatment on the BBB permeability 2, 4, and 8 h after the end of ischaemia. Permeability for sodium fluorescein (A,C,E,G) and Evan's blue-albumin (B,D,F,H) was determined in parietal cortex (A,B), hippocampus (C,D), striatum (E,F), and cerebellum (G,H) of newborn pigs. Extravasations were expressed as 10^{-2} g dye/ mg brain tissue x (µg dye/µl serum)$^{-1}$ for both dyes. Letters indicate significant differences ($P < 0.05$) compared to values measured at the following time-points in the same brain region: a, 0 h; b, 2 h, c, 4 h, respectively.

4.3.3. Other effects of Hic-receptor antagonist DPPE

The administration of intravenous 5 mg/kg DPPE was not followed any major behavioural or postural change in the rat. The dose of 20 mg/kg, however, proved to be toxic, and resulted in rapid elevation of muscle tone: extension in the forelegs and flexion in the hindlegs with immobility in the first 10-20 min. Gradual recovery was seen in 5 of 6 animals, while 1 rat died.

4.4. Bilateral carotid occlusion

4.4.1. Clinical data

There was a transient significant ($P < 0.05$) increase in the mean arterial blood pressure of female Sprague-Dawley CFY rats during the first 5 min after the bilateral carotid occlusion, but later the

blood pressure did not differ from the control value (**Fig. 12A**). Arterial blood chemistry values, such as pH, standard bicarbonate, pCO_2, pO_2, oxygen saturation, and haematocrit, were in the control range 2 h after the beginning of ischaemia (data not shown).

4.4.2. Cerebral blood flow in parietal cortex

Bilateral carotid occlusion resulted in a rapid and highly significant change in the regional blood flow (**Fig. 12B**). The cerebral blood flow in parietal cortex remained below the one third of the initial value all through the 2 h observation.

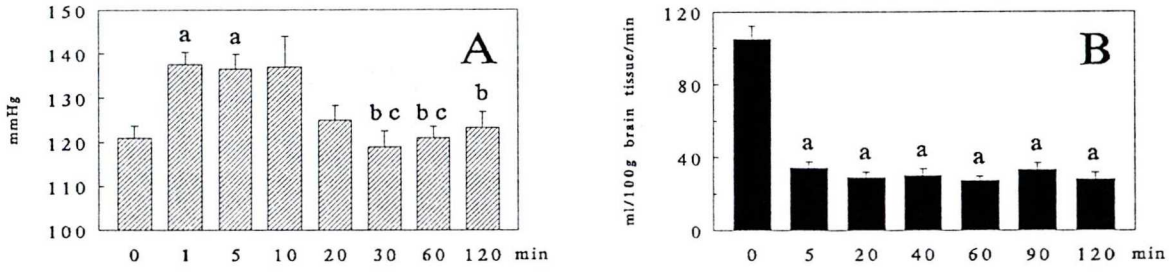

Figure 12. The effect of bilateral carotid occlusion on the mean arterial blood pressure (A), and parietal CBF (B) in the rat. Letters indicate significant differences ($P < 0.05$) compared to values measured at the following time-points: a, 0 min; b, 1 min; c, 5 min; respectively.

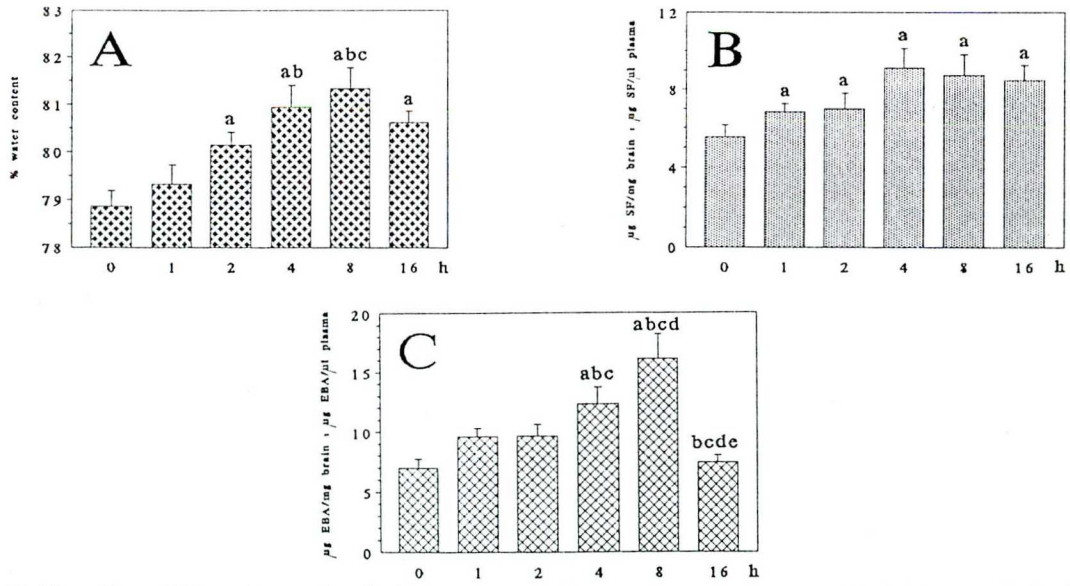

Figure 13. The effect of bilateral carotid occlusion on the cortical percentage water content (A), BBB permeability for sodium fluorescein (B), and Evan's blue-labelled albumin (C) in the rat. Extravasations were expressed as 10^{-2} µg dye/ mg brain tissue x (µg dye/µl serum)$^{-1}$ for both dyes. Letters indicate significant differences ($P < 0.05$) compared to values measured at the following time-points: a, 0 h; b, 1 h; c, 2 h; and d, 4 h; respectively.

4.4.3. Brain oedema: water content and BBB permeability changes

After the carotid occlusion a progressive increase in brain tissue percentage water content was seen (**Fig. 13A**) with a peak at 8 h. BBB permeability for sodium fluorescein was significantly increased compared to the control value all through the experiments (**Fig. 13B**). In that model, Evan's blue extravasation became significantly ($P < 0.05$) increased only 4 h after the ligation, and reached its maximum with a 2-fold increase at 8 h (**Fig. 13C**).

4.4.4. Acid phosphatase isoenzymes in cerebral microvessels and brain tissue

A significant ($P < 0.05$) increase in total acid phosphatase enzyme activity was demonstrated 2 h after the beginning of cerebral ischaemia in isolated cerebral microvessels (**Fig. 14A**), but there was no change in that in homogenised brain tissue (**Fig. 14B**).

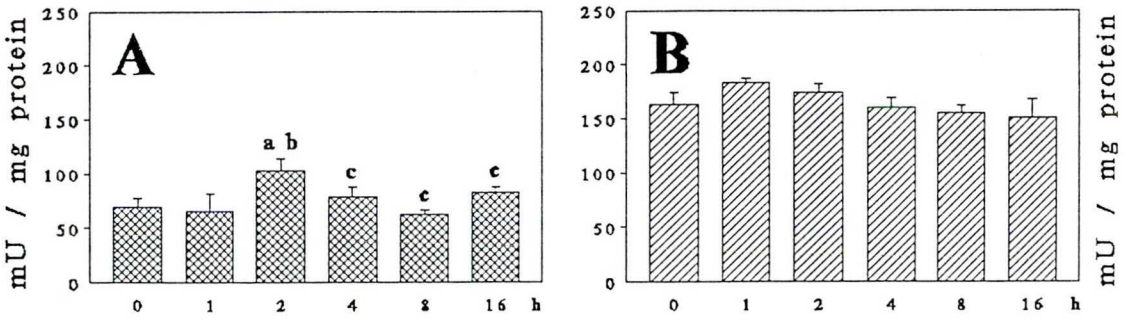

Figure 14. The effect of bilateral carotid occlusion on the total acid phosphatase activity in isolated cortical microvessels (A), and homogenised cortical tissue (B). Enzyme activity was expressed as mU/mg protein. Letters indicate significant differences ($P<0.05$) compared to values measured at the following time-points: a, 0 h; b, 1 h; c, 2 h; respectively.

Isoenzyme study revealed that there was a similar increase in enzyme activity of microvascular high-molecular-weight acid phosphatase (HMW AcP)after 2 h of ischaemia (**Fig. 15A-C**). At that time, both the tartrate- (**Fig. 15A**) and fluoride-sensitive (**Fig. 15C**) activities proved to be elevated. However, there was also a transient increase in brain HMW AcP activity 1 h after the carotid occlusion (**Fig. 15 B-D**).

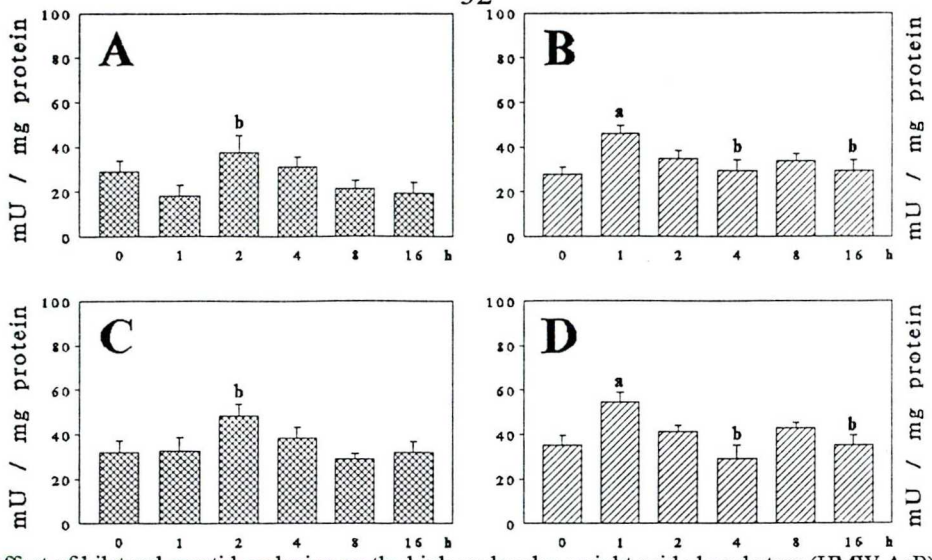

Figure 15. The effect of bilateral carotid occlusion on the high-molecular-weight acid phosphatase (HMW AcP) enzyme activity in isolated cortical microvessels (A-C), and homogenized cortical tissue (B-D). HMW AcP activity was measured as tartrate-sensitive (A-B), or fluoride-sensitive (C-D) enzyme activity which was expressed as mU/mg protein. Letters indicate significant differences ($P < 0.05$) compared to values measured at the following time-points: a, 0 h; b, 1 h; respectively.

Low-molecular-weight acid phosphatase (LMW AcP) enzyme activity did not change significantly in the homogenised brain tissue during the observation period (**Fig. 16 B-D**). In isolated cerebral microvessels, however, tartrate-resistant acid phosphatase activity was significantly ($P < 0.05$) elevated 2 and 16 h after the beginnning of ischaemia (**Fig. 16A**). A similar tendency to increase was also seen in case of fluoride-resistant acid phosphatase, but it failed to reach the limit of statistical significancy (**Fig. 16C**).

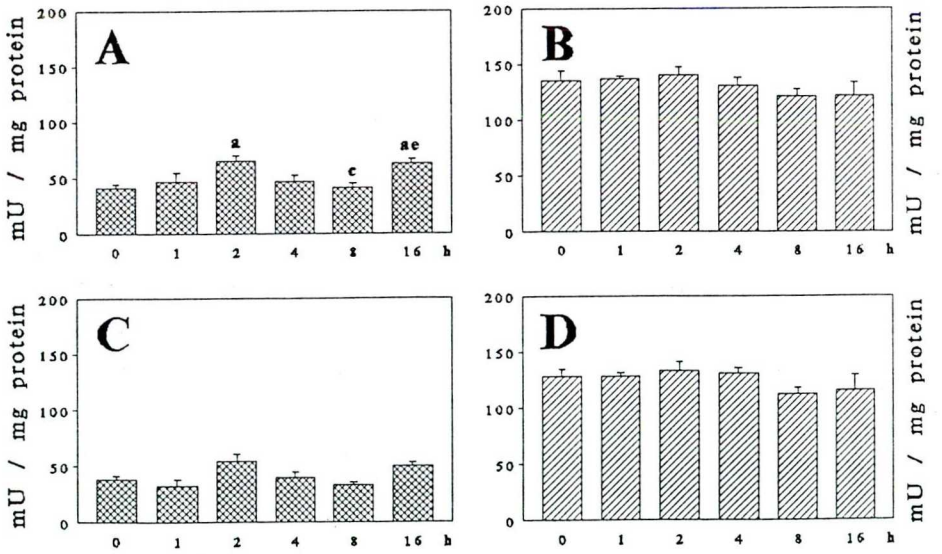

Figure 16. The effect of bilateral carotid occlusion on the low-molecular-weight acid phosphatase (LMW AcP) enzyme activity in isolated cortical microvessels (A-C), and homogenized cortical tissue (B-D). HMW AcP was measured as tartrate-resistant (A-B), or fluoride-resistant (C-D) enzyme activity which was expressed as mU/mg protein. Letters indicate significant differences ($P<0.05$) compared to values measured at the following time-points: a, 0 h; b, 1 h; respectively.

No ischaemia-induced change in Zn^{2+}-induced acid phosphatase activity was detected in cerebral capillaries (**Fig. 17 A-C**), while 2 h bilateral carotid occlusion caused a highly significant ($P < 0.05$) decrease both in the total enzyme activity in the presence of zinc (**Fig. 17B**), and practically abolished the Zn^{2+}-induced increase in acid phosphatase activity (**Fig. 17D**) in homogenised brain tissue. The cerebral activity of this isoenzyme, however, recovered soon (**Fig. 17 B-D**).

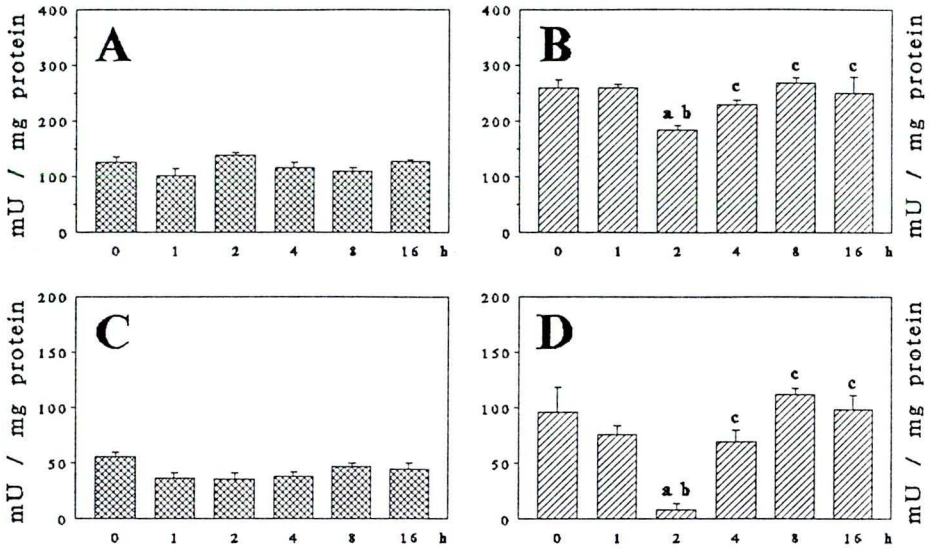

Figure 17. The effect of bilateral carotid occlusion on the Zn^{2+}-dependent acid phosphatase enzyme activity in isolated cortical microvessels (A-C), and homogenized cortical tissue (B-D). Zn^{2+}-dependent AcP was measured as total AcP activity in the presence of Zn^{2+} (A-B), or as a Zn^{2+}-induced increase in AcP activity which was expressed as mU/mg protein. Letters indicate significant differences ($P < 0.05$) compared to values measured at the following time-points: a, 0 h; b, 1 h; and c, 2 h; respectively.

5. DISCUSSION

5.1. BBB permeability changes induced by histamine, TNF-α, and cerebral ischaemia-reperfusion: major new findings of our study

5.1.1. Histamine

In newborn pigs, intracarotid administration of exogenous histamine resulted in a dose-dependent increase in the BBB permeability for intravascular tracers in each brain region examined. Histamine also induced the acid phosphatase enzyme activity in isolated cortical capillaries, and a linear correlation was found between the activity of acid phosphatase enzyme and the permeability of the BBB. A marked, dose-dependent increase was seen in the activity of tartrate-

resistant, low molecular weight isoform, while the induction of tartrate-sensitive, high molecular weight form was more moderate.

5.1.2. TNF-α

In our neonatal porcine model, intracarotid TNF-α administration caused a time-, dose-, and brain region-dependent increase the extravasation of the intravascular BBB permeability markers used. Prae-treatment with AEBSF, a water-soluble serine protease inhibitor, protected against the TNF-α-induced increase in the permeation of sodium fluorescein, a suggested paracellular marker, but it could only slightly attenuate that in the permeability for albumin.

5.1.3. Cerebral ischaemia-reperfusion induced by 4-vessel occlusion

Total cerebral ischaemia induced by 4-vessel occlusion and the consequent reperfusion resulted in a highly significant increase in the BBB permeability for albumin, but it had only a minor effect on the extravasation of sodium fluorescein. The Hic antagonist DPPE could not prevent the cerebral ischaemia-reperfusion-induced BBB damage, in fact it made the increase in permeability more prolonged. Moreover, DPPE alone could dose-dependently induce the BBB opening, and it also had some toxic side effects.

5.1.4. Forebrain ischaemia induced by 2-vessel occlusion

Bilateral carotid occlusion reduced the cortical blood flow by 70 %, and produced time-dependent increases both in brain water content and BBB permeability. A transient increase in the total activity of acid phosphatase, as well as that of each isoenzyme except for the Zn^{2+}-induced form, was seen in the isolated cortical microvessels 2 h after the beginning of the ischaemia. In carotid-occluded rats, the activity of high molecular weight isoform was elevated at 1 h, while that of Zn^{2+}-induced acid phosphatase was sharply reduced at 2 h.

5.2. Vasoactive and inflammatory mediators in neonatal and childhood brain injuries

Recent experimental and clinical studies revealed that vasoactive substances, lipid mediators, free radicals, cytokines, and inflammatory mediators play a role in the pathogenesis of injuries in developing brain, such as hypoxic-ischaemic CNS injuries of praemature infants (Silverstein et

al., 1997; Volpe, 1998; Taylor et al., 1999), perinatal asphyxia-related cerebral damages (Robertson and Edwards, 1998), head and brain trauma (Adelson and Kochanek, 1998), human immunodeficiency virus-1-induced encephalopathy (Epstein and Gelbard, 1999), cerebral malaria (Beutler and Grau, 1993), sepsis syndrome (Sáez-Llorens and McCracken, 1993), and bacterial meningitis (McCracken, 1992). On the other hand, a wide variety of systemic diseases in paediatrics may result in release of inflammatory mediators, including histamine and TNF-α, into the bloodstream which, if the amount is high enough, can provoke BBB changes. Increased histamine levels could be measured not only in the blood of patients with childhood asthma or allergy (Ennis et al., 1999), but also in that of neonates with respiratory distress syndrome (Drew and Arroyave, 1982), or of newborns undergoing the arterial switch operation for simple transposition of the great arteries (Seghaye et al., 1996). Sunder et al. (1982) described children suffering from angioedema with high blood histamine level, who developed neurological manifestations (seizures, headaches, focal and generalised deficits), and extensive clinical and laboratory investigations failed to reveal a separate cause for the CNS changes. It is suggested that histamine-induced brain oedema with increased BBB permeability contributed to the appearance of these cerebral symptoms. Elevated serum TNF-α levels were found in neonatal sepsis (de Bont et al., 1994), in neonates with cardiopulmonary bypass (Seghaye et al., 1996), in childhood malaria (Grau et al., 1989), acquired immunodeficiency syndrome (Mintz et al., 1989), and X-linked adrenoleukodistrophy (McGuinness et al., 1995). In cases of human immunodeficiency virus-1 related encephalopathy and cerebral malaria, TNF-α-induced BBB changes proved to be involved into the pathogenesis of CNS injury (Beutler and Grau, 1993). In neonates undergoing cardiac operations a peripheral capillary leak syndrome was described (Seghaye et al., 1996), but one can not exclude that elevated histamine and TNF-α concentrations also caused a BBB opening.

5.3. Cerebral ischaemia and reperfusion: BBB permeability changes in the animal models used

In the present study, increased BBB permeability was found both in the 4-vessel occlusion model of total cerebral ischaemia and reperfusion, and in the 2-vessel occlusion model of subtotal forebrain ischaemia in the rat. According to the classical neuropathological views (reviewed by Joó and Klatzo, 1989; Wahl et al., 1989), the essential events in the development of vasogenic

brain oedema formation are increased BBB permeability, enhancement of driving forces including a bulk flow into the interstitial space of the CNS, and retention of fluid. These changes can also be regarded as an "open-barrier oedema" (Betz et al., 1989) because the permeability of the BBB is increased and brain oedema results from the oncotic forces generated by an influx of serum proteins into brain. Surprisingly, the increase in the extravasation of Evan's blue-labelled albumin was much higher (3- to 10-fold) in both animal models, than the change in the permeability of sodium fluorescein. Another unexpected finding was that bilateral carotid occlusion, which similarly to literature data (Iwasaki et al., 1989), reduced the regional CBF to one-third of its original value resulted in relatively higher permeability rate for the paracellular permeability marker than transient total ischaemia and reperfusion. Though these differences may also originate in differing strain or gender of rats in the two models used, we assume that they are primarily related with the CBF changes in distinct models. On the other hand, the dramatic increase in the BBB permeability for albumin in both models is in accordance with the in vitro data of Plateel et al. (1995; 1997).

5.4. The role of histamine in BBB changes

Histamine has long been known to increase the BBB permeability by H_2-receptor-dependent ways, most probably by the activation of adenylate cyclase enzyme (Edvinsson et al., 1993; Tósaki et al., 1995; Joó, 1996). Histamine, similarly to cyclic adenosine 3'5'-monophosphate (cAMP), increased the formation of pinocytotic vesicles in cerebral endothelium in vivo (Joó, 1996). However, in an in vitro reconstituted model of the BBB, cAMP treatment resulted in a rapid decrease in paracellular permeability (Deli et al., 1995a), while histamine administration did not alter significantly the permeability of tight junction markers with a concomitant increase in albumin transport (Deli et al., 1995b). It was proved that histamine might affect the BBB permeability by an H_1-receptor dependent, phosphoinositol-mediated mechanism, too (Edvinsson et al., 1993; Joó, 1996). On the other hand, histamine could increase the BBB permeability through the nitric oxide-mediated activation of guanylate cyclase enzyme (Mayhan, 1996). In peripheral endothelial cells, a similar mechanism involving consequent phospholipase C activation, release of Ca^{2+} from intracellular stores, induction of nitric oxide synthase, stimulation of guanylate cyclase, and the formation of cyclic guanosine 3'5'-monophosphate (cGMP) is proved to be responsible for the histamine-induced increase in albumin permeability (Yuan et al., 1993). In rat cerebral endothelial cells, histamine elevated the intracellular Ca^{2+} concentrations in

vitro, while cGMP increased the rate of pinocytosis in vivo (Joó, 1996). It is supposed that histamine can also increase the albumin permeability by a re-arrangement of endothelial actin cytoskeleton in the CNS, similarly to that in peripheral endothelium. In this study, a linear relationship was found between the activity of acid phosphatase enzymes and increased permeability, which may support a role for these isoforms in the regulation of the BBB.

Though the emerging role of histamine, as an intracellular second messenger, has been established in the regulation of cellular processes in a wide variety of cell types (Brandes et al., 1990), no information was published about the role of Hic receptors in the regulation of the BBB properties until recently. Karlstedt et al. (1999) found lack of histamine synthesis in immortalised brain endothelial cells, but provided evidence that the internalised histamine was distributed in the cytoplasm and nucleus of the cells. It is known, however, that polyamines synthesised by ornithine decarboxylase can act as intracellular messengers and mediate the BBB breakdown (Koenig et al., 1983). In the present study, Hic antagonist DPPE induced an increased BBB permeability for intravascular tracers, which supports a role for the intracellular histamine in the maintenance of the barrier properties. To reveal the exact molecular mechanisms responsible for this phenomenon observed in our present experiments is a fascinating scientific challenge.

Clinical studies have also started recently with DPPE, a compound that antagonises histamine binding at Hic sites in the micromolar range, because of its ability to potentiate chemotherapy cytotoxicity to malignant cells (Brandes et al., 1995). However, after receiving a maximally tolerated parenteral dose of DPPE (6 mg/kg), acute treatment toxicity consisted of nausea/vomiting and ataxia, as well as delayed effects (tiredness and mild nausea) were seen (Brandes et al., 1995). The acute side effects were correlated with the peak serum levels of DPPE in the patients. The present findings about the DPPE-induced BBB disruption may partly explain these clinical observations. DPPE, in a dose of 20 mg/kg, proved to be toxic in our study on the rat, and potentiated drug-induced seizures in mice (Sturman et al., 1994). Surprisingly, Cramer and Toorop (1998) reported on a significant protection by 30 mg/kg DPPE against cerebral infarction without any side effects in the mouse.

5.5. The role of TNF-α in BBB changes: protective effect of the serine protease antagonist AEBSF

This study confirms the previous observation (Ábrahám et al., 1996) that intracarotid TNF-α administration results in BBB opening for sodium fluorescein and Evan's blue-albumin, and extends the scope of the observation from the cortex to the whole brain of newborn pigs. Though AEBSF prae-treatment dose-dependently inhibited the TNF-α-induced BBB opening for sodium fluorescein, its effect on the increase in albumin permeability was moderate. However, it is suggested that permeability tracers used can pass through the BBB by different permeation pathways: sodium fluorescein through the opened interendothelial tight junctions (Thompson et al., 1994), while albumin by transcytosis (Banks and Broadwell, 1994). It is hypothesised that AEBSF may primarily act on the BBB permeability by the prevention of disruption of tight junctions and not by attenuating transendothelial transport. In accordance with this presumption, Winton et al. (1998) found that AEBSF inhibited the serine protease-induced breakdown of tight junctions of epithelial monolayers and significantly decreased the paracellular permeability. Similarly, Nagy et al. (1995) published that serine proteases, e.g. thrombin, plasmin, or urokinase, could induce a contraction of brain endothelial cells, which might result in increased paracellular BBB permeability. Moreover, TNF-α induces serine protease gelatinase B, a matrix metalloproteinase, which can cause delayed opening of BBB (Rosenberg et al., 1995).

It is known that free radicals are also involved in the mediation of the TNF-α-induced brain injuries (del Zoppo, 1994; Feuerstein et al., 1994; Tureen, 1995). AEBSF may decrease the development of brain oedema, at least partly, by blocking the serine protease responsible for the proteolysis of inhibitory protein κB- (IκB-α), thereby preventing the activation of nuclear factor κB (NF-κB) and inhibiting the free radical production of activated cells (Megyeri et al., 1995; Remold-O'Donnell and Parent, 1995; Hecker et al., 1996). Serine protease inhibitors may have a therapeutical role in the treatment of infections, ischaemia-reperfusion injuries, and trauma in the near future. Recent reports have revealed some beneficial effects of AEBSF on brain cells: it could inhibit amyloid β-protein production (Citron et al., 1996), and could protect against glutamate-induced programmed cell death (Tan et al., 1998) in neuronal cell lines. The observation that a serine protease inhibitor can reduce the TNF-α induced vasogenic brain oedema formation may help us in understanding the role of serine proteases in the regulation of

the BBB.

The role of TNF-α in the cerebral ischaemia-reperfusion-induced brain damages is well established (del Zoppo, 1994, Feuerstein et al., 1994, Pantoni et al., 1998). Recently, Yang et al. (1999) proved that TNF-α expression produced increased BBB permeability following temporary focal cerebral ischaemia in mice, and treatment with anti-TNF-α monoclonal antibody in the beginning of reperfusion could reduce the BBB disruption. This observation suggesting TNF-α might be an important mediator in altering BBB permeability during postischaemic reperfusion hopefully gives a boost to the research of those drugs, which are capable to prevent the TNF-α induced BBB opening.

5.6. Acid phosphatases in brain injuries

The present results raise the possibility that acid phosphatase enzyme isoforms may have an effect on the regulation of the BBB permeability after different noxious stimuli. Both intracarotid histamine administration in newborn pigs and bilateral carotid occlusion in adult rats induced the activity of HMW and LMW AcP isoforms, concomitantly with the opening of the BBB. In order to reveal the potential effect of AcP isoenzymes, the possible connections between them are outlined (Table 3). Lysosomal HMW AcP may have a role in the regulation of transendothelial macromolecular transport. Broadwell and Salcman (1981) suggested that lysosomal system of organelles with acid hydrolase activity in cerebral capillaries would function as a deterrent to the BBB transport of blood-borne substances. Endothelial lysosomes fusing with AcP positive transcytotic structures were proposed to play a role in the increased macromolecular transport in brain microvessels of stroke-prone rats (Tagami et al., 1983), and brain-injured rats (Lossinsky et al., 1981). There is an assumption that cytosolic LMW AcP, an enzyme with known phosphotyrosine protein phosphatase activity, may also have an effect on the BBB permeability. However, a 56 kDa phosphoprotein phosphatase is also present in cerebral capillaries (Weber et al., 1987). Tyrosine phophorylation of proteins associated with intercellular tight junctions could increase the paracellular permeability (Staddon et al., 1995; Rubin and Staddon, 1999). On the other hand, phosphotyrosine protein phosphatase activation might tighten the junctions and decrease the paracellular flux in vitro (Anderson and Van Italie, 1995; Staddon et al., 1995; Gloor et al., 1997). Moreover, the endogenous substrate of LMW AcP in the brain was an epidermal growth factor receptor (Shimohama et al., 1994), through which it may influence the tight junction barrier (Gloor et al., 1997). However, protein tyrosine phosphatase activity might also

alter the transendothelial albumin permeability. Histamine treatment, similarly to the inhibition of this enzyme, stimulated tyrosine phosphorylation of two focal adhesion-associated proteins paxillin and pp125^{FAK}, and produced a high albumin permeability through coronary endothelium (Yuan et al., 1998). In summary, the assumption that HMW AcP may be involved in the increased transendothelial transport, while LMW isoform may tighten the interendothelial junctions can serve as an explanation for the histamine-induced selective albumin permeation without an increase in paracellular permeability in vitro (Deli et al., 1995b). In case of cerebral ischaemia, there is no explanation available at present, except for the histamine-related increase suggested, for the connection between the cerebral ischaemia-related changes in AcP enzyme activity and BBB permeability.

Table 3. **Possible functional connection between acid phosphatase enzyme (AcP; EC 3.1.3.2.) family and central nervous system injury**

Blood-brain barrier:

High molecular weigth AcP:
- a role for lysosomal system in transendothelial macromolecular transport
  - under physiological conditions, in stroke-prone rats, in brain injured mice
- induction by histamine in cultured rat brain endothelial cells

Low molecular weight AcP:
- a role in the regulation of paracellular permeability
  - Epidermal Growth Factor receptor: ZO-1, ZO-2, actin filaments
- induction by histamine in cultured rat brain endothelial cells

AcP isoenzymes with phosphotyrosyl protein phosphatase activity:
- a potential role in the regulation of paracellular permeability
  - tyrosine phosphorylation of ZO-1, ZO-2, and β-catenin
- a potential role in transendothelial macromolecular transport
  - tyrosine phosphorylation of paxillin, and pp125^{FAK}

Brain tissue:

High molecular weight AcP:
- a role for lysosomal system activation in different brain injuries
  - in gerbils and rats with cerebral ischemia
  - in stroke-prone spontaneously hypertensive rats
  - in scrapie-infected mice and hamsters
  - in mice exposed to brain trauma

myo-inositol monophosphatase:
- increased protein level and enzyme activity in the Alzheimer's disease brains
- a potential role in bipolar mood disorder

Low molecular weight AcP:
- reduced enzyme activity in the brain of patients with Alzheimer's disease

AcP isoenzymes with phosphotyrosyl protein phosphatase activity:
- a potential role in cerebral ischemia

References: Atack et al., 1995; Broadwell and Salcman, 1981; Chue et al., 1993; du Bois et al., 1985; Lossinsky et al., 1981; Rubin and Staddon, 1999; Shimohama et al., 1993; Shimohama et al., 1994; Shimohama et al., 1998; Staddon et al., 1995; Szabó et al., 1996; Tagami et al., 1983; Yuan et al., 1998;

Though a correlation was suggested between cerebral ischaemia and the elevated activity of AcP enzyme in brain tissue (Table 3), similar rapid induction has not been reported previously. In a morphological study, du Bois et al. (1985) could find an increase in the acid phosphatase histological reaction only 5-8 days after the carotid occlusion in gerbils. In stroke-prone spontaneously hypertensive rats, an age-dependent increase in lysosomal AcP activity was seen even without ischaemic stimulus, which correlated with the increased pinocytosis due to the BBB dysfunction (Chue et al., 1993). In the present study, the transient increase in lysosomal HMW AcP activity in brain tissue, preceded the similar ischaemia-induced change in cortical capillaries, but the process is not completely understood yet. This study is the first to describe an early and transient decrease in cerebral Zn^{2+}-induced AcP activity in carotid-occluded rats. According to recent papers (Caselli et al., 1996; Fujimoto et al., 1998), Zn^{2+}-dependent acid phosphatase has immunological identity with myo-inositol 1-phosphatase, but their pH- and ion-dependency is different. There are some speculations whether the two different activities in the same protein might work in different cell compartments or not, but a role in signal transduction is suggested for this 62 kDa enzyme. In the CNS, this phosphatase is thought to be mainly responsible for the supply of myo-inositol, the precursor for the inositol phosphate second-messenger signalling systems, because of the limited passage of myo-inositol through the BBB (Spector, 1988). However, the role of Zn^{2+}-dependent AcP in cerebral ischaemia remains to be elucidated.

5.7. Modification of the BBB permeability in clinical practice: therapeutical considerations

The homeostasis of the CNS is protected from external chemical insults and cellular elements by the BBB, a complex system of morphological, functional, and biochemical properties (reviewed by Deli and Joó, 1996). The BBB is formed by the cerebral capillary endothelial cells working in close co-operation with the neighbouring astrocytes, neurones, pericytes, microglial and smooth muscle cells. A wide variety of cerebral and systemic diseases, e.g. tumours, trauma, infections, hypoxic, toxic, and metabolic disorders may result in cerebral oedema formation characterized with abnormal accumulation of fluid in brain parenchyma, and extravasation of serum components (Joó and Klatzo, 1989). Elucidation of cellular and molecular mechanisms leading to the formation of oedematous swelling has an obvious importance in designing rational and effective therapies. The aim of these studies is to strengthen the barrier mechanisms of the CNS in

such cases where pharmacologically active or neurotoxic products possibly alter some BBB properties and are perilous for the immature or adult brain. On the other hand, the protective effect of this active interface between blood and brain tissue may play a role in restricting delivery of several type of therapies thereby limiting the effective treatment. To enhance drug penetration by temporary BBB disruption provides a method in the treatment of inflammatory brain diseases and cerebral malignant diseases.

6. CONCLUSIONS

6.1. Both histamine and TNF-α can induce an increase in the BBB permeability in neonates.

6.2. Intracellular histamine, as a second messenger, may have been involved in the maintainance of the BBB characteristics.

6.3. Prae-treatment with AEBSF, a water-soluble serine protease antagonist, can attenuate the TNF-α-induced BBB-opening.

6.4. In animal models of cerebral ischaemia and reperfusion, a disproportionally huge increase in the BBB permeability for albumin, a transendothelial marker, was found compared to that in the extravasation of sodium fluorescein, a paracellular tracer.

6.5. DPPE, an intracellular histamine binding site antagonist failed to prevent the cerebral ischaemia-reperfusion-induced BBB opening.

6.6. Cerebral microvascular acid phosphatase isoenzymes may have a role in the intracarotid histamine administration- and cerebral ischaemia-induced BBB damages.

7. THE POSSIBLE THEORETICAL AND CLINICAL IMPORTANCE OF THESE EXPERIMENTS

The intact cellular membrane structures and functional properties of the BBB have a major role in the protection of the developing and adult brain. The damages of the BBB are among the most

common occasions of acute neurological disorder in children, and remain important causes of long-term disability and mental handicap. Neurological complications have become more frequent in the last few decades, caused by a variety of diseases directly or by neurotoxicity induced by several chemicals or drugs. The prevention of these sequelae and better treatment of the problematic patient groups has become an important issue recently.

Investigations on the BBB have taken various approaches. Studies on the damaged BBB produced a thorough description of abnormalities in animal models with ischaemia- reperfusion. The second line of investigations is to induce these damages by chemical way to prove their role in this process. The third and most important group of the studies is to test pharmacological substances on the animal models to investigate the direct acute and late effects of possible new medical treatments. The increasing number of research projects that is carried out by a variety of disciplines is leading to a greater understanding of these pathological and therapeutic processes. Hopefully, the findings presented in this thesis might contribute to this.

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ABBREVIATIONS:

AcP, acid phosphatase

AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride

ATP, adenosine triphosphate

BBB, blood-brain barrier

cAMP, cyclic adenosine 3'5'-monophosphate

CBF, cerebral blood flow

cGMP, cyclic guanosine 3'5'-monophosphate

CNS, central nervous system

CSF, cerebrospinal fluid

DPPE, N,N-diethyil-2-(4-(phenyl-methyl)phenoxy)ethanamine

Hic, intracellular histamine binding sites

HDC, L-histidine decarboxylase

H_1 receptor, Histamin type 1 receptor

H_2 receptor, Histamin type 2 receptor

H_3 receptor, Histamin type 3 receptor

HMW, high-molecular-weight

IκB-α, inhibitory protein κB-α

LMW, low-molecular-weight

NF-κB, nuclear factor κB

P-creatine, phosphocreatine

RBE4, rat brain endothelial cell line

TNF-α, Tumour necrosis factor α

ZO, zonula occludens

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