REGULATION OF THE BLOOD-BRAIN BARRIER PERMEABILITY: STUDIES ON *IN VITRO* AND *IN VIVO* MODELS

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Abbreviations

6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$
12-HHT	12-L-hydroxy-5,8,10-heptadecatrienoic acid
AA	arachidonic acid
APase	acid phosphatase
B_{max}	binding capacity
BBB	blood-brain barrier
BCCAL	bilateral common carotid artery ligation
bFGF	basic fibroblast growth factors
CEC	cerebral endothelial cell
CNS	central nervous system
DMEM	Dulbecco's modified Eagle's medium
DPPE	N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine
DTT	dithiothreitol
EBA	Evan's blue labelled albumin
ECGF	endothelial cell growth factor
EDRF	endothelium-derived relaxing factor
FBS	fetal bovine serum
HRP	horseradish peroxidase
ICMV	isolated cortical microvessels
K_D	dissociation constant
KH	Krebs-Henseleit
LT	leukotrien
NO	nitric oxide
NOLA	N^{ω}-nitro-L-arginine
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDS	plasma derived serum
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin, prostaglandin I_2
RBEC	rat brain endothelial cell
SF	sodium fluorescein
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2

1. Rationale

The characterization of the blood-brain barrier (BBB) is undergoing a paradigmatic shift. The century-old concept of a massive, impermeable barrier that segregates blood and brain interstitial fluid is giving way to the idea that the BBB is a dynamic conduit for the transport between blood and brain. BBB has multiple functions. It protects the brain from many exogenous toxins and sudden high levels of systemic neurotransmitters but also excludes a large number of potentially therapeutic agents from it. The BBB modulates the entry of metabolic substrates such as glucose, lactate and amino acids. It forces all substances entering the brain to be exposed to endothelial cell cytoplasmic enzymes, either producing metabolites that cannot pass the abluminal capillary membrane or allowing loss of the metabolite back to blood. The barrier funcion of the BBB can change dramatically during various diseases.

Under several pathological conditions such as cerebral ischemia, hyperosmolarity, tumors of central nervous system (CNS), cerebral inflammation, hypertension, seizures and neurodegeneretive diseases an opening of the BBB may occur. Several mediators like histamine, arachidonic acid (AA), bradykinin, free radicals, leukotrienes (LT), and serotonin can be released in these illnesses, which in turn induce vasogenic edema (Wahl *et al.*, 1988), the extravasation of serum components, ions and water to the brain interstitium.

The *in vitro* study of the BBB barrier started in the 70s. As pioneers of the field, Joó and Karnushina developed a method to isolate fractions enriched in microvessels from rat brain cortex (1973). A decade later, a new method was described to cultivate endothelial cells from isolated brain capillaries (Bowman *et al.*, 1981; 1983). Recently, immortalized cerebral endothelial cell (CEC) lines have been established and characterized (Roux *et al.*, 1994; Greenwood *et al.*, 1996). These new model systems provided a better insight into the morphology, biochemistry and physiology of the CEC and new means to study the transport processes of nutrients and drugs across the BBB.

Elucidation of cellular and molecular mechanisms regulating BBB permeability is important in order to design effective therapy for the prevention and treatment of brain edema, inflammatory and immunological diseases of the CNS.

2. Aims

1. At the beginning of our studies, the first goal was to find an optimal culture protocol for rat CEC in our laboratory. For this reason, we compared different techniques.

2. Histamine is an important vasoactive mediator and plays a role in brain edema formation by increasing the number of pinocytotic vesicles and the macromolecular transport in brain capillary endothelium (Dux and Joó, 1982; Dux *et al.*, 1987). Histamine administration induced a selective albumin permeation in cultured CECs (Deli *et al.*, 1995b). Acid phosphatases (APase), as members of specific lysosomal enzyme systems, also identified in cerebral endothelium, take part in regulating entry of internalized macromolecules into CNS. Increased transendothelial transport and APase activity was described in stroke-prone hypertensive rats (Tagami *et al.*, 1983). Therefore, the effect of histamine on APase activity was investigated in cultured CECs *in vitro*. APase activity was also determined in isolated cortical microvessels (ICMV) and in cortical brain tissue from newborn pigs after intra-carotid histamine administration and from rats in which cerebral ischemia was induced by bilateral common carotid artery ligation (BCCAL). BBB permeability changes were quantified parallelly.

3. The carrier-mediated histamine uptake and release in cultured CECs has been described recently (Huszti *et al.*, 1995). Organic and inorganic mercurials were shown to interact with protein thiols and have neuropathologic effect. We decided to examine if mercury can cause any changes in histamine uptake and binding of CECs.

4. Prostanoids are involved in the regulation of regional cerebral blood flow and BBB permeability (Joó, 1993) and may be responsible for the permeability-increasing effect of angiotensin in CECs (Guillot and Audus, 1990). Immortalized cell lines of CECs have became available lately and proved to be useful models in basic research. Since they are good candidates for gene delivery to the CNS, these cell lines are subjects of thorough investigation. We compared the vasoactive substance synthesis and the eicosanoids production of primary and immortalized CECs.

3. Introduction

Cerebral endothelial cell culture

Specialized characteristics of CEC, which form the BBB including the occurrence of tight intercellular junctions, high transendothelial electrical resistance, few pinocytotic vesicles as well as the presence of specific, polarized, and highly discriminatory membrane transport systems. The CECs are in close interaction with the neighbouring brain cells, e.g. neurons, astrocytes, microglia, pericytes, vascular smooth muscle cells.

In order to obtain CEC cultures porcine (Deli *et al.*, 1997; Tewes *et al.*, 1997), murine (Tontsch and Bauer, 1989), bovine (Méresse *et al.*, 1989), human (Vinters *et al.*, 1987; Kása *et al.*, 1991; Vastag and Nagy, 1997) and rat brains (Abbott *et al.*, 1992) are used most frequently in different laboratories. The possibility to prepare relatively easily syngeneic co-cultures of rat brain endothelial cells (RBEC) and other cell types like astrocytes, smooth muscle cells, pericytes, neurons is an advantage of the rat model. For molecular biological studies, rodents are widely used, this is also favour to choose rat CECs. Almost all methods use gray matter of brain and enzymic digestion step(s) for dissociation of microvessels. The capillary fragments are separated by Percoll gradient (Bowman *et al.*, 1981), by column of glass beads (Diglio *et al.*, 1982) or by series of centrifugation steps (Tontsch and Bauer, 1989).

For characterization of the primary culture of CEC, it is necessary to check the purity and the expression of specific markers of endothelial cells. The primary cultures are usually not pure, pericytes, astrocytes, fibroblasts, smooth muscle or leptomeningeal cells may appear among endothelial cells. There are different methods to increase the purity of brain endothelial cell cultures like the subcloning of endothelial cell islands by microtrypsinisation as described by Méresse *et al.* (1989) or the complement mediated specific cytolysis (Risau *et al.*, 1990).

The growth and differentiation of endothelial and the other contaminant cell types depend on the presence of different mitogenic factors. The endothelial cell growth factor (ECGF) (Folkman and Haudenschild, 1982) or the acidic and the 10-30 times more potent basic fibroblast growth factors (bFGF) (Gospodarowicz *et al.*, 1986) were found to be necessary for the continuous growth and the expression of the phenotypic features of the endothelial cells *in vitro*. The quality of serum is also very important. There is a variability in the growth rate stimulating effect of different types or batches of serum. Fetal bovine serum (FBS) may contain different amount of platelet-derived growth factor (PDGF) which

stimulates proliferation of contaminating cells as well. The plasma derived serum (PDS) is free from PDGF.

Rat cerebral pericytes and astrocytes express the cell surface differentiation antigen Thy 1.1 while endothelial cells do not. Thy 1.1 antigen can be used to reduce the non-endothelial contamination of CECs (produce optimal purity of CEC cultures) by selectively lysing by antibody/complement treatment the pericytes and astrocytes (Risau *et al.*, 1990).

Histamine, acid phosphatase enzymes, and blood-brain barrier permeability

CECs have relatively few lysosomes and the number of these compartments seems to parallel the presence of intracellular vesicles in different endothelia (Audus *et al.*, 1993). On the other side, there is a growing evidence that endothelial lysosomes play a role in regulating entry of internalized macromolecules into the CNS despite limited expression, and the significance of lysosomes is increased during pathological conditions (Audus *et al.*, 1993; Tagami *et al.*, 1983). Specific lysosomal enzyme systems have been identified in CEC, such as APase, trimethaphosphatase, phosphoprotein phosphatase, β-galactosidase and aryl sulphatase (Audus *et al.*, 1993). Phosphoprotein phosphatases and protein kinases are important components of signal transduction in the cells of the CNS. APase enzyme (orthophosphoric monoester hydrolase, EC 3.1.3.2) not only plays a role in the protein degradation, but it also participates, similarly to other protein phosphatases, in the signal transduction through the dephosphorylation of certain proteins. It is known that APase isoenzymes differ in their subcellular localization, molecular weight, sensitivity to inhibitors, and substrate requirements (Rehkop and Etten, 1975; Taga and Etten, 1982; Vincent and Averill, 1990). The high molecular weight form of the enzyme (> 100,000 Da) is present mainly in the lysosomal fraction and nonspecifically hydrolyses phosphomonoesters. The activity of this isoform can be blocked by either tartrate or fluoride, while the low molecular weight form (< 20,000 Da) found predominantly in cytosol is resistant to these inhibitors. Low molecular weight APase also has phosphotyrosyl-protein phosphatase activity (Chernoff and Li, 1985). Another form of APase (62,000 Da) requires zinc ions to be activated and it has been recently described that this isoenzyme is identical with myo-inositol 1-phosphatase (Caselli *et al.*, 1996; Fujimoto *et al.*, 1996). Biochemical and morphological studies have revealed the presence of multiple molecular forms of APase in the CNS (Krizbai *et al.*, 1997; Shimohama *et al.*, 1993). However, the specific activity of izoenzymes in different cells of brain or the pathophysiological role of the APase isoforms is still poorly understood. Acid hydrolase

activity was first demonstrated in primary cultures of CEC by Baranczyk-Kuzma *et al.* (1989). These enzymes may participate in the alteration of the BBB permeability and the pathogenesis of brain edema during different diseases. Particularly, increased APase activity was shown to be involved in the enhancement of transendothelial transport of macromolecules through the BBB in stroke-prone spontaneously hypertensive rats (Tagami *et al.*, 1983). However, it remained to be seen if the enzyme activity could be modified in or released from CECs by vasoactive substances.

The existence of three histamine pools in CNS (histaminergic neurons, perivascular mast cells, cerebral endothelium) suggests involvement of histamine released after different physiological and pathological stimuli in neuronal transmission, regulation of cerebral blood flow and brain edema formation (Edvinsson *et al.*, 1993). The presence and function of histamine receptors on brain microvessels has been being studied in detail by the group of Joó (Joó, 1993). Studies with antihistamines suggested that H_2 receptors play a major role in the development of brain edema in asphyxiated newborn pigs H_1 receptors also contribute (Dux *et al.*, 1987). The direct effect of histamine in neonates has not yet been reported. We designed an experiment to study the effects of intracarotid histamine administration on brain edema formation and endothelial APase activity in newborn pigs. The changes in endothelial APase activity by histamine receptor ligands were also investigated in CEC cultures. Among other deleterious events ischemia leads to modifications in the function of BBB resulting in brain edema (Joó and Klatzo, 1989). The simultaneous occlusion of both common carotid arteries in female Sprague-Dawley rats of CFY strain was reported to induce cerebral ischemia (Tósaki *et al.*, 1985). We used this well-characterized *in vivo* model of postischemic edema to measure APase activity and BBB permeability changes.

A novel intracellular affinity site for histamine (Hic) different from H_1, H_2, H_3, membrane receptors was described, which, among other functions, participates in tumour cell division and growth regulation. N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine (DPPE) a compound that antagonizes histamine binding at the Hic sites in the micromolar range, potentiates chemoterapy cytotoxicity to malignant cells but protects normal tissue in clinical studies (Brandes *et al.*, 1995). In higher doses, DPPE might have acute neurological side-effects in humans (Brandes *et al.*, 1995), and potentiated drug-induced seizures, and produced abnormal behaviour in mice (Sturman *et al.*, 1994). The possible effect of DPPE on BBB permeability and postischemic brain edema seemed to be interesting to be revealed.

Histamine uptake and binding

A rapid and high affinity, Na^+-dependent histamine uptake and release was described in cultured rat CECs (Huszti *et al.*, 1995). Astroglial histamine uptake, which is also a high affinity, carrier-mediated system operating bidirectionally was similar in many respects to that of found in endothelium. However, histamine, taken up by CECs either from the luminal and abluminal side was released from the cells preferentially through the luminal membrane representing the "blood side" of the system. This asymmetrical function of the histamine specific carrier on CECs may help to eliminate this well-established mediator of vasogenic brain edema from the CNS.

Mercurials were shown to interact with protein thiols within the cell membranes (Haas and Schmidt, 1985) which may account for their neuropathologic effect. The initial rate of histamine uptake reflecting a high affinity interaction could be inhibited by heavy metals, Pb^{2+} and Hg^{2+} in low 1-10 μM concentration. A preliminary study indicated that mercury applied at higher concentrations can strongly stimulate the histamine uptake (Huszti and Balogh, 1995) which prompted us to investigate in detail the effect of mercurials on the histamine uptake carrier in cultured CECs.

Vasoactivity of the endothelial cells

The brain microvascular endothelium, like other endothelial cells, is also capable to produce several substances mediating endothelium-dependent vasorelaxation and contraction (Kontos *et al.*, 1990). The released vasoactive agents can modulate the endothelial second messenger systems, regulate the BBB permeability (Joó and Klatzo, 1989) and play a key role in the regulation of the vascular tone of cerebral vessels.

An important molecule in the determination of vascular tone synthetized by endothelial cells is the endothelium-derived relaxing factor (EDRF), which has been identified as nitric oxide (NO), or a closely related compound (Ignarro *et al.*, 1987). In different cell types, a variety of stimuli are known to activate phospholipase A_2 which leads to the release of free fatty acids, including AA, the precursor of the family of eicosanoids. Prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) are known to mediate vascular relaxation, while tromboxane A_2 (TxA_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are implicated in the vasoconstriction in different vessels (Hintze and Kaley, 1984; Toda, 1980; Van Diest *et al.*, 1986). Previous studies revealed the eicosanoid profile of isolated cerebral microvessels (Dux *et al.*, 1981; Gecse *et al.*, 1982; Joó *et al.*, 1981) and it was also suggested that pure cultures of rodent CECs produced

predominantly the vasodilatory PGE_2 and PGI_2 (Moore *et al.*, 1988; De Vries *et al.*, 1995). The eicosanoids released in cerebral microvessels play also a role in the changes of BBB permeability (Villacara *et al.*, 1990), cerebral edema formation, and inflammatory reactions in the CNS (Chan *et al.*, 1983). Significant differences in the type, amount, and ratio of vasoactive eicosanoids released under basal condition or upon chemical or physical stimulation have been demonstrated, depending on the localization of the endothelium within the circulatory system.

Recently, a growing number of immortalized CEC lines has been produced. CEC lines RBE4 and GP8 display a non-transformed, well differentiated endothelial phenotype and express several structural and pharmacological characteristics of the BBB (Durieu-Trautmann *et al.*, 1993; Roux *et al.*, 1994; Greenwood *et al.*, 1996). Their potential usefulness as gene-therapy vectors to the CNS was investigated in rat models (Lal *et al.*, 1994; Johnston *et al.*, 1996). For this reason, the comparison of the eicosanoid profile, and vasoactivity on a bioassay model of primary RBCs to that of immortalized cell lines may provide useful new pieces of information.

4. Materials and methods

4.1. Preparation of primary cultures of rat brain endothelial cells

Two-week-old Sprague-Dawley CFY rats of either sex were anesthetized with ether. After thorough rinse with 70% ethanol, than with iodine in 70% ethanol, heads were cut, and placed into a sterile glass Petri dish. In the laminar flow box, forebrains were removed from the skulls (cerebellum not) with sterile microdissecting forceps and scissors, and collected in cold sterile phosphate buffered saline (PBS, without calcium and magnesium, pH 7.4). Meninges were removed on sterile filter paper (Whatman 3M) from each brain hemisphere while at the same time white matter was "peeled off" with the aid of fine curved forceps. Gray matter was carefully collected from the filter paper (meninges stuck to it) and minced to approximately 1 mm^3 pieces by sterile disposable scalpels in the incubation medium (270 U/ml collagenase, 1 mg/ml dispase, DMEM-F12 containing antibiotics) in a sterile glass Petri dish. Incubation media for enzymic digestion was always prepared freshly from lyophilized enzymes, then sterilized by filtration. Their pH was adjusted to 7.4.

The minced tissue was transferred into a centrifuge tube with the rest of the collagenase-dispase solution (total: 2 ml/brains) and triturated with a pipette (10 up and down), than incubated at 37 °C for 1.5 h in shaking waterbath. After this incubation, cold DMEM-F12 was added to the homogenate and centrifuged at 1000 *g* for 8 min. The supernatant was aspirated and 20% bovine serum albumin contained DMEM-F12 (2 ml/brain) was added to the homogenate, mixed well by trituration and centrifuged at 1000 *g* for 15 min. The myelin layer and the supernatant was aspirated, the pellet washed once in DMEM-F12 (700 *g* for 5 min) then further digested in waterbath for an other 1 h in the incubation medium 1ml/brain. The cell suspension was centrifuged (700 *g* for 5 min). The pellet was suspended in 2 ml DMEM-F12 and carefully layered on a continuous 33% Percoll gradient and centrifuged at 1000 *g* for 10 min. For the gradient 10 ml Percoll, 18 ml PBS, 1 ml FBS and 1 ml 10× concentrated PBS were mixed, sterile filtered and centrifuged at 4 °C, 30000 *g* for 1 h.

The band of the endothelial cell clusters (clearly visible as a white-grayish layer above the red blood cells) was aspirated, washed twice in DMEM-F12 (at first 1000 *g*, 8 min, then 700 *g*, 5 min). The cells were suspended in culture medium (DMEM-F12 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 2 mM glutamine, 20 % heat inactivated FBS or PDS and from the second day 1 ng/ml bFGF) and were seeded onto rat tail collagen-coated 35 mm plastic dishes. Starting culture from 10 brains, we could obtain

confluent primary culture of rat CECs in 10 pieces of 35 mm tissue culture dish, equivalent approximately to 100 cm^2 surface area. The medium was changed on the next day, later on every third day.

Complement killing: At the third day RBEC cultures were washed twice with PBS, then incubated with monoclonal anti-mouse Thy 1.1 antibody (antibody : DMEM-F12 = 1:500) for 1 h at 37 °C. After washing twice with PBS, the cultures were incubated in the presence of rabbit complement (complement : DMEM-F12 = 1:3) for 2 h at 37 °C, which mediated the cytolysis of those cells which expressed on their surface the antigen Thy 1.1. After washing in PBS the cells were cultured in the previously described complete medium.

4.2. Immortalized brain endothelial cell lines

RBE4 cells are derived from rat brain microvessel endothelium immortalized with the plasmid pE1A-neo and characterized in respect to BBB properties (Durieu-Trautmann *et al.*, 1993; Roux *et al.*, 1994). The cells were passaged twice a week in DMEM/F12, supplemented with 10% heat-inactivated FBS, 2mM glutamine, 1 ng/ml bFGF and 300 μg/ml G418 onto rat tail collagen coated dishes and used between passages 30 and 50. GP8 is a temperature sensitive SV40 large T immortalized rat brain capillary cell line described by Greenwood *et al.* (1996). These cells, used between passages 10 and 20, were cultured in 20% PDS, 2mM glutamine, 1ng/ml bFGF and 200 μg/ml G418 in DMEM/F12.

4.3. Treatment of RBE4 cells with histamine receptor ligands

RBE4 cells were cultured in 6 cm petri dishes and stimulated in 1 ml serum free DMEM/F12 for 1 hour at 37°C with 10^{-5} M histamine with or without 10^{-6} M mepyramine (H_1-receptor blocker), 10^{-6} M ranitidine (H_2-receptor blocker) or in the presence of the antagonist only. The used concentations were selected on the basis of the affinity of agents to the receptors. After the incubation, the cells were washed with PBS, collected and homogenized in 500 μl PBS.

4.4. Intracarotid histamine administration to newborn pigs

Newborn pigs of either sex (age: 4-8 h, weight: 1,130-1,600 g) were included in the study. The animals were given intramuscular pentobarbital anesthesia (30 mg/kg), then an umbilical artery was catheterized and physiological parameters were monitored (Ábrahám *et al.*, 1996). The right internal carotid artery of the animals was catheterized through the external branch and histamine was given in slow intraarterial injection (0.5 ml isotonic saline)

in the following doses: 0 mol; 10^{-6} mol; 5×10^{-6} mol; 10^{-5} mol; 5×10^{-5} mol; and 10^{-4} mol (6 groups, n=4 in each). Then the catheter was removed and the external carotid artery was ligated.

4.5. Bilateral common carotid artery ligation in rats

A closed colony of randomly bred Sprague-Dawley CFY rats weighing 200-250 g were used. Cerebral ischemia was induced by ligation of common carotid arteries of female rats on both sides (Tósaki *et al.*, 1985). The animals were subjected to ischemia of different duration (1, 2, 4, 8 and 16 h; n=12 in each). At the end of the experiments cortical tissue were taken from each animal and cerebral microvessels were isolated.

4.6. Blood-brain barrier permeability measurements *in vivo*

The development of vasogenic brain oedema was measured as the extravasation of two intravascular tracers: sodium fluorescein (SF) (mw: 376) and Evan's blue labelled albumin (EBA) (mw: 67,000). The animals were given a solution of both dyes (2%, 5 ml/kg) in an intravenous injection 30 min before the end of the experiments. Then blood samples were taken and rats or pigs were perfused with 200 ml/kg isotonic saline. Serum as well as tissue samples from cerebral cortex were homogenized in 3 ml of cold 7.5% trichloroacetic acid and centrifuged with 10,000 *g* for 10 min. The concentration of tracers was measured in supernatants by a Hitachi fluorimeter, the absorbance of EBA at 620 nm, while the emission of SF at 525 nm after excitation at 440 nm, and the extravasation was expressed as brain tissue concentration divided by serum concentration, as described earlier in detail (Ábrahám *et al.*, 1996).

4.7. Isolation of cerebral microvessel

Cerebral microvessels were isolated using the method of Tontsch and Bauer (1989). The cortices were rinsed in ice cold isotonic sucrose buffer (0.32 M sucrose, 3 mM Hepes, pH 7.4) the pia mater was removed and cut into pieces of about 1 mm^3. After two times homogenization and centrifugation (4 °C for 10 min at 1000 *g*) the supernatant was discarded. The pellet was homogenized and after a series of centrifugation steps (4 °C for 45 sec at 100 *g*) the supernatants were pooled and washed several times (4 °C for 1 min at 200 *g*). The resulting pellet almost exclusively contained microvessels.

4.8. Measurements of acid phosphatase activity

APase activity was measured by the rate of hydrolysis of *p*-nitrophenylphosphate both in homogenized cultured CECs, brain tissue and ICMV (Shimohama *et al.*, 1993). Different samples 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 30 min with or without L-(+)tartrate (10 mM), sodium fluoride (5 mM), or zinc sulphate (2.5 mM). After incubation, 45 µl of 1 M NaOH was added to stop the reaction and the absorbance was read at 450 nm by an ELISA reader (Labsystems Multiskan Biochromatic type 348). Enzyme activity, expressed in mU/mg protein, was determined from a calibration curve using increasing concentrations of purified APase (AcP Lin-trol, Sigma) of known activity. Protein concentration was determined by a dye-binding method (Bradford, 1976).

4.9. [^{3}H]-histamine uptake and binding to uptake carrier in cultured cerebral endothelial cells

[^{3}H]-histamine uptake measurments were carried out as described by Huszti *et al.* (1990). Briefly, after removal of the growth medium from the cell cultures (RBEC and RBE4) and several washing with 1-2 ml Krebs buffer, the cells were preincubated in the presence of tested compounds in Krebs buffer at 37 °C for 15 min. The assay was started by the addition of [^{3}H]-histamine (0.1-0.2 µCi) containing unlabelled amine (final concentration: 0.04 µM) and incubated further at 37 °C for 3-20 min. The incubation was determined by adding 1 ml ice-cold Krebs buffer and rapid removal of the medium. After 3 times washing, the cells were lysed in 500 µl of 1 N NaOH (containing 0.1 % Triton-X-100) and 1-2 h later samples were taken from the cell lysates for scintillation counting. The protein contents were determined by the method of Lowry (1951).

Histamine binding to uptake carrier was measured in RBEC and RBE4 cells cultured in 24-well tissue cultured plates. After removal of the growth medium from the cell cultures and several washing with 1-2 ml of ice cold PBS, the cells were stored frozen at -20 °C until use or they were separated from the washing solution and used freshly. The cells were incubated in Ca^{2+} (2.5 mM) and Mg^{2+} (2.5 mM)-rich sodium phosphate buffer (50 mM, pH 7.4) with 10-100 nM of [^{3}H]-histamine (0.5 µCi) in the presence or absence of 100 µM $HgCl_2$ and/or 4 µM impromidine, in the presence of H_1, H_2 and H_3 antagonists, triprolidine, cimetidine, and thioperamide (40 µM each) in a total volume of 500 µl at 37 °C for 30 min. The reaction was stopped by dilution with 1-2 ml of ice-cold medium. The unbound radioactivity was removed

by several washing with 3 ml ice-cold medium and the radioactivity, retained in the cell membranes was measured in 5 ml Aquasafe "300" (Zinsser Analytic, Frankfurt, Germany) after lysing the cells in 250 µl 1N NaOH containing 1% Triton-X-100. Non-specific binding was obtained by incubating the cells with the addition of 40 µM unlabelled histamine and substracted from "total" to get the "net bindings".

4.10. Bioassay for the vasoactive metabolites of brain endothelial cells

Mongrel dogs of either sex weighing 9-15 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.) and heparinized (1000 I.U./kg i.v.). The heart was excised and placed into a Krebs-Henseleit (KH) solution of the following composition (in mM): NaCl 120, KCl 4.2, $CaCl_2$ 1.5, $NaHCO_3$ 20, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 11. Rings (1.1-1.9 mm o.d., 5 mm widths) from the circumflex branches of left coronary artery were isolated. Endothelium was removed by gently rubbing the endothelial surface with a stainless steel wire covered with a cotton swab. Rings were mounted in water-jacketed baths containing 2 ml of KH solution bubbled with 95 % O_2 and 5% CO_2 gas mixture at 37 °C. The isometric tension was recorded with a force-displacement transducer (Hugo Sachs Elektronic, Type F30, Germany). Rings were stretched up to 10 mN and allowed to stabilize for 45 min. This tension was readjusted to 10 mN during equilibration. The arterial rings were exposed to 25 µM $PGF_{2\alpha}$ and at the maximal amplitude of contraction 1 µM acetylcholine was applied. Only those arterial preparations were used for the experiments that responded with contraction after addition of the endothelium dependent vasodilator, acetylcholine. This protocol served as an evidence for functionally deendothelialized arterial preparations. Confluent monolayers of cultured RBECs, RBE4 or GP8 were suspended in KH solution resulting in 5×10^6 cells/ml and 5×10^5 cells/ml. The cell suspensions were divided into two parts: one was treated with solvents (control), the other either with 100 µM NOLA (N^{ω}-nitro-L-arginine) or with 10 µM indomethacin for 30 min at 37 °C. Final concentration of indomethacin at the highest cell number in the organ bath was 0,3 µM. When $PGF_{2\alpha}$ induced contraction of coronary rings had reached the steady-state amplitude the control or treated endothelial suspensions were added cumulatively.

4.11. Assay of prostaglandin synthesis in cerebral endothelial cells

Confluent cultures of primary, RBE4 or GP8 cells in petri dishes (35 mm diameter, approx. 3×10^5 cells/dish) were incubated at 37 °C with the tracer substrate, 1-[^{14}C]-AA (0.172

pmol, 3.7 kBq) in 1 ml serum free DMEM-F12. Thirty minutes later the incubation medium of the cell culture was removed and acidified to pH 3 with formic acid. According to our preliminary experiments a period of 30 min was appropriate for labelling *in vitro* CECs. The arachidonate metabolites were immediately extracted with ethyl acetate (2×3 ml) from the samples, and the organic phases were pooled and evaporated to dryness under nitrogen. The residues were reconstituted in 150 µl ethyl acetate and quantitatively applied to silica gel thin-layer plates. The plates were developed to a distance of 15 cm in an organic phase of ethyl acetate : acetic acid : 2,2,4-trimethylpentane : water (110:20:30:100) by means of overpressure thin-layer chromatography. The radiolabelled products of AA were identified with unlabelled authentic standards, which were detected with anisaldehyde reagent. Each 3 mm band of the chromatograms was scraped off and the radioactivity was determined in a Packard Tri-Carb 2100TR liquid scintillation analyser, using 5 ml toluene containing 0.44% w/v 2,5-diphenyloxazole, 0.02% w/v 1,4-di-[2-(5-phenyl)-oxazole]benzene and 10% v/v ethanol.

4.12. Statistical analysis

The values measured in different groups (APase activity and BBB permeability) were compared using the Kruskal-Wallis one way analysis of variance (ANOVA) on ranks followed by the Dunn's test. Enhancement or reduction of arterial tone was calculated as percent of maximum increase (+) or decrease (-) of contractile force compared to the pre-drug values. Results are expressed as mean ± S.E.M. and n refers to the number of experiments. ANOVA followed by Newman-Keuls multiple range test (bioassay) or the Tukey-B multiple comparison *post hoc* test (eicosanoids) was used to determine the significance of differences between the corresponding mean values. $P < 0.05$ values were considered significant differences.

4.13. Animals

Sprague-Dawley CFY rats, fed commercial food pellets and tap water were obtained from the Institute's animal house, while newborn pigs used in the experiments, from a local cooperative farm. Animal experiments were performed according to the NIH Guidelines and were approved by the Ethical Committee of the Biological Research Center.

5. Results

5.1. Primary rat brain endothelial cell cultures

The small vessel fragments obtained at the end of the isolation procedure attached rapidly to collagen coated surfaces, and with in 2-3 days colonies of RBECs emerged. A non-overlapping continuous monolayer with some swirling patterns was formed at the end of the first week. RBECs displayed a so-called "fibroblast-like" morphology: cell-shape is fusiform with an oval nucleus in the center, neighbouring cells tightly attached to each other in such a way that no intercellular space could be observed. RBECs gave specific immunohistochemical staining with anti-FVIII antibody, bound the galactose-specific BS-I-B_4 isolectin and showed positive histochemical staining for alkaline phosphatase enzyme (Publ. II. Fig. 1).

In FBS containing medium, CECs lose the elongated, spindle-shape fenotype, the cells move away from each other and pericytes become the dominant cell type. Pericytes appear as large spreading cells with highly irregular edges which do not express FVIII (Shepro and Morel, 1993).

When the cultures were fed with PDS which does not contain PDGF, the endothelial cells showed healthy, uniform phase-bright appearance. RBECs were tightly packed against each other without intercellular gaps (Publ. V. Fig. 2B). Less contaminating cells could be found than in those cultures which received FBS. There was also a difference in RBECs number between the cultures which were treated with different sera. In the presence of PDS we counted 962 ± 65 cells/mm^2 while in FBS containing medium the cells grew more sparsely: 807 ± 53 cells/mm^2. From the second day on, bFGF (1 ng/ml) was added to the cultures, which greatly improved the growth rate of the endothelial cells.

We could considerably reduce the number of contaminating cells by selective cytolysis using anti-Thy1.1 antibody and complement (Fig.1) resulting in more than 95% pure RBEC cultures. If non-endothelial cells were not removed in time, they could overgrow the RBECs in a couple of days.

5.2. Regulation of acid phosphatase enzyme activity by histamine in RBE4 cell line

Histamine increased significantly the total APase activity as well as tartrate-sensitive, lysosomal form in cultured CECs. The effect of histamine on total but not tartrate-sensitive activity could be inhibited by ranitidine (Fig. 2.B). The stimulatory effect of histamine on total acid phosphatase activity seemed, therefore, to be mediated by H_2-receptors. On the other

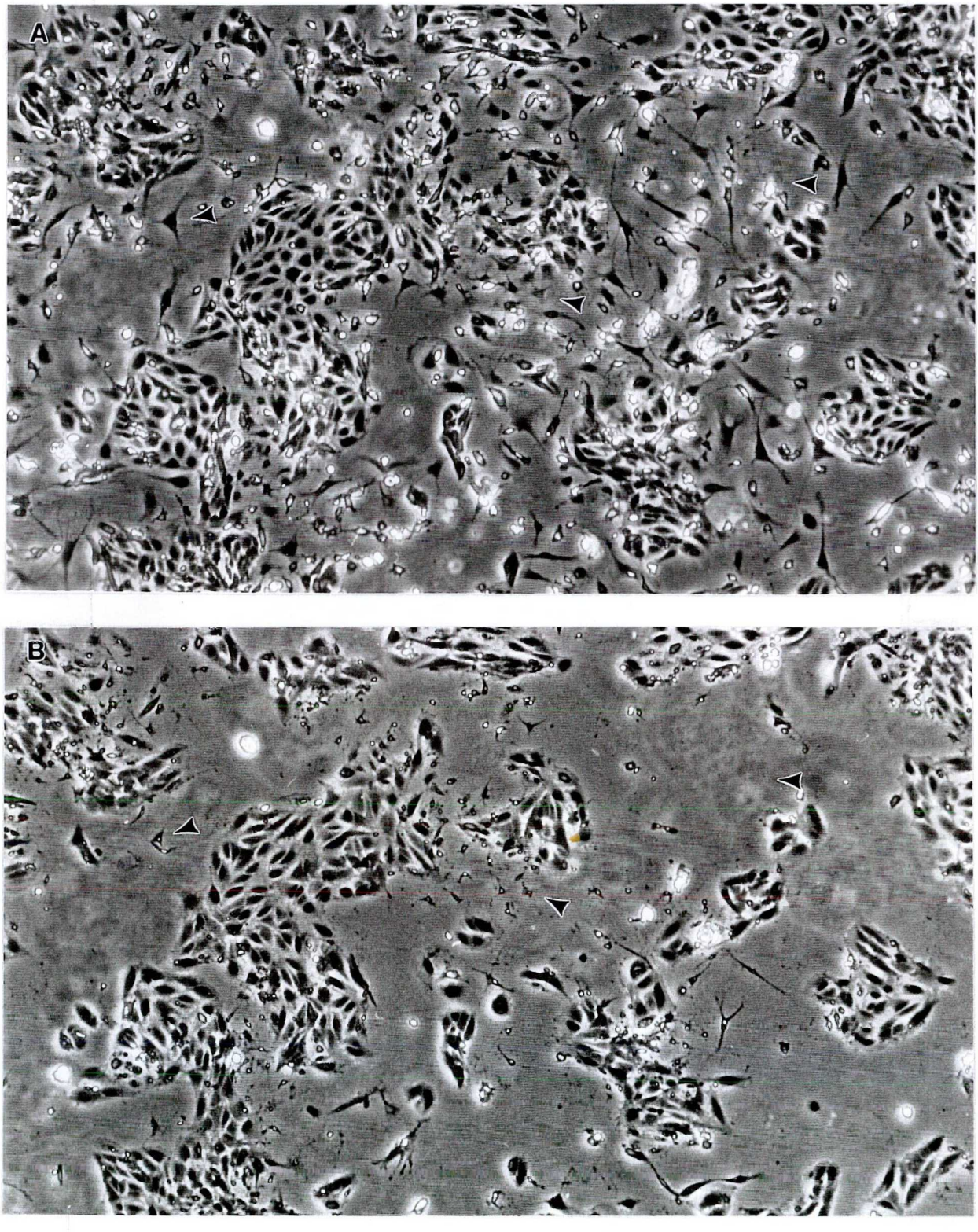

Fig. 1. Complement killing. **(A)** Before the cytolysis the contamining cells look as multipolar or round flat cells around or on the top of the endothelial cell clusters. **(B)** Immediately after the killing we can see only the shadows or the nucleus of the lysed pericytes and astrocytes, sometimes the whole cell disappears during the process.

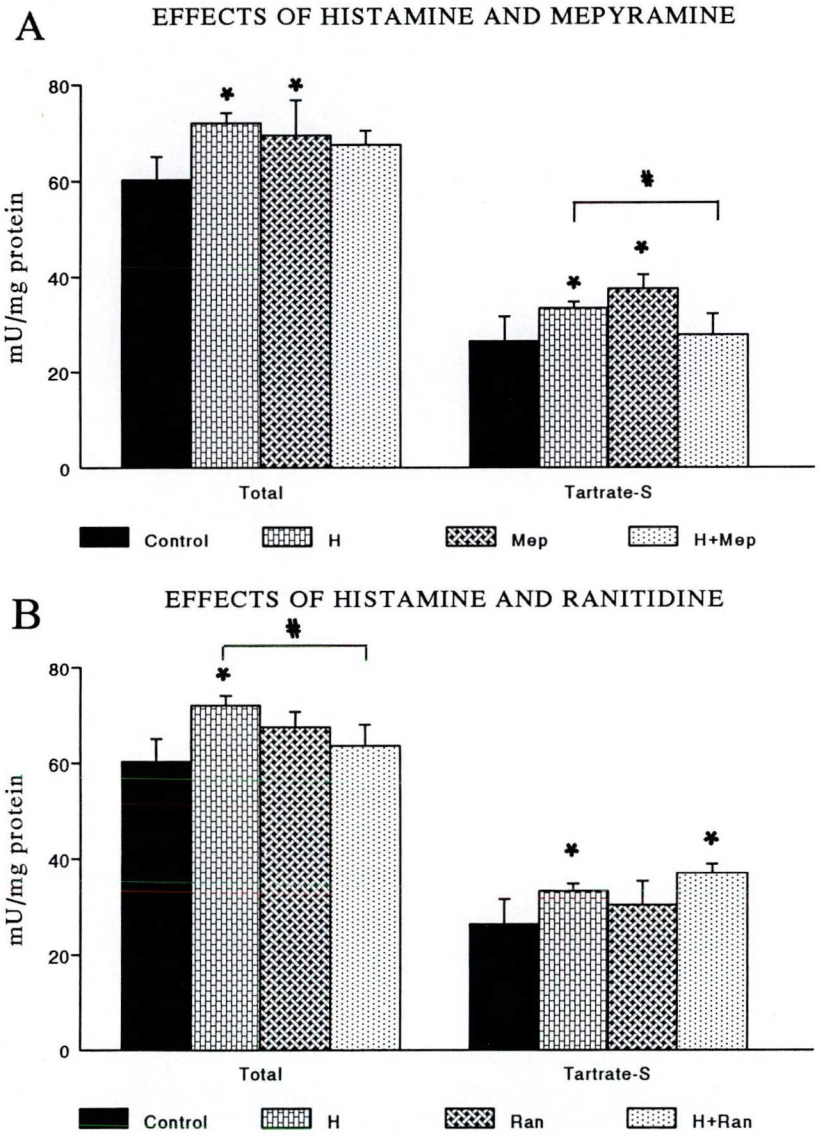

Fig. 2. Effect of histamine, mepyramine **(A)** and ranitidine **(B)** on the activity of total and tartrate-sensitive form of acid phosphatase on RBE4 cell line. In these experiments, the cells were treated with 10^{-5} M histamine (H), 10^{-6} M mepyramine (Mep), 10^{-6} M ranitidine (Ran), or solvent in the control groups. The error bar represents the S.E.M. and n=6. Significant differences: * = p <0.05 compared to control values, while # = p <0.05 compared to histamine-treated values.

hand, mepyramine blocked the effect of histamine on tartrate-sensitive form only (Fig 2.A). Therefore, H_1-receptors are apparently involved in the regulation of activity of tartrate-sensitive, lysosomal isoforms. Mepyramine and ranitidine alone also increased the activity of enzyme which may be explained by the partial agonist effect of the drugs.

5.3. Blood-brain barrier permeability and acid phosphatase enzyme activity changes after intracarotid histamine administration in newborn pig

A significantly increased transport of both tracers, SF and EBA was found in cerebral cortex of newborn pigs 1 h after histamine injection in 5×10^{-5} mol and 10^{-4} mol histamine-treated groups compared to that in control group (Fig. 3.A and B). Significant linear correlations were found between dose of histamine and extravasation of both SF (Publ. VI., Fig. 2.A) and albumin (Publ. VI., Fig. 2.B).

Intracarotid histamine administration resulted in a dose-dependent increase in both total and tartrate resistant APase activity in homogenized ICMV (Fig. 4.B and D), but not in cortical tissue samples (Fig. 4.A and C). Total APase activity was significantly ($P < 0.05$) higher in the cerebral capillary endothelium of 10^{-4} mol histamine-treated animals than that in vessels of control (0 mol histamine-treated) pigs. There was a linear correlation between the dose of histamine and total enzyme activity (Publ. VI., Fig. 1.A). Each dose of histamine significantly ($P < 0.05$) increased the tartrate-resistant acid phosphatase activity in cortical microvessels of new-born pigs compared to that measured in control animals. Publ. VI., Fig. 1.B shows that the dose of vasogenic amine injected and tartrate-resistant APase phosphatase activity are also correlated. There was no change in tartrate-sensitive and zinc-induced APase activity. We could detect neither a dose-dependent change in the enzyme activity between different groups (Fig. 4.A and C) nor any correlation between treatments and activities in brain tissue homogenates (Publ. VI., Fig. 1.C and D).

5.4. Blood-brain barrier permeability and acid phosphatase enzyme activity changes after cerebral ischemia in rats

In rats, BBB permeability for SF was increased all through the ischemic period, while EBA transport was elevated more than 2 h after the carotid occlusion and fell back to the level of the control valves at 16 h (Fig. 5). Total and tartrate resistant, cytosolic type, APase activity in ICMV was significantly increased 2 h after the occlusion compared to the values measured in other groups. Lysosomal, tartrate sensitive APase activity was significantly elevated in cortical tissue 1 h after the occlusion but not in ICMV. Zinc-induced APase activity was

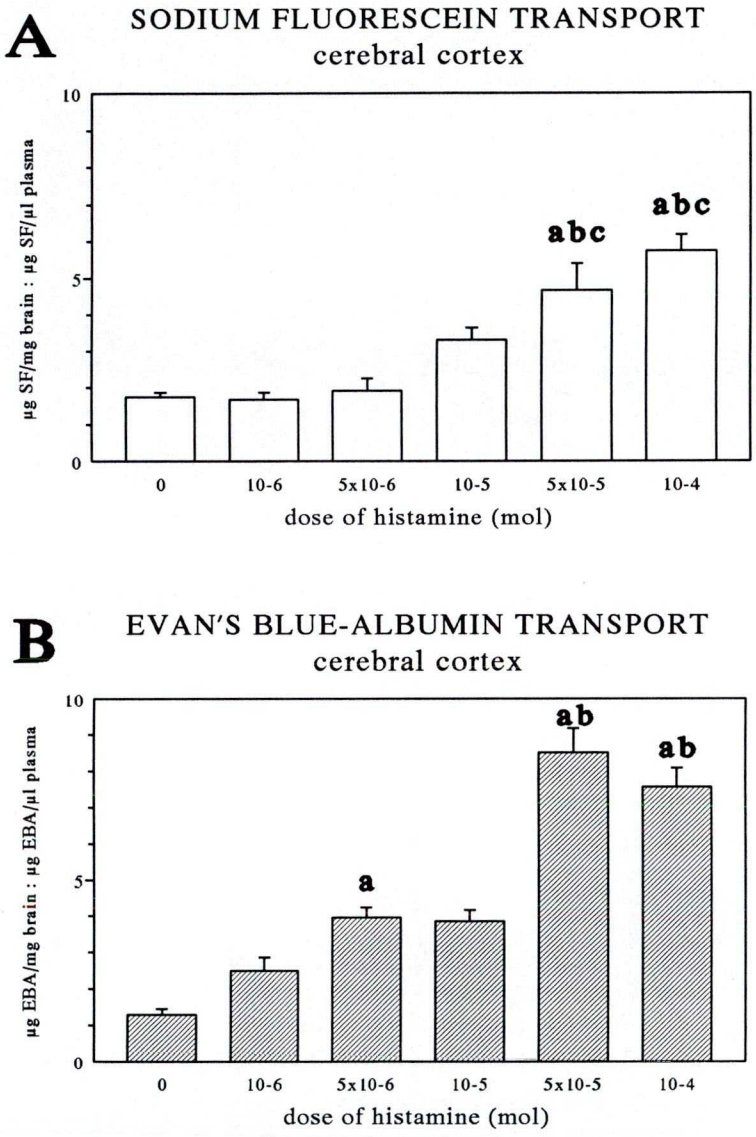

Fig. 3. Development of brain edema in newborn pigs 1 h after intracarotid histamine challenge. BBB permeability markers were SF **(A)** and EBA **(B)**. Each value represents mean ± S.E.M. (n=6). Symbols indicate significant differences ($p<0.05$) compared to the following treatments: a=0 mol, b=10^{-6} mol, c=5×10^{-6} histamine.

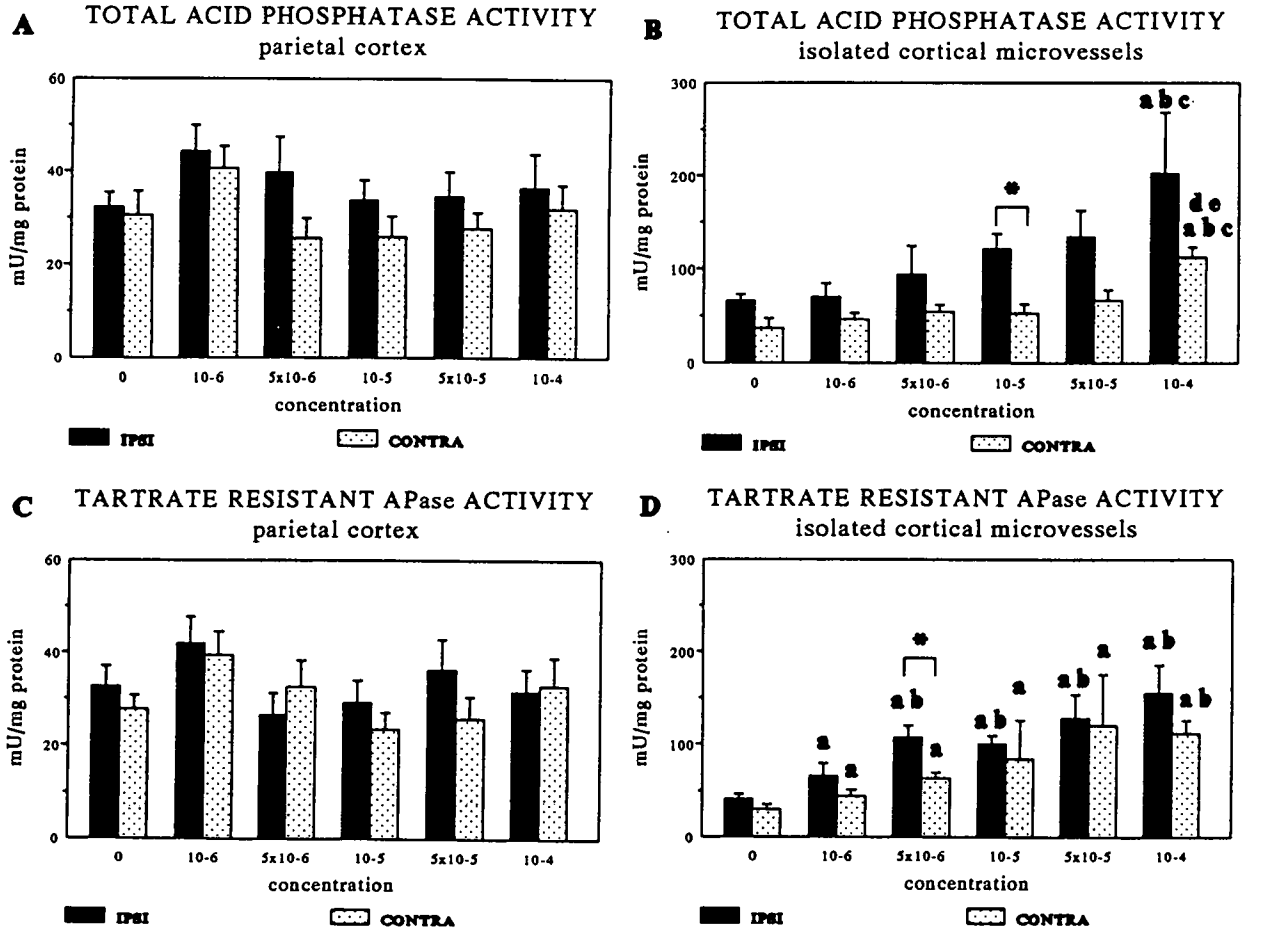

Fig. 4. Total **(A,B)** and tartrate-resistant **(C,D)** APase activity in cerebral cortex **(A,C)** and in homogenized ICMV **(B,D)** 1 h after intracarotid administration of 0, 10^{-6}, 5×10^{-6}, 10^{-5}, 5×10^{-5} and 10^{-4} mol of histamine in the new-born pig. Each value represents mean ± S.E.M. (n=6). Symbols indicate significant differences ($p<0.05$) compared to the following treatments: a=0 mol, b=10^{-6} mol, c=5×10^{-6} mol, d=10^{-5} mol, e=5×10^{-5} mol histamine.

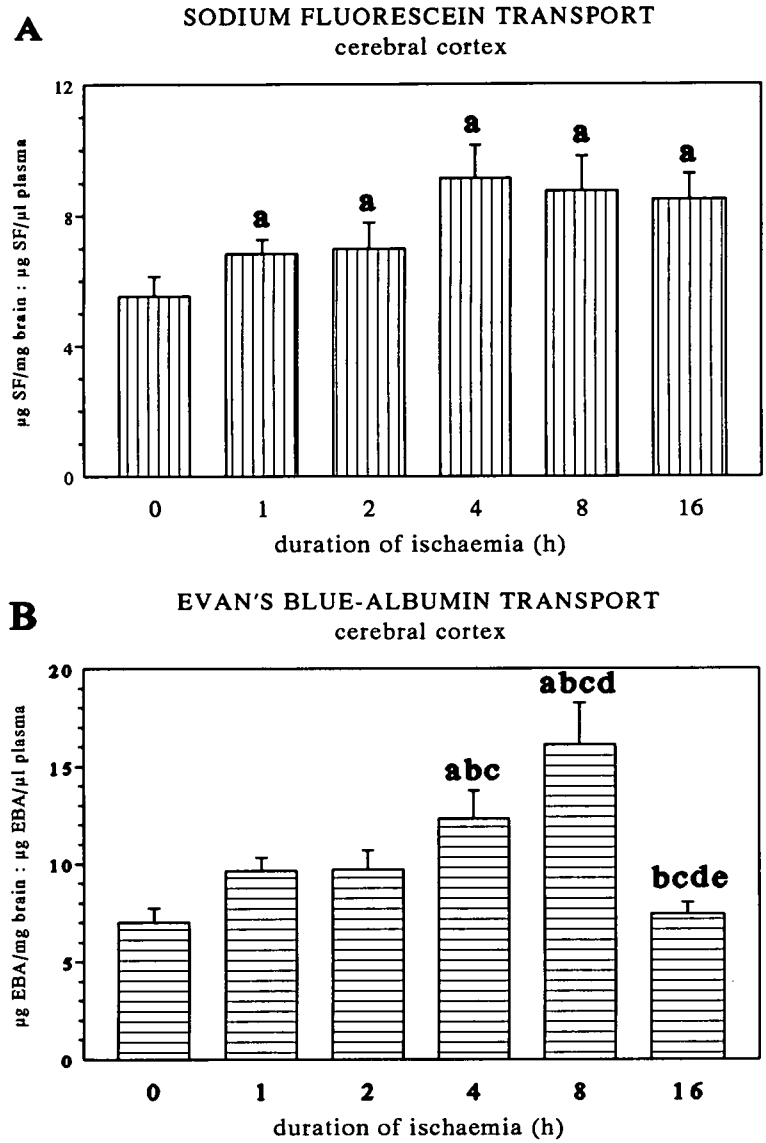

Fig. 5. Development of brain edema in rats during cerebral ischemia induced by BCCAL. BBB permeability markers were SF **(A)** and EBA **(B)**. Each value represents mean ± S.E.M. (n=6). Symbols indicate significant differences ($p<0.05$) compared to the duration of ischemia: a=0 h, b=1 h, c=2 h, d=4 h, e=8 h.

unchanged during global ischemia in ICMV, but it decreased sharply at 2 h in cerebral cortex (Fig. 6).

5.5. Mercury-stimulated histamine uptake and binding in brain endothelial cells

The histamine uptake was stimulated by 100 μM $HgCl_2$ in RBEC (Fig. 7.A). Pretreatment of the cells with ouabain (1 mM), a potent Na^+/K^+ ATP-ase blocker suppressed mercury-stimulated uptake (Fig. 7.A). Potent sulfhydryl agents 1 mM DTT (dithiothreitol) and 0.1 mM cysteamine completely inhibited the histamine uptake increased by mercury (Fig. 7.B) thereby reversing the action of $HgCl_2$ and not simply providing a protection from it. In the absence of mercury, DTT or cysteamine has no effect on histamine uptake (data not shown). The histamine uptake was markedly inhibited by impromidine (Fig. 7.C), a potent H_2 agonistic and H_3 antagonistic compound (Huszti *et al.*, 1994). The concentration required for 50% inhibition was less than 4 μM (K_i=2.8 μM). Moreover, after 10 min incubation the corresponding uptake rates were lower than in the control.

As detected in astroglial cell membrane (Publ. III.) binding sites of histamine carrier were also found in RBE4 cell line (Fig. 8). The binding capacity (B_{max}) increased markedly from 0.18±0.03 to 1.20±0.36 pmoles/mg protein (Fig. 8. I.A). The apparent dissociation constants (K_D) for basic and Hg^{2+}-stimulated binding were 22.5±3.2 nM and 26.5±3.6 nM, respectively (Fig. 8. II.B and C). Impromidine (4 μM) decreased the Hg^{2+}-stimulated B_{max} by about 50% (Fig. 8. II. A) with a slight modification of the K_D values (Fig. 8. II. B and C).

5.6. Vasoactive action of cerebral endothelial cells

In the first part of the experiments, the vasoactive action of endothelial cell culture suspensions was determined. Fig. 9.A demonstrates the effect of primary RBEC on the tone of isolated coronary artery precontracted with $PGF_{2\alpha}$. The primary cells decreased the vascular tone which correlated with the cell number and the maximal relaxation was 32.5 % after administration of 2×10^5 cells/ml. RBE4 cells caused a small but not significant contraction (Fig. 9.B) Enhancement of arterial tone by these cells showed no cell-response relationship between 0.2-6.2$\times10^5$ cells/ml with an average of 15-18 % contraction. GP8 cells, like primary cells decreased markedly the tone of precontracted arterial rings (Fig. 9.C) which depended on the number of cells with a maximum of 33.2 ±8.2% (n=7) decrease of $PGF_{2\alpha}$ induced tone.

In the second part of the bioassay experiments, the possible involvement of NO as an endothelium dependent vasodilator in the changes of coronary tone was investigated. After 30

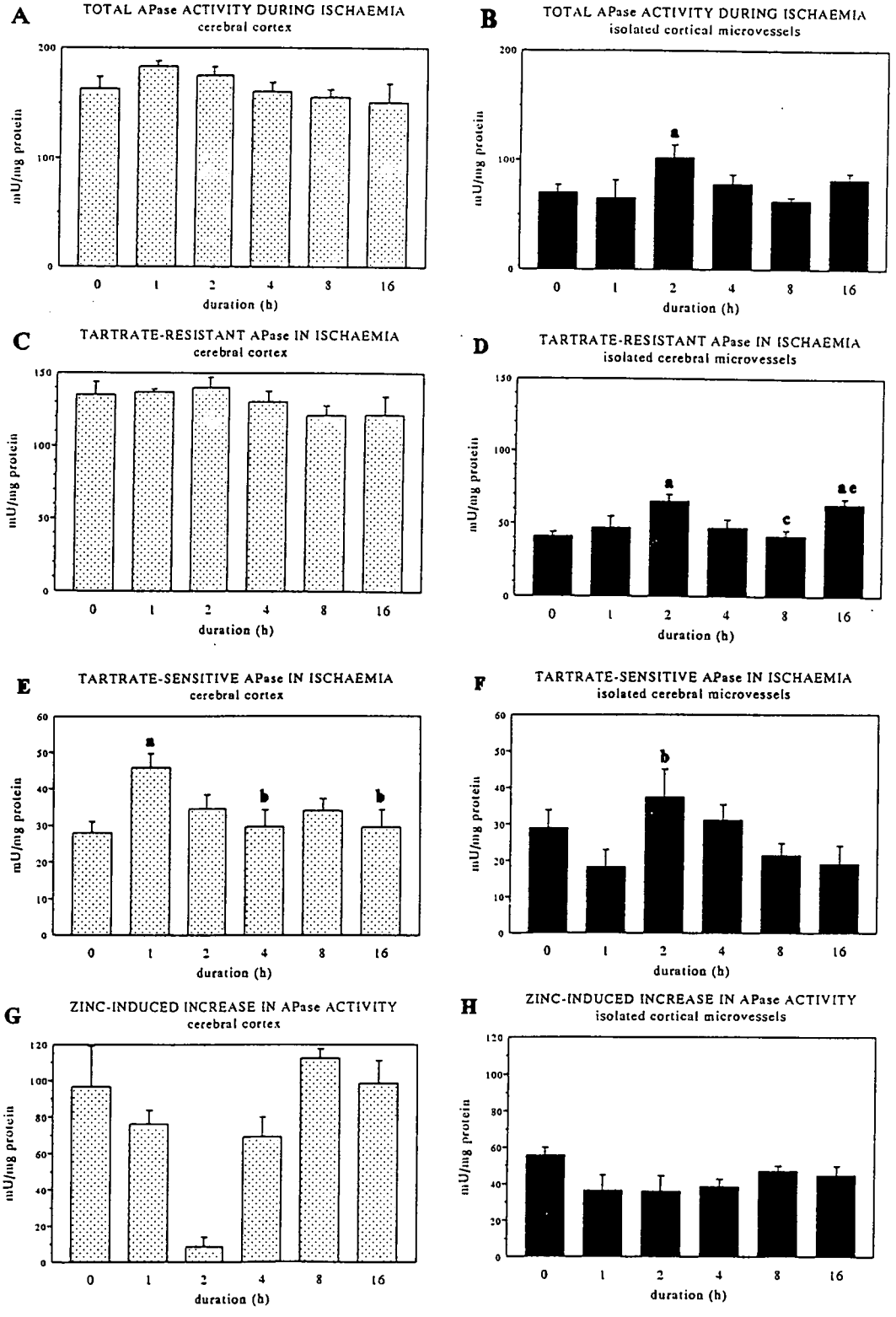

Fig. 6. Effect of cerebral ischemia induced by BCCAL on the total **(A,B)**, tartrate-resistant **(C,D)**, tartrate-sensitive **(E,F)** and zinc-inducable **(G,H)** APase activity in cerebral cortex **(A,C,E,G)** and homogenized ICMV **(B,D,F,H)** in rats. Each value represents mean ± S.E.M. (n=6). Symbols indicate significant differences ($p<0.05$) compared to the duration of ischemia: a=0 h, b=1 h, c=2 h, e=8 h.

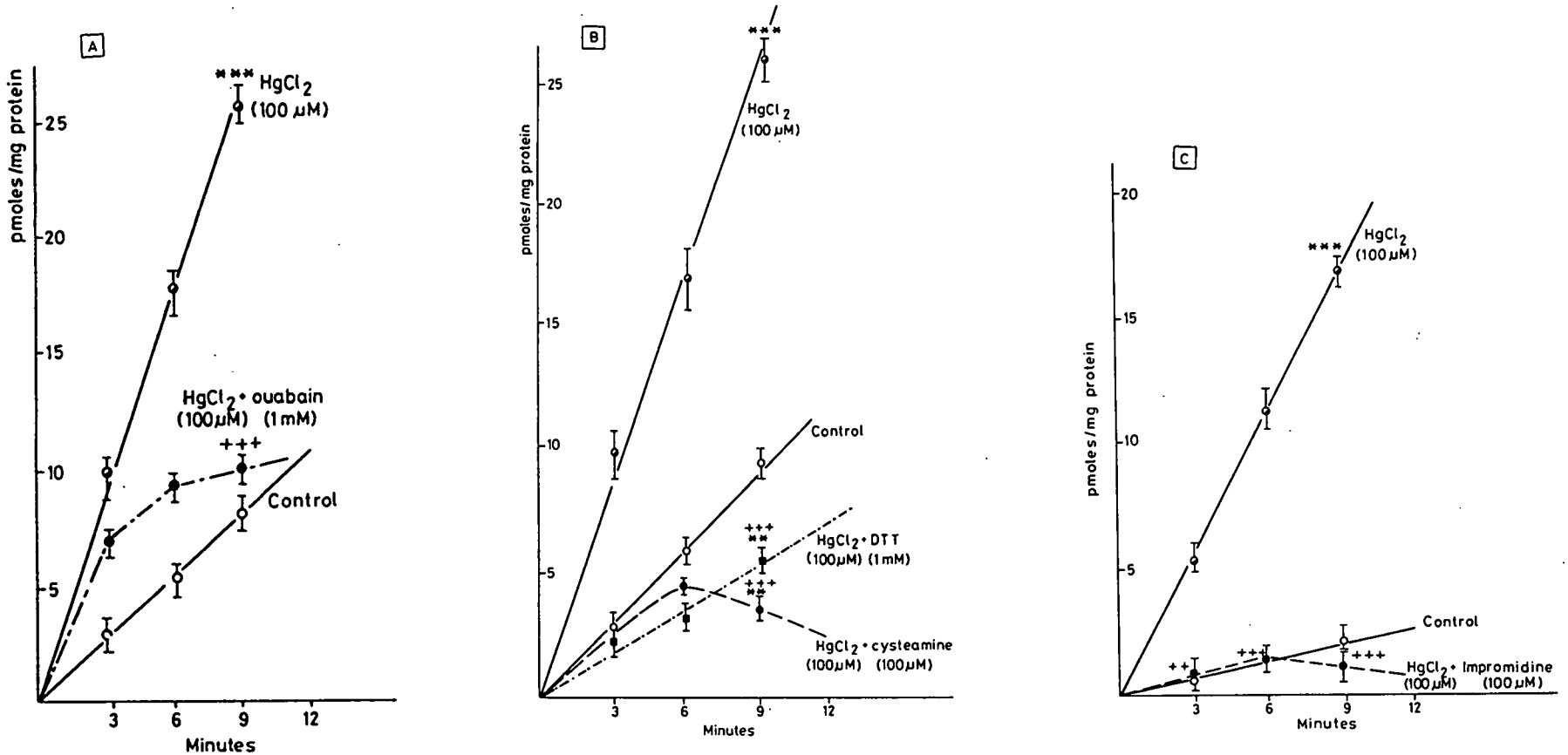

Fig. 7. Time course of basic and mercury-stimulated [^{3}H]-histamine uptake by RBEC: effect of ouabain **(A)**, sulfhydryl agents (DTT and cysteamine), **(B)** and impromidine **(C)**. Data points represent mean values of 6 individual experiments. S.E.M. were indicated by vertical bars. Significant differences between basal and mercury stimulated uptake are: $^{***}p<0.001$; between mercury stimulated uptake in the absence and presence of inhibitors are: $^{++}p<0.01$; $^{+++}p<0.001$; between basal and mercury stimulated uptake in the presence of inhibitors are: $^{**}p<0.01$.

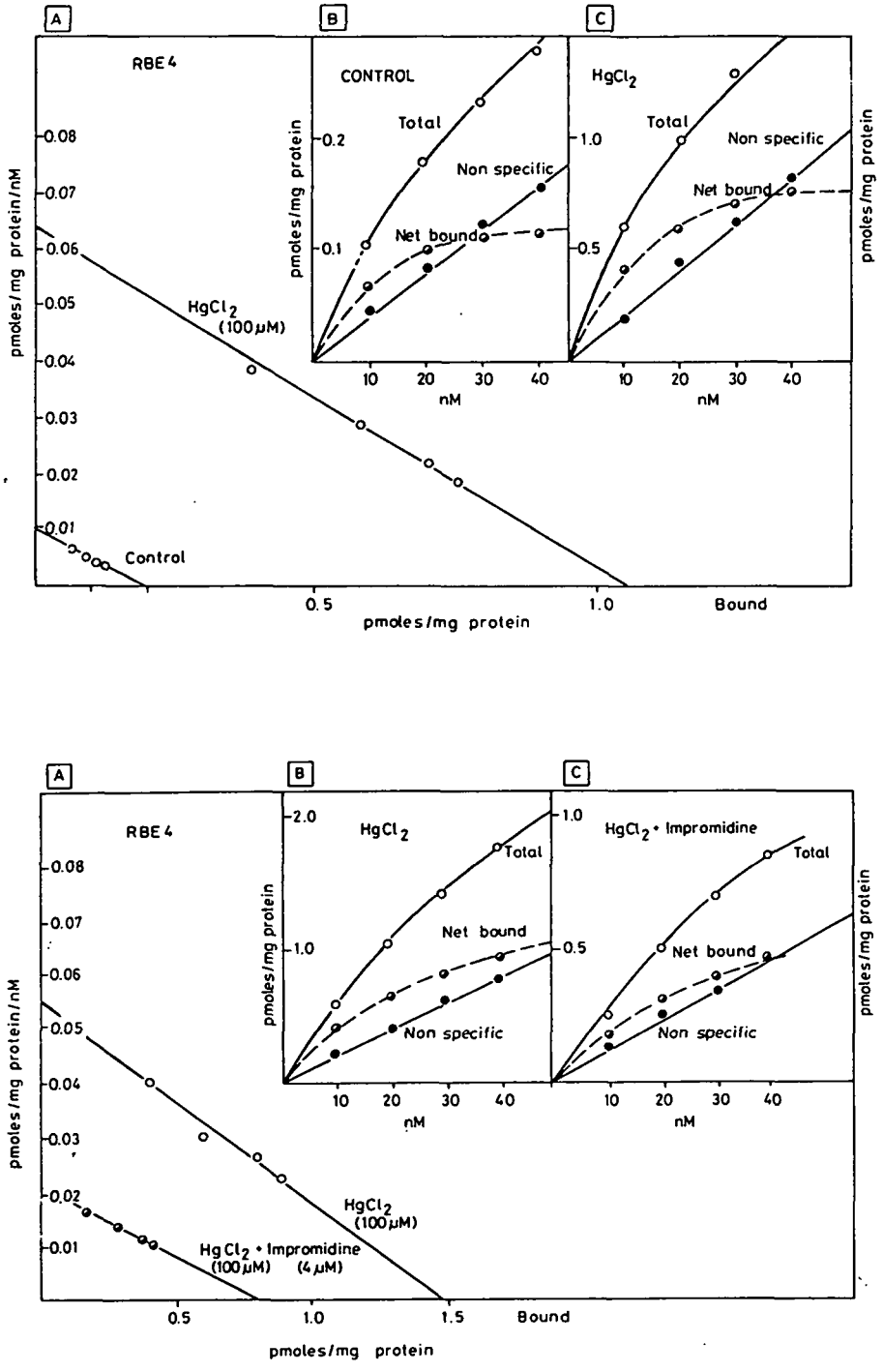

Fig. 8. Kinetics of **(I.)** basic and mercury-stimulated and **(II.)** mercury-stimulated [^{3}H]-histamine binding in the presence and absence of impromidine in RBE4 cell line. **I.A:** Scatchard plots of [^{3}H]-histamine binding, the effect of mercuric chloride; **II.A:** the effect of impromidine on mercury induced stimulation. Saturation curves of control **(I.B)** and mercury-stimulated **(II.B)** histamine bindings in equilibrium (30 min incubation at 37 °C). Saturation curves of mercury-stimulated **(I.C)** histamine bindings and in presence of $HgCl_2$ and impromidine **(II.C)** in equilibrium (30 min incubation at 37 °C). Specific binding (broken line ◒), total binding (○) and non-specific binding (●). Data in **I.** and **II.** represent measurements made with different batches of RBE4 cell line, (triplicated determinations). Experiments were repeated twice. Lines were fitted by linear and non-linear regression.

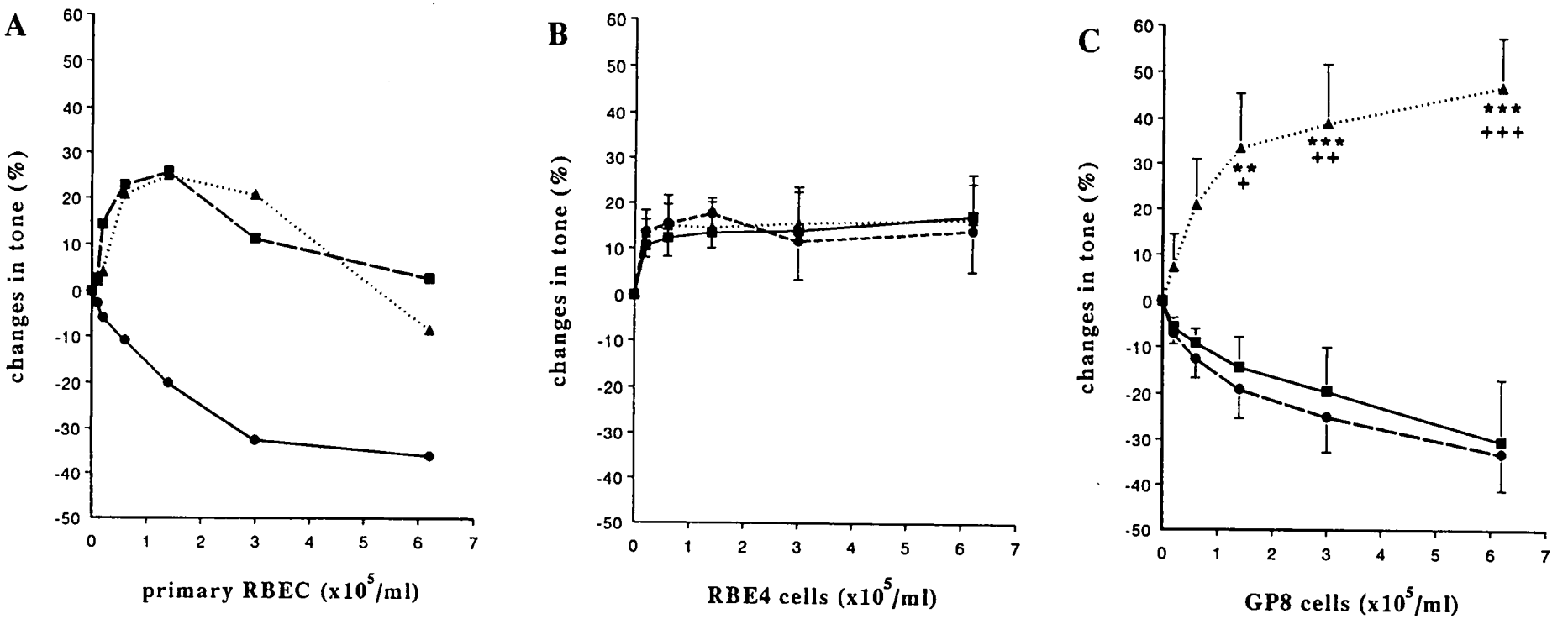

Fig. 9. Effect of primary RBECs **(A)**, RBE4 cell line **(B)** and GP8 cell line **(C)** on the tone of endothelium denuded coronary artery isolated from dogs after the steady-state contraction by $PGF_{2\alpha}$. The data are shown in control condition (——), pretreatment of the cells with 100 μM NOLA (-----) or 10 μM indomethacin (·····).Each value represents the mean ± S.E.M. **(B,C)**, n=6 **(B)**, n=7 **(C)**, n=1-3 **(A)**. Significant differences between the control and NOLA treated cells are: *$p<0.05$, **$p<0.01$, ***$p<0.001$; between the NOLA and indometacin treated cells are: $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$.

min incubation of primary endothelial cells with the inhibitor of nitric oxide synthesis, NOLA, the RBEC induced vasorelaxation was decreased (Fig. 9.A). On the contrary, no significant change in the vasoactivity of the two immortalized cell lines was achieved by NOLA (Fig. 9.B,C). This suggests the absence or the very low level of basal NO production by constitutive endothelial nitric oxide synthase (NOS) enzyme.

In the third series of bioassay experiments the cells were pretreated with the cyclooxygenase enzyme inhibitor, indomethacin, for assessing the role of PGs in the vasoactive potency of cerebral microvascular endothelium. The largest concentration of indomethacin (0.3 μM) by itself caused a small but significant increase of the acceptor vascular tone (from zero to +9.6 ± 1.4%, n=6, $p<0.05$). This increase reflects the known basal release of vasodilator prostaglandins from the smooth muscle of the acceptor coronary artery (Sakanashi *et al.*, 1980). The small enhancement of tone by indomethacin was deduced from the effect of the endothelial suspension in Figure 9. Indomethacin did not influence the contraction induced by RBE4 cells (Fig. 9.B) but turned the primary RBEC (Fig. 9.A) and GP8 cells (Fig. 9.C) induced vasorelaxation into contraction. These results suggest a higher activity of cyclooxygenase enzyme in primary RBEC and GP8 cells.

5.7. Production of eicosanoids by cerebral endothelial cells

The cyclooxygenase pathway of arachidonate cascade, 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2), PGD_2, PGE_2, $PGF_{2\alpha}$, TxB_2 (the stable metabolite of TxA_2) and 12-HHT were identified in the incubation medium of three different types of non-stimulated RBEC cultures. The profile of eicosanoid production by primary RBEC, RBE4 and GP8 cells is shown in Table I.

Each type of RBECs was found to be capable of synthesizing prostaglandins. Significant ($p < 0.05$) differences in total cyclooxygenase metabolite release were found, the synthesis rate in increasing order was as follows: RBE4 < primary cells < GP8 cell line. The predominant vasodilator substance was PGE_2 both in primary cells and GP8 cell line, 28.51 and 42.56 % of total eicosanoid production, respectively. PGE_2 release (9.38 % of total), from RBE4 cells was significantly ($p < 0.05$) lower than that from the other cells, and the main metabolites formed by RBE4 cells were $PGF_{2\alpha}$ and TxB_2, (25.43 and 24.39 %), respectively. While primary and GP8 cells released more than three times more PGE_2 than PGI_2, the ratio of PGE_2:PGI_2 produced by RBE4 cell line was approximately 1:2. The percentage distribution of TxB_2 and 12-HHT was in similar range in all three cell types.

TABLE I.

The profile of eicosanoid production by primary RBEC, RBE4 and GP8 cells.

	primary RBEC		RBE4		GP8	
	in DPM/ 3×10^5 cells/30 min and in percent of the total production					
	DPM	%	DPM	%	DPM	%
6-keto-$PGF_{1\alpha}$	427 ± 67	8,1	481 ± 12	16,6	635 ± 17	8,4
PGE_2	1500 ± 17	28,5	271 ± 41‡	9,3	3186 ± 41†*	42,5
PGD_2	734 ± 15	13,9	368 ± 16	12,7	408 ± 73	5,4
$PGF_{2\alpha}$	626 ± 84	11,9	735 ± 74	25,4	711 ± 15	9,5
TxB_2	1166 ± 28	22,1	705 ± 74	24,3	1476 ± 14*	19,7
12-HHT	889 ± 13	16,9	338 ± 12	11,7	1086 ± 31	14,5
TOTAL	5261 ± 60	100	2890 ± 33‡	100	7486 ± 91†*	100

The prostanoid production was measured from the incubation solution (1ml DMEM/F-12) of 3×10^5 cells at 37 °C 30 min after the administration of 1-[^{14}C]-AA (0.172 pmol, 3.7 kBq). Each value represents the mean ± S.E.M. (n=6). Significant difference between primary RBEC and RBE4 is indicated by ‡ ($p<0.05$), between primary RBEC and GP8 by † ($p<0.05$) and between RBE4 and GP8 by * ($p<0.05$). TOTAL - amount of all prostanoids measured.

6. Discussion

Cerebral endothelial cells in culture

During the production of purified RBEC cultures contamination from pericytes is the most frequent problem. *In vivo* they are integrated in the capillary wall and wrapped by the basement membrane of the endothelium. Pericytes play a role in the proliferation and differentiation of endothelial cells, secrete vasoactive agents, release structural components of the basement membrane. The endothelium and pericytes form a morphological and functional unit (Shepro and Morel, 1993). Pericytes inhibited adrenal capillary endothelial cell proliferation *in vitro* in a contact dependent manner and they became the dominant cell type in the culture. (Olridge *et al.*, 1989). We also observed that pericytes tend to overgrow primary RBECs in longterm culture especially if the serum is not free from PDGF.

The optimal duration of enzymic digestion can keep the ratio of pericytes and astrocytes to a minimum level. Some of the contaminating non-endothelial cells, pericytes and astrocytes, express Thy 1.1 antigen, whereas CECs do not, therefore these cell types can be removed by selective cytolysis using anti-Thy 1.1 antibody and complement (Risau *et al.*, 1990). It is important to find the optimal time-point for complement killing at each culture. This depends on the ratio of CECs and pericytes and the absolute number of endothelial cells. If this treatment is done too early, we can kill the young and weak endothelial cells, while later we can not remove the pericytes. We found that the optimal time is about 2-3 days after the plating of the cells.

Reduction of the percentage of smooth muscle cells and fibroblasts in the cell culture can be attained by careful removal of large vessels, meninges and choroid plexus tissue during the dissection. These contaminanting cells, like pericytes, grow faster than the endothelial cells do and FBS stimulates fibroblast and smooth muscle cell proliferation in addition to promoting endothelial cell growth.

Several laboratories (Tontsch and Bauer, 1989; Dehouck *et al.*, 1990; Vastag and Nagy, 1997) described the cloning of CECs to remove the contaminating cell types and enrich the culture. Although this method is suitable for porcine, murine, bovine and human brain microvascular endothelial cells can not be adapted for rat CECs. Primary RBECs can not be subcultured because the subclones are not viable and are not able to form spontaneusly growing cell lines.

The CECs may lose the expression of some differentiated functions during long culture period, especially after subcultivation or cloning. Interendothelial tight junctions may be present but not assembled into complex structures in cell culture (Wolburg *et al.*, 1994). On the "filter model" - CEC monolayers grown on porous membranes - these interendothelial junctions inhibit the passage of proteins but do not restrict the movement of smaller molecules and ions as strictly as *in vivo*. The endocytotic capacity is also elevated *in vitro* (Rupnick *et al.*, 1988). It has been observed by Goetz *et al.* (1985) that γ glutamyl transpeptidase and alkaline phosphatase activity may be lost with increasing time in culture. The reason of these changes might be the absence of associated cells (e.g., pericytes, astroglia, neurons). Many of the barrier features lost during culture can be reinduced by co-culture with astrocytes (Tao-Cheng *et al.*, 1987), or by addition of astrocyte-conditioned medium (Rubin aet al., 1991) or neuronal membrane fraction (Tontsch and Bauer, 1991) Intracellular cyclic adenosine 3`,5`-monophosphate elevating drugs can further increase the tightness of the intercellular junctions and decrease the permeability of the CEC monolayers for small molecular weight substances (Rubin *et al.*, 1991; Wolburg *et al.*, 1994; Deli *et al.*, 1995a)

Primary cultures of rat CECs expressing many of the so-called BBB characteristics, like transferrin receptor, P-glycoprotein, alkaline phosphatase activity etc. are widely used for morphological, functional and pharmacological studies as an *in vitro* BBB model system (Abbott *et al.*, 1992; for review see Joó, 1992; 1996). The yield of rat CECs is relatively low as compared to that obtained from bovine or porcine brain, but sufficient amounts of endothelial cells can be obtained for Western-blot or PCR techniques (Deli *et al.*, 1993; Krizbai *et al.*, 1995). Using the above described protocol, feeding the cells with special serum and performing the complement killing of contaminating cells, we succeeded to obtain routinely primary RBEC cultures in our laboratory that are more than 95% pure and suitable for most biochemical and molecular biological experiments.

Immortalized cell lines present several advantages: the time consuming and expensive procedure of preparing primary cultures can be avoided, and it is easy to produce large amount of cells for genetic, immunological or biochemical experiments. Both RBE4 and GP8 cells were well characterized and expressed several endothelial markers, among them Factor VIII-related antigen (Roux *et al.*, 1994; Greenwood *et al.*, 1996) - like primary CECs. They also possess BBB phenotype in many respect. The presence of γ-glutamyl transpeptidase and alkaline phosphatase in RBE4 cells (Roux *et al.*, 1994) and that of glucose transporter, P-glycoprotein, transferrin receptor in GP8 cells (Greenwood *et al.*, 1996) was demonstrated.

Besides using in basic research a new area of application of CEC lines can be gene therapy. These cells are good candidates for gene delivery to the nervous system because of their normal localization at the blood-parenchyma interface and their ability to proliferate *in vivo* and *in vitro*. It has been recently reported that RBE4 cells modified to express β-galactosidase reporter can be stably engrafted to growing gliomas in rats (Johnston *et al.*, 1996). Altered RBE4 cells expressing both β-galactosidase and human fibroblast growth factor-1 gene survived following implantation to neonatal and adult rat brain (Lal *et al.*, 1994).

Regulation of the blood-brain barrier permeability by histamine and acid phosphatases

It is well known, that histamine plays an important role in the CNS (Schwartz *et al.*, 1991). The concentration of histamine in different brain compartments is elevated during cerebral injuries (Mohanty *et al.*, 1989; Kovács *et al.*, 1995). Previous studies revealed that both H_2-receptor-dependent adenylate cyclase-mediated and H_1-receptor-dependent phosphoinositol-mediated mechanisms participate in the histamine-induced brain edema formation (Joó, 1993). H_1- and H_2-histamine receptor antagonists could prevent the development of brain edema in experimental models (Edvinsson et al., 1993; Joó, 1993), but there is no information about the effect of H_3 and Hic receptor antagonists on BBB permeability. Previous *in vivo* studies revealed that histamine increased the number of pinocytotic vesicles and the macromolecular transport (Dux and Joó, 1982; Joó, 1993). It was also suggested that APase isoenzymes may play a role in the regulation of transendothelial macromolecular permeability (Audus and Raub, 1993).

In CECs approximately 52% of APase activity was associated with the microsome fraction and acid hydrolases were regarded potential factors in the endocytic pathway for transport of proteins through the BBB and as contributors to the BBB's enzymatic barrier function (Baranczyk-Kuzma *et al.*, 1989). In an *in vitro* model, the H_2-receptor antagonists could reduce the tartrate-resistant, while H_1-receptor antagonists decreased the tartrate-sensitive increase in APase activity after histamine treatment in RBE4 cell line (Publ. I).

The precise role of the APase isoforms in cellular functions is still poorly understood. Changes in APase activity under different conditions have been demonstrated in several tissue types such as in gastric mucosa in response to indomethacin treatment (Nosalova and Navarova, 1994), in hepatocytes in response to insuline and cAMP (Stvolinskaya *et al.*, 1992) or in brain in Alzheimer disease (Shimohama *et al.*, 1993).

In cerebral endothelium, the role of different isoforms of APase enzyme in the process

of histamine-induced brain edema remains to be elucidated. Intracarotid histamine administration and the cerebral ischemia resulted in an activation of APase enzyme in cortical capillaries concomitantly with a dose-dependent increase in the BBB permeability, but no change was found in the enzyme activity in the whole cortical tissue in case of histamine administration. After cerebral ischemia, the activity of tartrate-sensitive and zinc-inducable APase activity was increased only in cortical tissue. Isolated microvessels contain endothelial cells in vast majority as well as a few percents of pericytes, while neurons and astrocytes are present only in trace. It seems probable for us that CECs are responsible for the increased activity of APase. Tagami and colleagues (1983) described APase positive transendothelial channels in cerebral capillary endothelium of stroke-prone rats, while interendothelial junctions were well-preserved. They suggested that endothelial lysosomes fusing with these structures might play an important role in the transport of macromolecules. However, there is a debate about the creation of transcellular channels in CECs by other studies (Nagy, 1986). It was recently published that histamine administration induced a selective albumin permeation without affecting the transport of tight junction markers through monolayers of bovine CECs co-cultured with rat astrocytes (Deli *et al.*, 1995b). It is supposed that absorptive transcytosis of albumin through BBB involves the Golgi complexes, endosomes and transport vesicles while the tight junctions are not compromised (Banks and Broadwell, 1994).

We examined the effect of Hic receptor antagonist DPPE on the permeability of the BBB in rats (Publ. VII). In healthy animals, single bolus of DPPE (5mg/kg) resulted in a 5- to 12-fold increase in albumin transport. On the other hand, DPPE could not prevent the development of postischemic vasogenic brain edema in the 4-vessel-occlusion model of cerebral ischemia-reperfusion described by Pulsinelli and Brierley (1979). The paradoxical finding, that the transport of EBA, a high molecular weight intravascular tracer, was increased several-fold, while BBB permeability for the low molecular weight tracer was not changed may be explained by the selective stimulation of transendothelial macromolecular transport without affecting the tight interendothelial junctions and the paracellular flux. Histamine was also shown to induce high pinocytotic activity in brain capillaries (Dux and Joó, 1982) and elevated microvascular histamine was connected with triggering of selective albumin transport through the brain microvessels *in vivo* (Ádám *et al.*, 1987). This was also supported by an *in vitro* study, where histamine increased the transendothelial passage of EBA but not that of paracellular markers sucrose and inulin (Deli *et al.*, 1995b). We assume that Hic antagonist DPPE has a histamine-like effect on BBB permeability.

Mercury-stimulated histamine uptake and binding in cerebral endothelial cells

Our study shows that exposure of rat CEC and astrocytes to mercuric compounds in concentration range of 25-500 μM promotes carrier-mediated uptake of histamine. Since sodium is necessary for stimulated as well as basal uptake of the amine the enhanced transport did not appear to be a diffusional process caused by non-specific membrane leakage. Complete inhibition of stimulated uptake by impromidine (a potent histamine uptake blocker) also argues against a diffusional process. Organic and inorganic mercurials were shown to interact with protein thiols within the cell membrane (Sutherland *et al.*, 1967; Haas and Schmidt, 1985) and this may account for the neuropathologic effects. Concentration of inorganic mercuric salts in the 25-500 μM range appear to be sufficient for a reversible reaction between mercury and the distinct protein thiols at the carrier (increasing the binding capacity for histamine); and this offers a possible explanation for the observed stimulation of histamine uptake. Our finding that thiol reducing agents such as DTT and cysteamine could completely abolish this stimulation provides further evidence for this hypothesis. Below this concentration range, mercuric salts were found ineffective and in a narrow (1-10 μM) range, inhibitory for histamine uptake (Huszti and Balogh, 1995). These low concentrations of mercury were probably insufficient for reactions with distinct protein sulfhydryls at the carrier and thus for uptake stimulation; and at 1-10 μM, a non-specific inhibitory effect of mercurials may occur.

The pharmacological specificity of [^{3}H]-histamine binding to the sodium-dependent binding site of the histamine-carrier fits well with that observed for endothelial or glial histamine uptake. An assumption that measured histamine binding could represent accumulation of [^{3}H]-histamine in the cells or in membrane-bound saccules might be excluded since net binding was not reduced by freezing and thawing CECs.

It is also unlikely that [^{3}H]-histamine was bound to any of the three well-characterized subclasses of histamine receptors because assays were carried out in the presence of H_1, H_2, H_3 receptor antagonists. Mercuric chloride that had been identified as a stimulator of histamine uptake was effective in enhancing the capacity of Na^+-dependent histamine binding without affecting K_D values. Impromidine, the potent inhibitor of basal and stimulated histamine uptake, was the only coumpound to significantly decrease the enhanced binding sites, probable through specific interactions with the histamine-carrier.

These findings suggest that [^{3}H]-histamine binds to uptake carrier in both CECs and astrocytes. The close similarities in binding charracteristics and the closely equal apparent K_D

values obtained for different cell types suggest identical mechanisms for uptake and identical structure of the uptake carrier in astroglia and CECs. The increased binding capacities caused by mercurials suggest that the uptake carrier undergoes similar processes in both cell types: possible cross linking SH-protein groups may help to stabilize the carrier an activated state.

Vasoactive action of and eicosanoid syntesis by cerebral endothelial cells

The *in vitro* vasoactive properties of three types of RBECs, such as primary cells as well as two immortalized cell lines, RBE4 and GP8, were investigated. In order to analyse the composition of the paracrin signal derived from these cells in the absence of chemical stimulation, a bioassay system was constructed. The effect of vasoactive substances released by RBECs was investigated on the isometric tone of an endothelium-denuded canine coronary arterial *in vitro* preparation. The smooth muscle of this blood vessel is known to be very sensitive to PGs (Hyman *et al.*, 1978), and also has an active soluble guanylate cyclase enzyme for detecting NO *in vitro* (Hintze *et al.*, 1984; Feleton *et al.*, 1989). PGE_2 and PGI_2 have been found to be the main cyclooxygenase metabolites released by cultured RBECs (Moore *et al.*, 1988; De Vries *et al.*, 1995). Our findings obtained both in the bioassay experiments and determination of AA metabolites are in accordance with the previous observations. Vasorelaxation effect of primary cells and GP8 cell line corresponds well to the larger amount of PGE_2 release. Furthermore, the abundance of the dilator type of prostaglandins (PGE_2 and PGI_2) released by primary RBECs and GP8 cells in relation to the amount of the vasoconstrictor type of cyclooxygenase metabolites, such as $PGF_{2\alpha}$ (Miller *et al.*, 1989), PGD_2 (Sakanashi *et al.*, 1980) and TxA_2 (Toda *et al.*, 1986), also explains the vasorelaxant effect. The release of vasoactive eicosanoids from RBE4 was limited in agreement with the low-profile functional effect induced by these cells in bioassay.

The presence of functionally relevant quantity of AA metabolites released from primary and GP8 cells suggests that eicosanoids of cerebral endothelium may have physiological and/or pathophysiological significance *in vivo*. It has been recently demonstrated that vasodilator prostanoids derived from small cerebral blood vessels play roles in the regulation of cerebral circulation (Anzai *et al.*, 1995; Satoh *et al.*, 1995). Endogenous vasodilator prostanoids (PGE_2 and PGI_2) have been found to inhibit cerebral vasoconstriction induced by experimental hematoma (Yakubu *et al.*, 1995), while exogenous PGI_2 was succesfully used to protect against endothelial dysfunction during chronic cerebral vasospasm (Egemen *et al.*, 1995). Cultured brain microvascular endothelium was shown to produce less vasodilator prostanoids

than the endothelium of a large vessel (Satoh *et al.*, 1995). It is interesting that the higher PGE_2: PGI_2 ratio found in primary cells and GP8 cell line is similar to that seen in the endothelium of microvessels, while the predominance of PGI_2 measured in RBE4 cell line resembles to the eicosanoid profile of larger vessels (Renzi *et al.*, 1992). In our experiments, endothelial cell-induced vasodilation on precontracted canine coronary artery was turned to vasoconstriction when the endothelial cells were preincubated with indomethacin. We do not know which mediators are responsible for the *in vitro* contractile effect, but indomethacin may shift the arachidonate cascade to the lipoxygenase pathway releasing vasoconstrictor leukotrien (LT) C_4, LTD_4 and LTE_4 (Woodman and Dusting, 1982; Yakubu *et al.*, 1995). In addition indomethacin could enhance the vasoconstrictor effects of endothelin-1 on porcine pial arterioles *in vivo* (Yakubu *et al.*, 1995).

Both primary cells and the GP8 cell line significantly decreased the coronary vascular tone, while the RBE4 cell line was ineffective. In a previous study, only the inducible type of NOS was detectable in RBE4 cells (Durieu-Trautmann *et al.*, 1993). In accordance with these findings, our recent preliminary results indicated the expression of mRNA of constitutive, endothelial NOS in cultured primary RBECs and GP8 cell line, which was missing in RBE4 cells (unpublished data, Krizbai *et al.*). However, the NOS inhibitor, NOLA, applied into the organ bath decreased the vasorelaxation of canine coronary artery caused by primary cells, but failed to do so in GP8 cells. The ineffectiveness of NOLA on GP8 cell line might be explained by that NO plays only a minor role in the vasorelaxation observed compared to the eicosanoids. We can not exclude the ineffective basal release of NO in GP8 cells, at least up to the concentration of 1.2×10^6 cells in 2 ml volume. It is possible that this number of endothelial cells released insufficient amount of NO for producing relaxation. The volume of KH solution was four order of magnitude larger than the greatest estimated endothelial cell volume (approx. 150 μm^3/cell), and NO molecules produced with a half-life of shorter than a second, could lose activity before reaching the effector cells.

In the present study, we used primary cerebral endothelial cells as well as RBE4 and GP8 immortalized cell lines in order to determine their bioactivity and the production of vasoactive eicosanoids. We conclude that the eicosanoid profile of GP8 cell line is closer to that of primary CECs than that of RBE4 cells, which was also reflected by the results of the bioassay. Nervertheless both cell lines, which retain the majority of BBB characteristics (Roux *et al.*, 1994; Greenwood *et al.*, 1996) may be useful tools for biotechnological and therapeutic applications in the near future (Lal *et al.*, 1994; Johnston *et al.*, 1996).

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9. Annex

Full papers published in international journals referenced by Science Citation Index

I

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Receptor-mediated regulation by histamine of the acid phosphatase activity in cultured cerebral endothelial cells

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Introduction

Acid phosphatase (APase; othophosphoric monoester hydrolase, EC 3.1.3.2) is widely distributed in nature and has been studied in both plants and animals. Biochemical studies have revealed the presence of multiple molecular forms of acid phosphatase which differ in their molecular size, subcellular localization, sensitivity to inhibitors and substrate requirements [1–2]. The high molecular weight form of the enzyme (HMW; m.w. > 100,000 Da) can be completely inhibited by tartrate or fluoride and is present mainly in the lysosomal fraction nonspecifically hydrolysing various phosphomonoesters. A low molecular weight form (LMW; m.w. < 20,000 Da; tartrate-resistant form) is present predominantly in the cytosol and a third form of APase activity with a molecular weight of about 62,000 Da can be detected in the presence of Zn^{2+} ions [3].

Acid hydroloase activity was first demonstrated in primary cultures of cerebral endothelial cells (CEC) by Baranczyk-Kuzma et al. [4]. However, it remained to be seen if the enzyme activity could be modified in, or released from, the CEC by vasoactive substances. The present study was designed to check the possible effects of histamine on the activity of different molecular forms of acid phosphatase.

Materials and methods

Immortalized rat brain endothelial cells (RBE4 cell line) [6] were cultured in 6 cm petri dishes and stimulated in 1 ml serum free Dulbecco's modified Eagle's medium (DMEM) for 1 hour at 37 °C in the presence of 10^{-5} M histamine with or without 10^{-6} M mepyramine (H_1-receptor blocker), 10^{-6} M ranitidine (H_2-receptor blocker) or in the presence of the antagonist only. Drug concentrations were selected on the basis of the affinity of agents for the receptors. After incubation, the cells were washed with phosphate buffered saline (PBS), collected and homogenized in 500 µl PBS.

Acid phosphatase activity was measured by the rate of hydrolysis of *p*-nitrophenylphosphate (*p*-NPP) [3]. Different samples of endothelial cells were incubated in solution containing 1 ml 0.1 M acetate buffer (pH 5.5) and 2.5 mM *p*-NPP at 37 °C for 1 h with or without L-(+)tartrate (10 mM). After incubation, 0.3 ml of 1 M sodium hydroxide was added to stop the reaction and the absorbance was read at 410 nm in a Shimadzu spectrophotometer. Enzyme activity, expressed in mU/mg protein, was determined from a calibration curve constructed using increasing concentrations of purified acid phosphatase of known activity (AcP Lin-trol, Sigma). Protein concentrations were determined according to Bradford using bovine albumin as a standard [5].

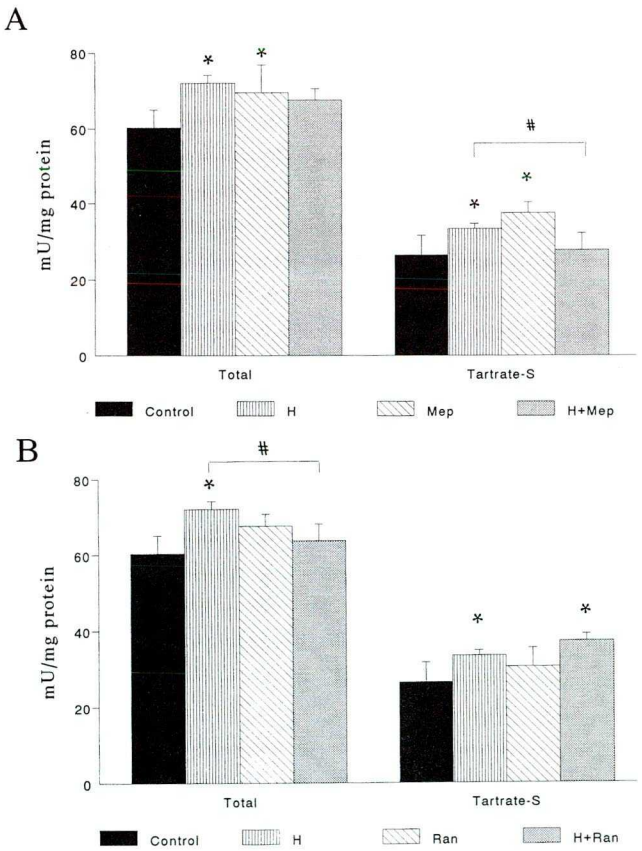

Fig. 1. Effects of histamine, mepyramine A) and ranitidine B) on the activity of total and tartrate-sensitive forms of acid phosphatase in RBE4 cells. Cells were treated with 10^{-5} M histamine (H), 10^{-6} M mepyramine (Mep), 10^{-6} M ranitidine (Ran), or solvent in the control groups. Error bars represent SEM and n = 6. Significant differences: $* = p < 0.05$ compared to control values, while $\# = p < 0.05$ compared to histamine-treated values.

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Statistical analysis was performed using Kruskal-Wallis one way analysis of variance on ranks followed by the Student-Neuman-Keuls test. A value of $p < 0.05$ was considered to be significant.

Results and discussion

Histamine increased both the total APase activity (72.02 ± 2.04 mU/mg protein vs. 60.24 ± 4.79 mU/mg protein, mean ± SEM, $n = 6$, $p < 0.05$) and that of the tartrate-sensitive form (33.33 ± 1.39 mU/m protein vs. 26.41 ± 5.24 mU/mg protein, mean ± SEM, $n = 6$, $p < 0.05$). Mepyramine blocked the effect of histamine on the tartrate-sensitive form only. Therefore, H_1-receptors are apparently involved in the regulation of activity of tartrate-sensitive isoforms (Fig. 1A). The effect of histamine on total but not tartrate-sensitive activity was inhibited by ranitidine (Fig. 1B). The stimulatory effect of histamine on total acid phosphatase activity seemed, therefore, to be mediated by H_2-receptors. Mepyramine and ranitidine alone also increased the activity of the enzyme which may be explained by the partial agonist effect of the drugs.

The precise role of the APase isoforms in cellular function is still poorly understood. Changes in APase activity under different conditions have been demonstrated in several tissue types such as in gastric mucosa in response to indomethacin treatment [7], in hepatocytes in response to insulin and cAMP [8] or in brain in Alzheimer's disease [3]. In cerebral endothelial cells, approximately 52% of APase activity was associated with the microsome fraction and acid hydrolases were regarded as potential factors in the endocytic pathway for transport of proteins though the blood-brain barrier and as contributors to the enzymatic barrier function [4]. Thus, histamine activation of acid phosphatase activity in the cerebral endothelial cells could possibly play a role during inflammatory reactions of the central nervous system.

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II

II.5. IMMUNOHISTOCHEMICAL AND ELECTRONMICROSCOPY DETECTIONS

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Primary cultures of endothelial cells were obtained from cortical grey matter of two-week-old rats using a two-step enzymic digestion followed by a Percoll gradient centrifugation. Rat cerebral endothelial cells (RCECs) formed a monolayer of spindle-shaped, tightly attached cells on rat tail collagen matrix. RCECs specifically stained for Factor VIII (FVIII) and alkaline phosphatase (AP), and bound *Bandeiraea simplicifolia* isolectin I-B_4 (BS-I-B_4). At electron microscopic level gap junction-like attachment sites were observed. Contaminating cells in the cultures were eliminated by complement mediated cytolysis using anti-Thy 1.1 antibody. On average, a value of 120 $\Omega.cm^2$ for transendothelial electrical resistance (TEER) was measured for RCEC monolayers. Primary cultures of RCECs were used to study regulatory enzymes in signal transduction.

INTRODUCTION

With the recognition that most of the endothelial cells resisted damage during isolation, remained viable, and could be maintained in tissue culture conditions (Panula *et al.*, 1978), a new generation of blood-brain barrier (BBB) model systems was developed, which seemed to be devoid of most of the problems experienced with the isolated cerebral microvessels. Several procedures and many modifications have been worked out for culturing RCECs in different laboratories since then (Phillips *et al.*, 1979; Spatz *et al.*, 1980; Bowman *et al.*, 1981; Diglio *et al.*, 1982; Rupnick *et al.*, 1988; Gordon *et al.*, 1991; Dux *et al.*, 1991; Abbott *et al.*, 1992). Except that of Phillips *et al.* (1979) all methods used grey matter as a starting material, collagenase and/or collagenase-dispase for enzymic digestion. In most of the protocols endothelial cells were separated by a Percoll gradient centrifugation step. In our method, which is very close to the method established in the laboratory of N.J. Abbott (see chapter II.1), first the microvessels were separated from brain tissue with a collagenase digestion, and then basement membrane and the majority of perivascular cells were removed by a second enzymic treatment. Endothelial cell clusters were further purified by Percoll gradient centrifugation before plating. Optimal purity of RCEC cultures was obtained by selectively lysing Thy 1.1 expressing contaminating cells.

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†Deceased.

MATERIALS AND METHODS

Animals

Two-week-old CFY rats of either sex were obtained from the Institute's animal house.

Chemicals

Products	*Supplier and catalogue No.*
Establishment of primary cultures	
Collagenase type CLS2	Worthington, NJ, USA
Collagenase-dispase	Boehringer 1097113
Percoll	Pharmacia 17-0891-01
Bovine serum albumin (BSA)	Sigma A 2153
L-glutamine	Gibco 15039-019
Dulbecco's modified Eagle's medium (DME)	Sigma D 5648
Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (DME/F-12)	Sigma D 8900
Penicillin-Streptomycin	Gibco 15145-014
Gentamicin	Gibco 15710-031
Fetal calf serum (FCS)	HyClone and Sigma
Complement mediated specific cytolysis	
monoclonal anti-mouse Thy 1.1 antibody	Sigma M 7898
complement serum HLA-ABC, rabbit	Sigma S 7764
Characterisation	
5-bromo-4-chloro-3-indolyl phosphate (BCIP)	Sigma B 6149
nitro blue tetrazolium (NBT)	Sigma N 6876
Bandeiraea simplicifolia isolectin B_4	Sigma L 5391
3,3′-diaminobenzidine (DAB)	Sigma D 5637
Fluorescein isothiocyanate-dextran (FITC-dextran)	Sigma FD-70S

Establishment of Primary Cultures

Dissection of brains

Rats were anaesthetised with ether. After a thorough rinse with 70% ethanol and then with iodine in 70% ethanol, the heads were cut, and placed in a sterile glass petri dish. In the laminar flow box forebrains were removed from the skulls (without the cerebellum) using sterile microdissecting forceps and scissors, and collected in cold sterile phosphate buffered saline (PBS, without calcium and magnesium, pH 7.4). Meninges were removed on sterile filter paper (Whatman 3M) from each brain

hemisphere and at the same time white matter was 'peeled off' with the aid of fine curved forceps. Grey matter was carefully collected from the filter paper (meninges tend to stick to it) and minced to approximately 1 mm^3 pieces using sterile disposable scalpels in the first incubation medium (3 mg/ml collagenase CLS2, 1 mg/ml BSA in DME containing antibiotics) in a sterile glass petri dish.

Enzyme digestions

The minced tissue was transferred into a centrifuge tube (35 ml, Oakridge-type with screw cap) with the rest of the collagenase solution (total: 15 ml/10 brains) and triturated with a pipette (10 up and down), and then incubated at 37 °C for 1.5 h in a shaking waterbath. After this incubation, 25 ml of cold 25% BSA-DME was added to the homogenate, mixed well by trituration and centrifuged at 1000 g for 20 min. The myelin layer and the supernatant was aspirated, the pellet washed once in DME (1000 g for 10 min) and then further incubated in the waterbath for a maximum of 2 h in 10 ml of the second incubation medium containing 1 mg/ml collagenase-dispase in DME.

Percoll gradient centrifugation

The cell suspension was centrifuged (700 g for 5 min). The pellet was suspended in 2 ml DME and carefully layered onto a continuous 33% Percoll gradient and centrifuged at 1000 g for 10 min. For the gradient 10 ml Percoll, 18 ml PBS, 1 ml FCS and 1 ml 10 × concentrated PBS were mixed, sterile filtered and centrifuged at 4 °C, 30000 g for 1 h.

Plating and feeding cells

The band of the endothelial cell clusters (clearly visible as a white-greyish layer above the red blood cells) was aspirated and washed twice in DME (1000 g, 10 min). The cells were then suspended in culture medium (DME/F-12 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 2 mM glutamine, 20% heat inactivated FCS) and were seeded onto rat tail collagen-coated 35 mm plastic dishes or 25 mm cell culture inserts (seeding density 10 cm^2/brain). The medium was changed the next day, later on, every third day.

Yield

Starting with the culture from 10 brains, we could obtain confluent primary culture of RCECs in 10 pieces of 35 mm tissue culture dish, equivalent approximately to 100 cm^2 surface area.

Comments

Incubation media for enzymic digestions were always prepared freshly with lyophilised enzymes, and then sterilised by filtration. Their pH was adjusted to 7.4. During the separation we used only plasticware. If it was necessary to use any kind of glassware, we coated it before use with BSA.

SPECIFIC APPLICATION

For RCEC monolayers, passage 1–2, grown on Falcon 25 mm inserts, 120 $\Omega.cm^2$ TEER was obtained. The passage of 70 kDa FITC-dextran was restricted through RCECs: 99.12 ± 8.79 $\mu g/cm^2/h$ *vs.* 655.80 ± 12.37 $\mu g/cm^2/h$ ($n = 6$) in the case of cell-free filters.

Using cells grown on collagen-coated dishes and/or coverslips, the production, presence, and phosphorylation of the α-subunit of calcium/calmodulin-stimulated protein kinase II have been described in primary cultures of RCECs (Deli *et al.*, 1993). The expression of seven different protein kinase C isoforms using reverse transcriptase-polymerase chain reaction was investigated in cerebral endothelial cells (Krizbai *et al.*, 1995). The expression patterns of brain tissue, isolated microvessels, RCECs, an immortalized cerebral endothelial cell line and aortic endothelial cells were determined and analysed.

PROBLEM SOURCES AND QUALITY CONTROL

Characterisation of the Cultures

Factor VIII-related antigen (FVIII) immunohistochemistry

After a brief washing in PBS and fixing in ethanol at 4 °C for 15 min, cells were treated with 1% H_2O_2 in PBS for 10 min, followed by washing in PBS. Non-specific binding sites were blocked by incubation in 3% normal goat serum in PBS at room temperature for 20 min. Anti-FVIII rabbit immunoglobulin (Dako) was used as primary antibody and biotin-labelled anti-rabbit IgG (Dako) as secondary antibody, both applied for 30 min. After a 30 min incubation with avidin-biotin-horseradish peroxidase (HRP) complex (ABC kit, Vector Lab, CA, USA), DAB was used as HRP substrate, followed by hematoxylin-eosin (HE) counterstaining.

Lectin binding

RCECs were washed in PBS, fixed in 4% formalin and 70% ethanol in PBS for 15 min, treated with 1% H_2O_2 in PBS for 10 min, washed again in PBS and then incubated in 15 $\mu g/ml$ HRP-conjugated BS-I-B_4 in 0.1% BSA-PBS for 90 min. DAB was used as HRP substrate. The preparations were counterstained with HE.

AP histochemistry

RCECs were washed in PBS, fixed in 2% paraformaldehyde-PBS for 2 min, washed again in PBS, and then incubated in 0.41 mM NBT, 0.40 mM BCIP in Tris buffered saline, pH 9.5 for 3 h. The reaction was stopped by washing in PBS, and subsequently subjected to graded dehydration and mounted in Entellan®.

Transmission electron microscopy

RCEC cultures were fixed for 30 min in long Karnovsky's fixative and then washed in 0.1 M phosphate buffer, pH 7.4. Cells were scraped off the surface, and centrifuged in the buffer. The pellet was post-fixed in 1% OsO_4, dehydrated and embedded in Spurr resin. Thin sections were stained in lead citrate and finally examined in a Zeiss 902 electron microscope.

Endothelial Cells

The small vessel fragments obtained at the end of the isolation procedure attached rapidly to collagen-coated surfaces, and in 2–3 days colonies of RCECs emerged (Figure 1a), and formed a non-overlapping continuous monolayer at the end of the first week (Figure 1c) with some swirling patterns. RCECs displayed a so called 'fibroblast-like' morphology: cell-shape was fusiform with an oval nuclei in the centre with neighbouring cells tightly apposed to each other. Cells gave specific immunohistochemical staining with anti-FVIII antibody (Figures 1b and d), bound the galactose-specific BS-I-B_4 isolectin (Figure 1f) and showed positive histochemical staining for AP. Cells were abundant in mitochondria and endoplasmic reticulum (Figures 2a and b). Some attachment sites were without specialisation (Figure 2c), while others were gap junction-like (Figure 2d).

Contaminating Non-Endothelial Cells

In primary cultures of RCECs the presence of contaminating non-endothelial cells could be occasionally observed (Figures 3a and c), which did not express FVIII (Figure 1d). These cells, mostly pericytes and very few astrocytes, expressed Thy 1.1 antigen and therefore could be removed by selective cytolysis using anti-Thy 1.1 antibody and complement (Risau *et al.*, 1990). For detailed description of the method see chapters A.II.1. and II.2. We have followed the method established in the laboratory of N.J. Abbott, and could considerably reduce the number of contaminating cells (Figure 3).

CONCLUDING REMARKS

The *in vitro* BBB model systems are applicable not only to biochemistry and physiology but also to drug research, and may contribute to the improvement of the transport of substances through the BBB (Joó, 1992). The *in vitro* approach has

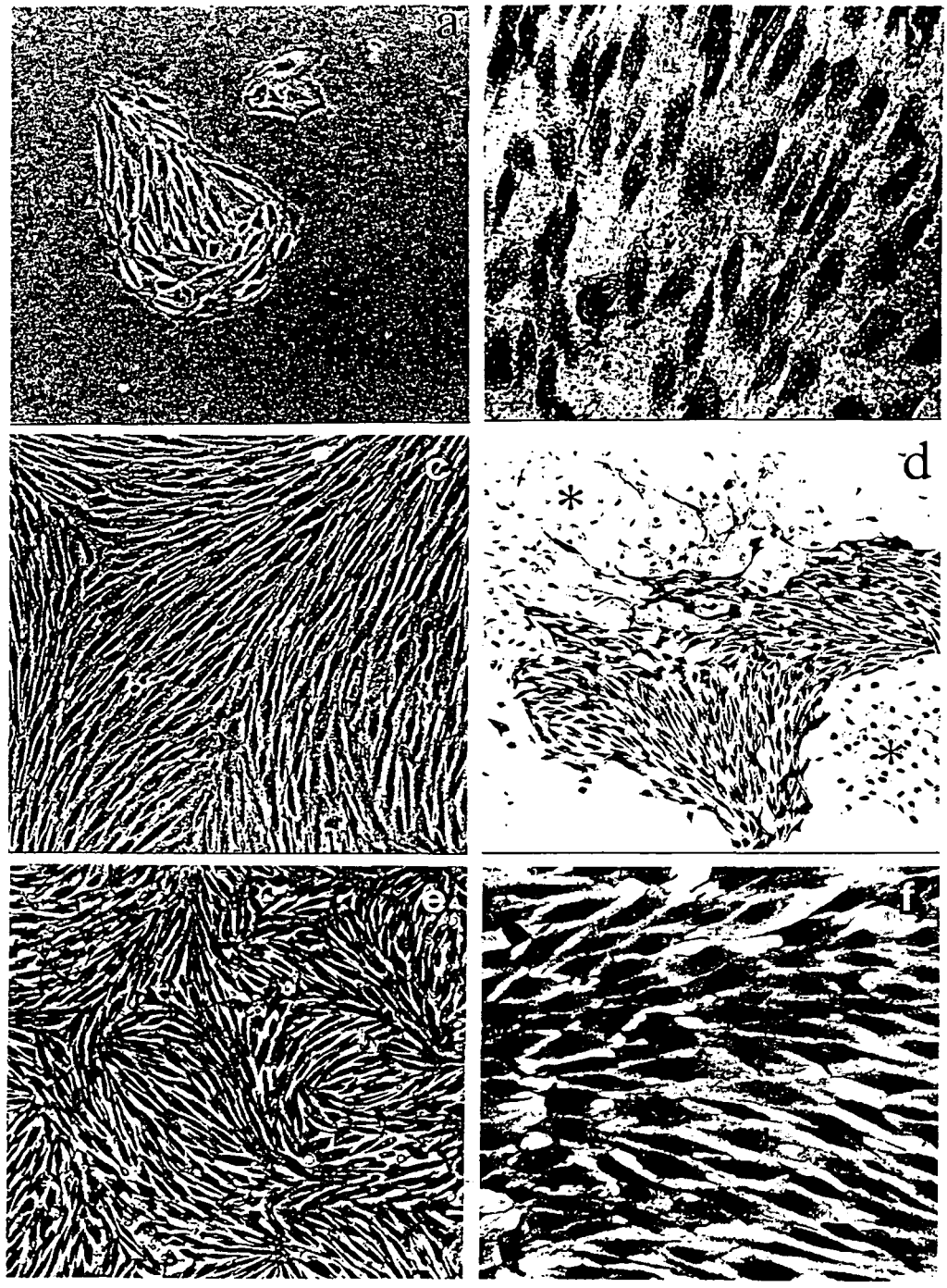

Figure 1 Light microscopy. Phase contrast photomicrographs from cerebral endothelial cells (a, c and e). A colony of primary RCECs 3 days after seeding (a), and a confluent culture 7 days after seeding (c). Confluent primary culture of PCECs, 7 days after seeding (e), magnification 200 ×. FVIII immunohistochemistry, HE counterstaining (b and d). Specific, perinuclear cytoplasmic dot-like staining of RCEC (b), 400 ×. An islet of RCECs was well stained for FVIII (d), while surrounding contaminating cells were not (asterisks), 80 ×. *Bandeiraea simplicifolia* isolectin B_4 binding to RCEC (f), 400 ×.

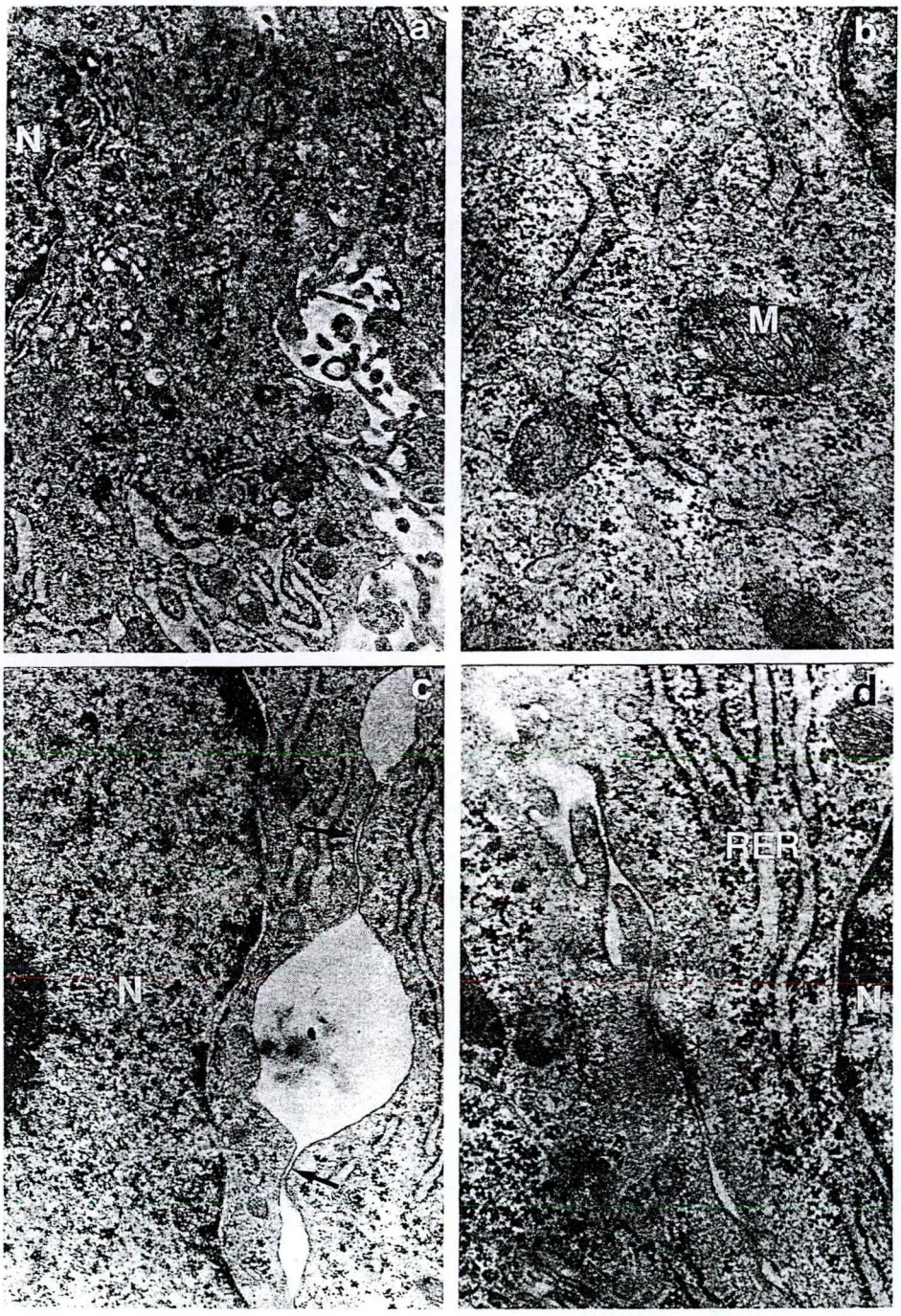

Figure 2 Electron microscopy. Low power view of RCECs (a), note the extended endoplasmic reticulum, numerous mitochondria; N = nucleus, magnification 15000 ×. High magnification of the cytoplasm (b); M = mitochondrion, 32000 ×. Attachment sites (arrows) of two RCECs without specialization (c); N = nucleus, 26000 ×. Gap junction-like specialized membrane attachment (asterisk) could be seen between two RCECs (d); RER = rough endoplasmic reticulum, N = nucleus, 38000 ×.

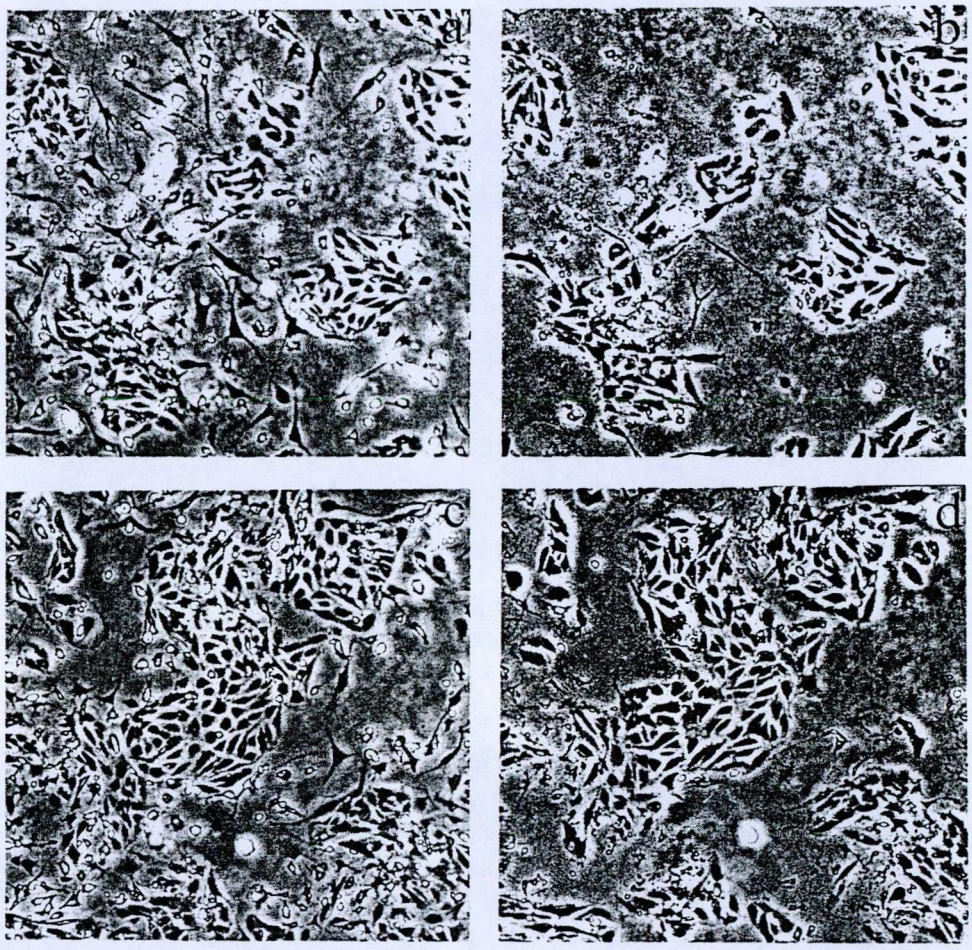

Figure 3 Selective cytolysis of contaminating cells in primary cultures of RCEC by anti-Thy 1.1 antibody and complement. Phase contrast microscopy pictures from primary cultures of RCECs 3 days after seeding, magnification 200 ×. Cultures before (a and c), and immediately after the treatment (b and d).

been and should remain an excellent model of the BBB to help unravel the complex molecular interactions underlying and regulating the permeability of the cerebral endothelium.

It should be stressed, however, that studies on permeability and transport across RCEC monolayers ought to be carried out in cultures having parameters resembling those *in vivo*, e.g. monolayers with TEER equal or higher than 500 $\Omega.cm^2$. Methods that co-cultivate cerebral endothelial cells with astrocytes (Dehouck *et al.*, 1990) and/or treatment of monolayers with compounds that increase intracellular cyclic AMP concentration, thereby enhance the 'tightness' of the tight junctions between endothelial cells (Rubin *et al.*, 1991; Deli *et al.*, 1995) could provide means to reach that goal.

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III

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Mercury-Stimulated Histamine Uptake and Binding in Cultured Astroglial and Cerebral Endothelial Cells

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The effects of mercuric compounds on histamine uptake and binding to uptake carrier in cultured rat astroglial and cerebral endothelial cells were investigated. Experimental results showed that mercuric compounds produced strong stimulation of glial and cerebroendothelial histamine uptake over a concentration range of 25–500 μM. The stimulated histamine uptake showed characteristics similar to those described for basal uptake in terms of sensitivity to inhibitory agents (e.g., impromidine) and the requirement of external Na^+. Mercury-induced stimulation of histamine uptake could be abolished by sulfhydryl agents, dithiotreitol and cysteamine, indicating a complete reversal of, and not simply a protection from, the action of mercury.

Basal and stimulated uptake of histamine represent bindings to uptake carrier with high and closely equal affinities but markedly higher capacities for stimulated uptake. In controls, the mean value of apparent K_D (derived from saturation kinetics at equilibrium) was obtained as 26.7 ± 3.9 nM for astroglial cells; and 100 μM mercuric chloride did not modify it significantly. In contrast, the apparent B_{max} values differed markedly; found as 0.63 ± 0.10 pmol/mg protein and 3.32 ± 0.47 pmol/mg protein in the absence and the presence of 100 μM mercuric chloride respectively.

For the cerebral endothelial cell line, RBE4, the apparent K_D was calculated as 22.5 ± 3.2 nM and was comparable to that obtained for astroglial cells in control and mercury-stimulated conditions. The apparent B_{max} values were less, but markedly different in these conditions, obtained as 0.18 ± 0.03 pmol/mg protein and 1.2 ± 0.36 pmol/mg protein in the absence and the presence of mercuric ion respectively. In both cells, impromidine, the potent inhibitor of basal and stimulated histamine uptake, decreased the enhanced capacities of histamine binding (B_{max}) (without affecting the dissociation constant, K_D) in micromolar range, comparable to its inhibiting potency.

Results confirmed that mercuric ion might enhance the binding capacity of histamine carrier and protein sulfhydryls might play a role in this effect. The observed stimulations by mercuric compounds suggest close similarities in the mechanism of histamine uptake and the structure of histamine carrier in astroglial and cerebral endothelial cells. J. Neurosci. Res. 48:71–81, 1997. © 1997 Wiley-Liss, Inc.

Key words: histamine; mercurials; stimulated uptake; binding

INTRODUCTION

A histamine uptake mechanism has been identified in chicken and rat cerebral astrocytes and also in cerebral endothelial cells on the basis of experiments in which virtually homogeneous cell cultures were incubated with the radiolabeled amine (Huszti et al. 1990, 1994, 1995; Huszti and Balogh, 1995). The initial rate of histamine uptake reflecting a high affinity interaction that could be described by Michaelis—Menten kinetics, required external Na^+ and was inhibited by histamine analogues (2-methylhistamine, 4-methylhistamine, impromidine), desmethylimipramine and depolarizing agents (barium chloride and sparteine) but also by heavy metals (Pb^{++} and Hg^{++}) in low 1–10 μM concentrations.

Strong stimulation of the uptake was observed by mercurials when they were applied at higher concentrations (Huszti and Balogh, 1995) but the interpretation of these experiments were limited. In the present paper, we combined the uptake and carrier-binding studies to deter-

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mine binding characteristics in basal and stimulated conditions and to establish if mercury induces changes in the binding parameters.

MATERIALS AND METHODS

Materials

[2,5-^{3}H]-Histamine dihydrochloride (1.67 TBq per mmole) was purchased from Amersham International Plc (Amersham Place, Little Chalfon, Buckinghamshire, England). Histaminergic agents were generously provided by SKF, Smith Kline and French Laboratories Ltd. (Welwyn Garden City, Herts. UK) except for thioperamide (Cookson Chemicals, Southampton, England) and tripolidine (Sigma Chemical Company, St. Louis, MO). Mercuric chloride and mercuric nitrate were of the highest analytical grade and were obtained from Reanal (Hungary, Budapest). All other chemicals were purchased from commercial sources and were of the highest purity available.

Primary Cultures of Astroglial Cells

Primary cultures from the cerebrum of postnatal (P_0–P_6) Wistar CFY Long Evans rats were prepared as described by Madarász (Madarász et al., 1991). Briefly: primary cultures were maintained in Minimum Essential Medium (MEM; GIBCO or SIGMA) supplemented with 4 mM glutamine, 10% fetal calf serum (CSF; BioSer), and 40 μg/ml gentamycin for several weeks and they were used for investigations on 19–21 days of the culture. The cell composition of the cultures was checked by immunocytochemical identification of cells containing glial fibrially acidic protein or neurofilament proteins in the cytoskeleton or neurofilament epitopes on the cell surfaces as it was described (Madarász et al., 1991). Cultures were composed of 95–98% astroglial cells, contained no neurons. Oligodendrocytes, identified with the myeline basic protein (MBP; Boehringer, Germany) appeared only incidentaly (<2%). Under conditions described, microglia could not be identified by using anti-Fc-receptor antibody (SIGMA Chemical Company, St. Louis, MO) and B4 isolectin for immunohistochemical staining.

Primary Cultures of Cerebral Endothelial Cells

Separation of brain microvessels and cultivation of endothelial cells from the isolated cerebral microvessels of 2-week-old rats were carried out as described by Deli (Deli et al., 1993).

Briefly: minced cortical tissue was first incubated with 0.1% collagenase-dispase enzyme mixture (Boehringer Manheim, Germany) for 1.5 hr, then the microvessels were collected by a centrifugation through 25% bovine serum albumin in the medium. The washed pellet was further digested in collagenase-dispase solution for an additional 1.5 hr. Endothelial cells were separated by a centrifugation step on a 35% continous Percoll gradient. The collected and washed cells were seeded onto collagen coated plastic dishes. Dulbecco's modified Eagle's medium was supplemented with antibiotics, 20% fetal calf serum, 2 mmol glutamine, and 10 mmol N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES). Fourteen-day-old cultures were used for the experiments. Cells gave positive immunohistochemical staining with anti-Factor VIII antibody (rabbit, Dako) which is a general marker for endothelial cells and bound the galactose specific BSL I B4 isolectin, considered to be a marker for rodent cerebral endothelium.

Endothelial Cell Line, RBE4

The RBE4 cell line from primary cultures of rat brain microvessels has been prepared and characterized by Durieu-Trautmann (Durieu-Trautmann et al., 1993) and kindly offered for these experiments.

The cells were passaged twice a week in a Medium/Ham's F10 (1:1; Seromed, France), supplemented with 2 mM glutamine, 10% heat-inactivated fetal calf serum, and 1 ng/ml basic fibroblast growth factor. They were spread at a density of 10^4 cells/cm^2 onto collagen-coated dishes (rat tail collagen, type I) and used between passages 30 and 60 in these experiments.

[^{3}H]-Histamine Uptake

[^{3}H]-histamine uptake measurements were carried out as described in our previous studies (Huszti, et al. 1990, 1994) in a Krebs buffered salt medium (pH 7.4). The cultured cells were preincubated with the compounds tested at 37°C for 15 min in 0.48 ml incubation medium. The assay was started by the addition of 20 μl of [^{3}H]-histamine (0.1–0.2 μCi) containing unlabelled amine (final concentration: 0.04 μM) and incubated further at 37°C for 3–20 min. The incubation was terminated by adding 1 ml of ice-cold Krebs buffered salt medium and rapid removal of the medium by aspiration. After several (at least 3) washings, cells were lysed in 500 μl of 1N NaOH (containing 0.1% Triton-X-100) and after 1–2 hr standing, aliquots were taken from the lysed cells for scintillation counting. The protein content was determined by the method of Lowry. (Lowry et al., 1951)

The cells in parallel wells were used for protein determination after complete solubilization in 1N NaOH. Results presented as the mean ± SEM of 3–4 individual experiments with at least 2-2 parallel aliquots prepared from individual cultures. Significant differences between groups were determined by analysis of variance (one-way

Anova) followed by Dunnett's t-test (Sokal and Rohlf, 1980).

[^{3}H]-Histamine Binding to Uptake Carrier

Histamine binding to glial histamine carrier was measured in cultured cells (astrocytes and RBE4 clone) grown on poly-L-lysine or collagen coated 24-well tissue culture plates. After rapid removal of the growth medium and two washings with 1–2 ml of cold buffered saline (PBS), the cells were stored frozen at −20°C until use (1–2 days) or they were separated from the washing solution and used freshly by incubating in a Ca^{++} (2.5 mM) and Mg^{++} (2.5 mM)-rich sodium phosphate buffer medium (50 mM; pH 7.4) with 10–100 nM of [^{3}H]—histamine (0,5 μCi) in the presence of histamine H_1, H_2, and H_3 antagonists, tripolidine, cimetidine, and thioperamide (40 μM each) in a total volume of 500 μl at 25°C or at 37°C for 30–60 min as indicated in the text. The reaction was stopped by dilution with 1–2 ml of ice-cold medium. The unbound radioactivity was removed by several (at least five) washings with 3 ml of ice-cold medium and the radioactivity, retained in cell membranes was measured in 5 ml Aquasafe "300" (Zinsser Analytic, Frankfurt, Germany) after lysing the cells in 250 μl 1 N NaOH containing 1% Triton-X-100. Non specific binding was obtained by incubating the cells with the addition of 40 μM unlabelled histamine and substracted from "totals" to get the "net bindings."

Analysis of Binding Data

The binding capacity (B_{max}) and the dissociation constant (K_D) were determined by Scatchard analysis. The untransformed data were analyzed by using linear and non-linear least-square curve fitting procedures (Sokal and Rohlf, 1980).

RESULTS

Mercury-Stimulated Histamine Uptake

Figure 1 shows the cumulative data relating to $HgCl_2$ concentration to the value of the initial uptake rate of rat astroglial histamine transport. The concentration dependence of the $HgCl_2$ effect on histamine uptake rates was measured in the presence and absence of $HgCl_2$ in the same batch of cultures. Mean data of such experiments are shown in the figure. The corresponding uptake rates differed statistically from that of controls in the concentration range studied, pointing to dual effects of mercury on histamine uptake: inhibitory at low and stimulatory at higher concentrations given a closely U-shaped dose-response curve with a maximum at 200 μM.

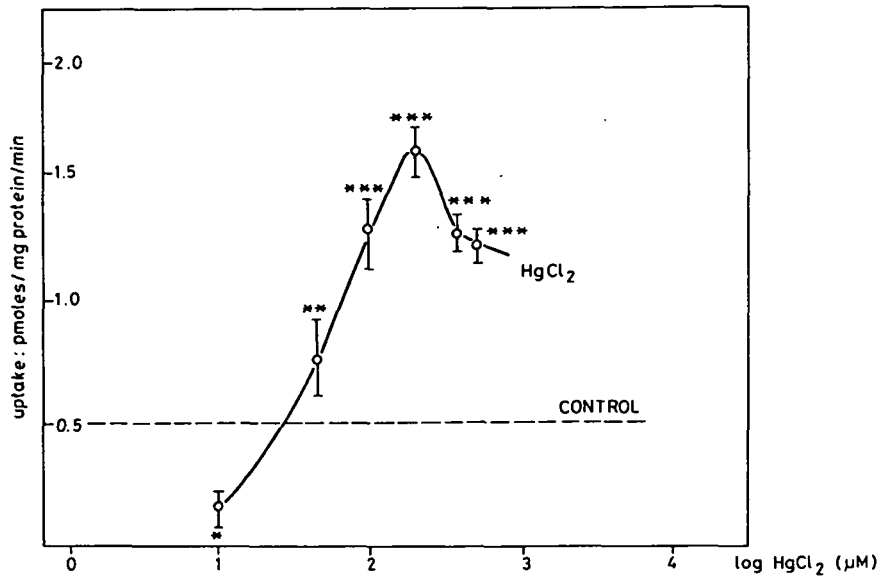

Fig. 1. Concentration dependence of mercury effect on astroglial histamine uptake. Plot: Uptake rate of 0.04 μM histamine (pmol/mg protein) vs. logarithm of $HgCl_2$ concentrations (μM). Data points represent mean values of 3 individual experiments with duplicates (n = 6). S.E.M. were indicated by vertical bars. $HgCl_2$ effects were compared to control (broken line). $^*P < 0.05$; $^{**}P < 0.1$; $^{***}P < 0.001$.

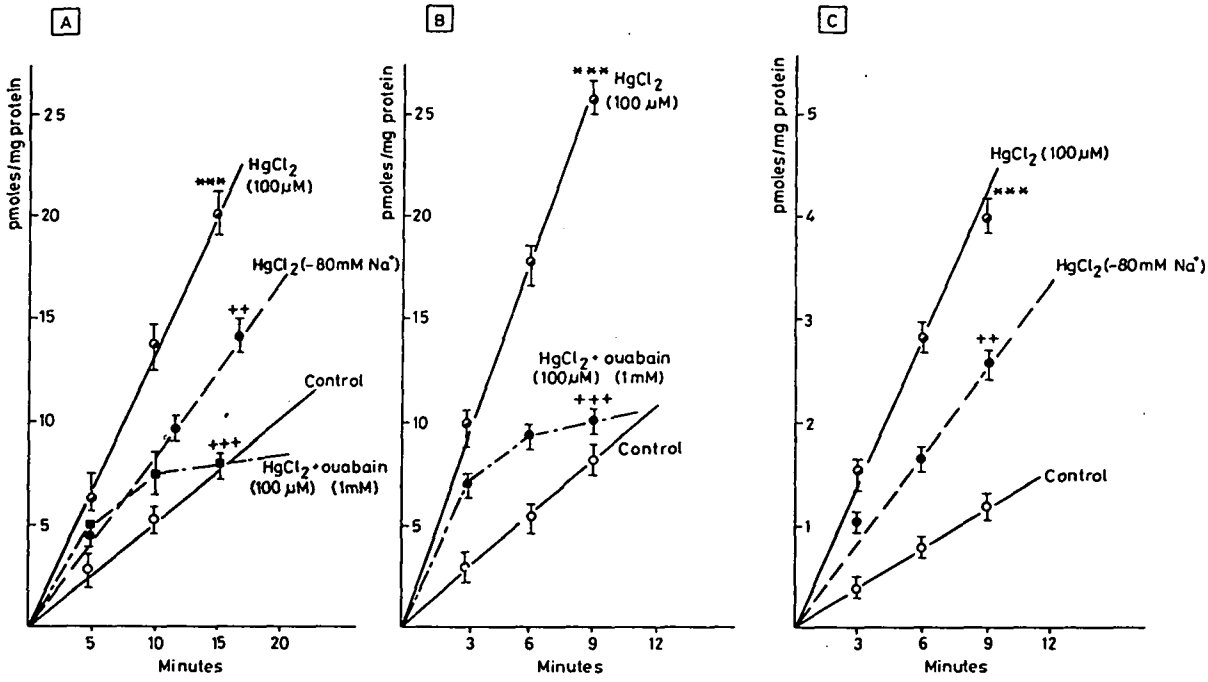

Fig. 2. Time course of basic and mercury-stimulated histamine uptake: effect of ouabain treatment and Na^+-deprivation. **A:** [^{3}H]-histamine uptake by cultured astroglial cells. **B:** [^{3}H]-histamine uptake by cultured cerebral endothelial cells. **C:** [^{3}H]-histamine uptake by RBE4 cell line. Data points represent mean values of three individual experiments with duplicates (n = 6). SEM were indicated by vertical bars: The histamine concentration in the medium was 0.04 μM, mercuric chloride, applied in 100 μM (final concentration) and in a 20 min preincubation period preceded the incubation. Sodium (80 mM) replaced by choline. Mercury effect was compared to control (***$P < 0.001$). Inhibiting effects obtained for ouabain treatment (dotted lines) or for Na^+ deprivation (broken lines) in the presence of mercury were compared to mercury-induced stimulation of histamine uptake ($^{++}P < 0.1$; $^{+++}P < 0.001$).

Figure 2A–C offer direct comparisons of the stimulatory effects of 100 μM $HgCl_2$ on histamine uptake in cultured astroglial, cerebral endothelial cells, and the RBE4 cell line showing marked stimulations by 100 μM $HgCl_2$. Basal and stimulated uptake rates were comparable in cultured astroglial and cerebral endothelial cells. The uptake by RBE4 cells was much less in both conditions. Pretreatment the cells with ouabain (1 mM) or sodium deprivation (−80 mM Na^+) suppressed mercury-stimulated uptake in both cell types.

Glial histamine uptake was markedly inhibited by impromidine, a potent H_2 agonistic and H_3 antagonistic compound (Huszti et al., 1994). The concentration required for 50% inhibition was less than 4 μM (K_i = 2.8 μM). Inhibiting effects of impromidine on mercury-stimulated histamine uptake in astroglial and cerebral endothelial cell cultures are shown in Figure 3A,B. A total of 100 μM impromidine resulted in complete inhibition of stimulated histamine uptake in both cell types. Moreover, after 10 min incubation the corresponding uptake rates were lower than that of the controls.

Experiments with potent sulfhydryl agents (1 mM DTT and 0.1 mM cysteamine) showed complete inhibition of stimulation indicating a reversal of, and not simply a protection from, $HgCl_2$ action (Figure 4A and B). In the absence of mercury, 1 mM DTT or 0.1 mM cysteamine has no effect on histamine uptake (unpublished results). Figure 4A also shows that mercuric chloride could be substituted by mercuric nitrate. Both 100 μM $HgCl_2$ and 100 μM $Hg(NO_3)_2$ reflect closely similar enhancements in glial histamine uptake.

Histamine Binding to Uptake Carrier: Mercury-Induced Modulations

Initial experiments were carried out to examine transport sites for histamine in cultured astroglial cells. In these kinetic studies, [^{3}H]-histamine binding was determined as described in Materials and Methods. Figure 5 shows the results of a typical experiment in previously frozen astroglial cultures. Specific binding in the presence of histamine receptor antagonists was saturable,

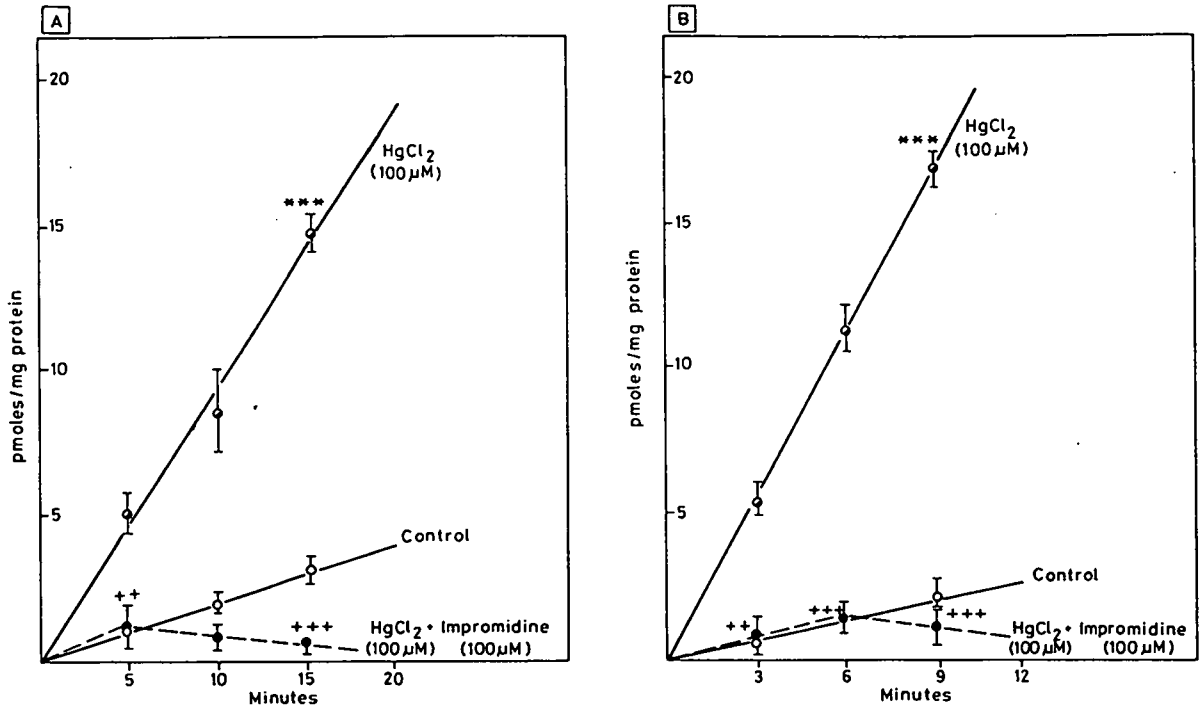

Fig. 3. Basic and mercury-stimulated histamine uptake: inhibition by impromidine (100 µM). **A:** [^{3}H]-histamine uptake by cultured asroglail cells. **B:** [^{3}H]-histamine uptake by cultured cerebral endothelial cells. Data points represent mean values of two individual experiments with duplicates (n = 4). S.E.M. were indicated by vertical bars. The histamine concentration in the medium was 0.04 µM, mercuric chloride was applied in 100 µM (final concentration) and a 20 min preincubation period preceded the incubation. Mercury effect was compared to control (***$P < 0.001$), whereas inhibiting effect of impromidine in the presence of mercury (broken lines) was compared to mercury-induced stimulation ($^{+++}P < 0.001$) and also to control ($P < 0.01$).

whereas non specific binding determined in the presence of 40 µM histamine, increased linearly and represented about 45% of total binding at 40 nM of [^{3}H]- -histamine. Analysis of the data indicated that the Hill coefficient was not significantly different from unity ($n_H = 0.90$). Under condition given in Materials and Methods, the binding of [^{3}H]-histamine was not reduced by prior freezing and thawing and did not change significantly when incubation was carried out at 25°C instead of 37°C. In individual batches of cultures, (n = 5) the apparent K_D and B_{max} ranged within 20%, yielding a mean value of 26.7 ± 3.9 nM (n = 5) for K_D (ranging from 21 nM to 36 nM) and 0.58 ± 0.18 pmol/mg protein for B_{max} (ranging from 0.53 pmol/mg protein to 0.70 pmol/mg protein).

The effect of 100 µM $HgCl_2$ on histamine binding was first assessed by measuring the saturation parameters in previously frozen astroglial cells, and compared to that of controls, in the same batch of cultures (Fig. 6). Analysis of the data indicated that $HgCl_2$ did not modify the linearity of the Scatchard plot, the Hill plot coefficient ($n_H = 1.16$), and the apparent K_D but increased the B_{max} markedly. In this series of experiments, the K_D ranged from 25 nM to 40 nM with a mean value of 28.2 ± 4.2 (n = 5), while the B_{max} ranged from 2.5 to 4.6 pmol/mg protein with a mean value of 3.32 ± 0.47 pmol/mg protein (n = 5) in the presence of 100 µM $HgCl_2$.

The analysis of mercury effect on [^{3}H]-histamine binding revealed specificity for impromidine to significantly decrease [^{3}H]-histamine binding, whereas impromidine did not modify affinity for uptake carrier (K_D). The half-maximum inhibiting effect of impromidine on $HgCl_2$-increased binding capacity (B_{max}) was observed at micromolar concentrations, similar to that observed on [^{3}H]-histamine uptake in astroglial cultures: B_{max} obtained in mercury-exposed astroglial membranes in the presence of 4 µM impromidine was about half of that obtained in the absence of the inhibitor (Fig. 7).

As detected with astroglial cell membranes, binding sites of histamine carrier were found to exist in the cerebral endothelial cell line RBE4. For comparative

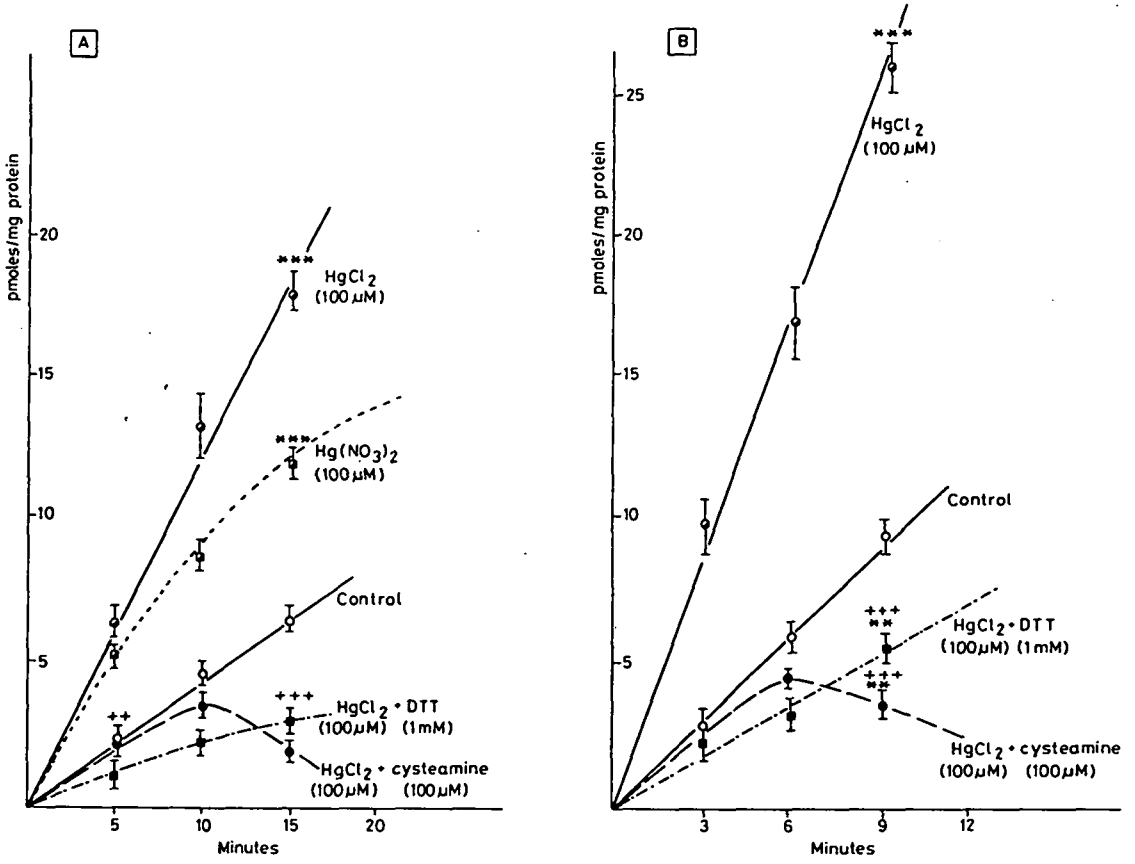

Fig. 4. Basic and mercury-stimulated histamine uptake: reversal by sulfhydryl agents, DTT (1 mM) and cysteamine (0.1 mM). **A:** [^{3}H]-histamine uptake by cultured astroglial cells. **B:** [^{3}H]-histamine uptake by cultured endothelial cells. Data points represent mean values of 3 individual experiments with duplicates (n = 6). SEM were indicated by vertical bars. The histamine concentration in the medium was 0.04 μM, mercuric chloride and mercuric nitrate (dotted line) were was applied in 100 μM (final concentration) and a 15 min preincubation preceded the incubation. Sulfhydryl reagents, DTT (1 mM: dotted lines) and cysteamine (100 μM: broken lines) were present during preincubation and also in incubation periods. Mercury effect was compared to control (***$P < 0.001$) whereas reversal (inhibiting) effects of DTT (■) and cysteamine (●) (in the presence of mercury) were compared to mercury-induced stimulation ($^{+++}P < 0.001$) and also to control (**$P < 0.01$).

studies, experiments were carried out with RBE4 cells to identify and analyze the effect of $HgCl_2$ on [^{3}H]-histamine binding. The analysis of the $HgCl_2$ effect indicated that the saturation curve and the corresponding Scatchard plot were monophasic and the slope of Hill plot was close to unity (Fig. 8). The apparent dissociation constants (K_D) obtained for basic and mercury-stimulated binding were equal within the range of standard error; calculated as 22.5 ± 3.2 nM (mean value of 4 individual experiments) and were comparable to that obtained for astroglial cells. The binding capacity (B_{max}) was significantly less that determined for astroglial cells but increased markedly when cells were exposed to 100 μM $HgCl_2$. The apparent B_{max} values, obtained for control and mercury-stimulated conditions were 0.18 ± 0.03 (n = 6) and 1.20 ± 0.36 (n = 4), respectively. Impromidine (4 μM) decreased the binding capacity (B_{max}) by about 50% with a slight modification of the dissociation constant (K_D), similar to that observed with astroglial cells (Fig. 9).

The apparent K_D and B_{max} values obtained for cultured astroglial cells and RBE4 cells in the presence and absence of 100 μM $HgCl_2$ are summarized in Table I.

DISCUSSION

The present study shows that exposure of rat astrocytes or cerebral endothelial cells to mercuric compounds in a concentration range of 25–500 μM promotes

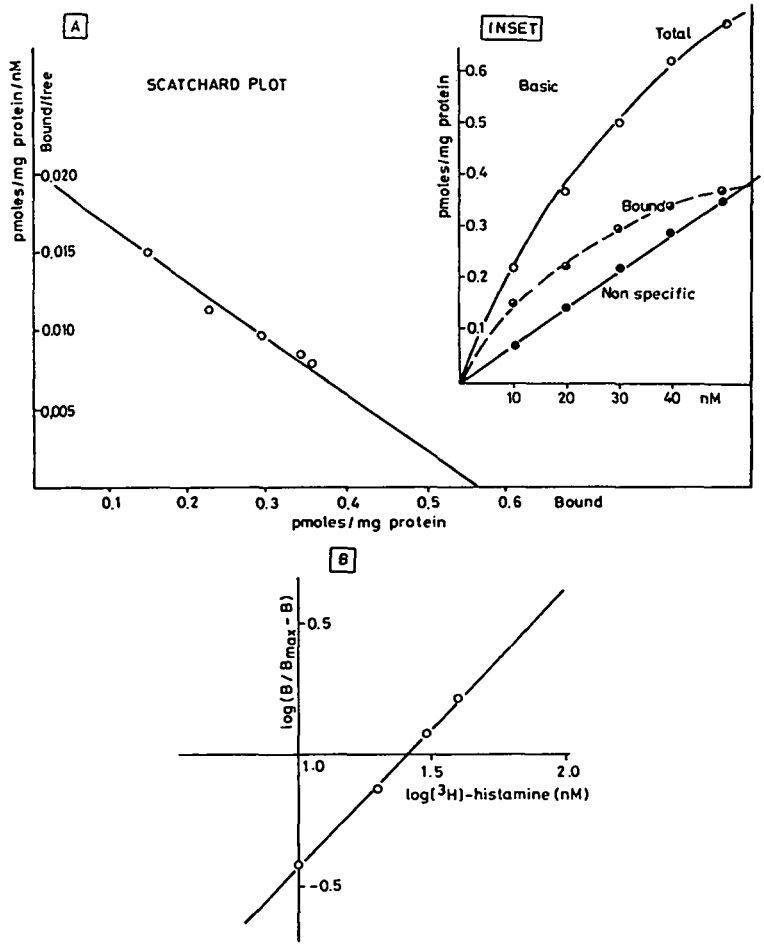

Fig. 5. Kinetics of [^{3}H]-histamine binding to uptake carrier in cultured astroglial cells. **A:** Scatchard plot of [^{3}H]-histamine binding to uptake carrier. **Inset:** Saturation curve of binding in equilibrium (30 min incubation at 37°C). Specific binding (broken line: ⊖), total binding (○), and nonspecific binding (●). **B:** Hill plot from the same data. Data represent measurements made with one batch of cultures (triplicate determinations). Experiments were replicated twice. Lines were fitted by linear and non-linear regressions.

carrier-mediated uptake of histamine. Since sodium is necessary for stimulated as well as basal uptake of the amine the enhanced transport did not appear to be a diffusional process caused by non-specific membrane leakage. Complete inhibition of the stimulated uptake by impromidine (a potent histamine uptake blocker) also argues against a diffusional process.

Organic and inorganic mercurials were shown to interact with protein thiols within the cell membrane (Sutherland et al., 1967; Haas and Schmidt, 1985) and this may account for the neuropathologic effects of mercury. Concentrations of inorganic mercuric salts in the 25–500 μM range appear to be sufficient for a reversible reaction between mercury and the distinct protein thiols at the carrier (increasing the binding capacity for histamine); and this offers a possible explanation for the observed stimulation of histamine uptake. Our finding that thiol reducing agents such as dithiotreitol and cysteamine could completely abolish this stimulation provides further evidence for this hypothesis. Protection by thiols does not appear to be caused by lowering effective mercurial concentration; but rather a complete reversal of the mercury-induced effect at the carrier. Direct measurement of mercury bindings to carrier thiols are however necessary to obtain direct evidence.

Below this concentration range, mercuric salts were found ineffective and in a narrow (1–10 μM) range, inhibitory for histamine uptake (Huszti and Balogh,

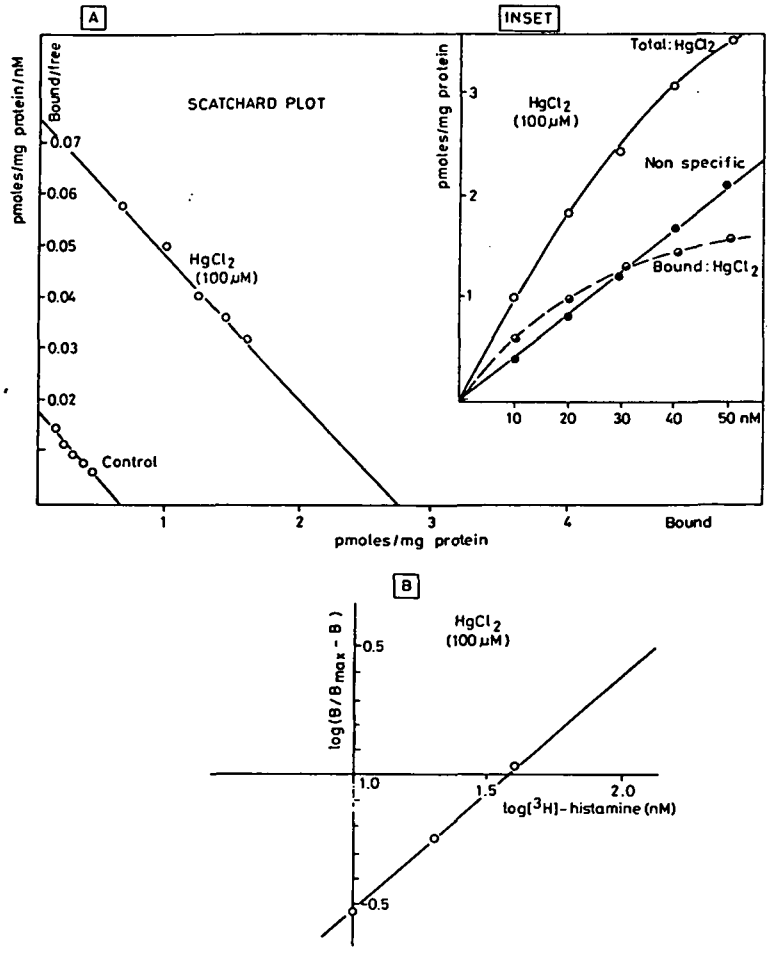

Fig. 6. Kinetics of [^{3}H]-histamine binding in the presence of mercuric chloride (100 μM) in cultured astroglial cells. **A:** Scatchard plots of [^{3}H]-histamine binding to uptake carrier in the presence and absence of mercuric chloride. **Inset:** Saturation curves of histamine bindings in equilibrium (30 min incubation at 37°C). Specific binding (broken line: with $HgCl_2$) total binding in the presence of HgCl2 (○) and nonspecific binding (●). **B:** Hill plot from the same data (mercury-induced changes in binding). Data represent measurements made with one batch of cultures (triplicate determinations). Experiments were replicated twice. Lines were fitted by linear and non-linear regressions.

1995). These low concentrations of mercury were probably insufficient for reactions with distinct protein sulfhydryls at the carrier and thus for uptake stimulation; and at 1–10 μM, a non-specific inhibitory effect of mercurials may occur. Alternatively, stimulation and inhibition lead to a compensation or to an under expression, showing ineffectiveness or moderate inhibitory effects on the uptake. This later assumption may explain the bidirectional effect of mercuric salts on glial and cerebroendothelial histamine uptake. At high (above 200 μM) concentrations, the toxic effects of mercury might prevail and suppress the induced stimulation resulting in an U-shaped dose-response curve (Fig. 1). Over a wide range of concentration, organic and inorganic mercurials were found exclusively inhibitory for glial glutamate uptake (Brookes, 1988; Aschner et al., 1990, 1993). This indicates marked differences in glutamate and histamine uptake systems especially in the structure of carrier proteins.

The sodium-dependent binding site of the histamine carrier was identified first in this study. The pharmacological specificity of [^{3}H]-histamine binding fit well with that observed for glial or endothelial histamine uptake. An assumption that measured histamine binding could represent accumulation of [^{3}H]-histamine in the cells or in membrane-bound saccules might be excluded since net

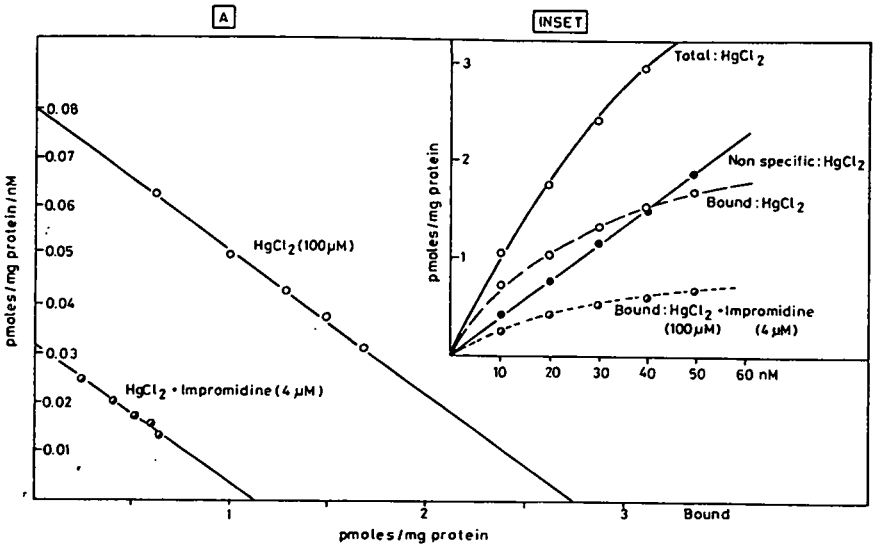

Fig. 7. Kinetics of mercury-stimulated [^{3}H]-histamine bindings in the presence of impromidine (4 μM) in cultured astroglial cells. **A:** Scatchard plots of mercury-stimulated [^{3}H]-histamine binding to uptake carrier in the presence and absence of impromidine. **Inset:** Saturation curves of histamine bindings in equilibrium (30 min incubation at 37°C). Specific bindings (broken lines: without impromidine ○, with impromidine ◒), Total binding (○) and nonspecific binding (●) without impromidine. Data represent measurements made with one batch of cultures (triplicate determinations). Experiments were replicated twice. Lines were fitted by linear and nonlinear regressions.

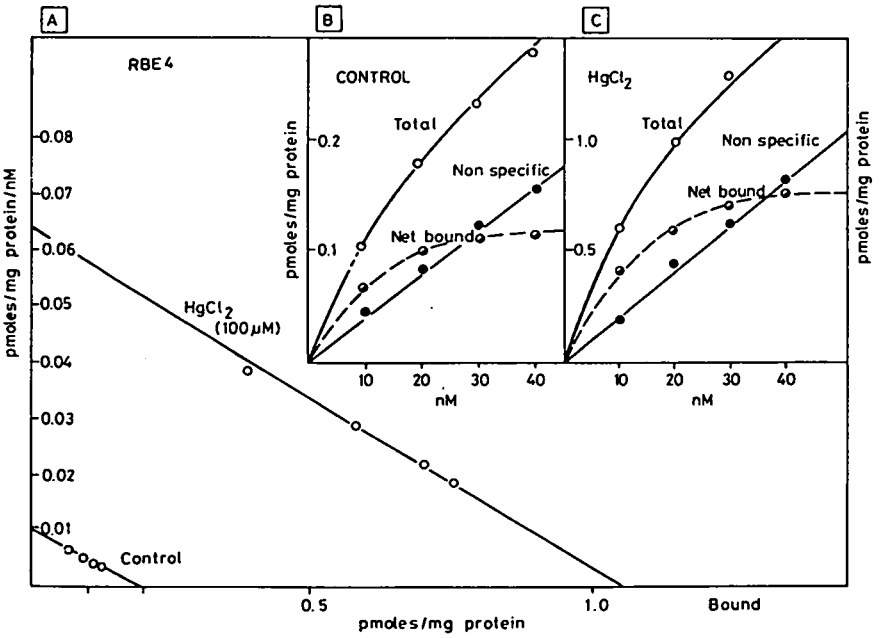

Fig. 8. Kinetics of basic and mercury-stimulated [^{3}H]-histamine binding in RBE4 cell line. **A:** Scatchard plots of [^{3}H]-histamine binding in the presence and absence of mercuric chloride. **B:** Saturation curves of histamine bindings in equilibrium (30 min incubation at 37°C). Specific binding (dotted line ◒), total binding (○) and nonspecific binding (●). **C:** Saturation curves of mercury-stimulated histamine bindings in equilibrium (30 min incubation at 37°C). Specific binding (broken line ◒), total binding (○) and nonspecific binding (●). Data represent measurements made with one batch of RBE4 cell line, treated as given in Materials and Methods. (triplicate determinations). Experiments were replaced twice. Lines were fitted by linear and non-linear regressions.

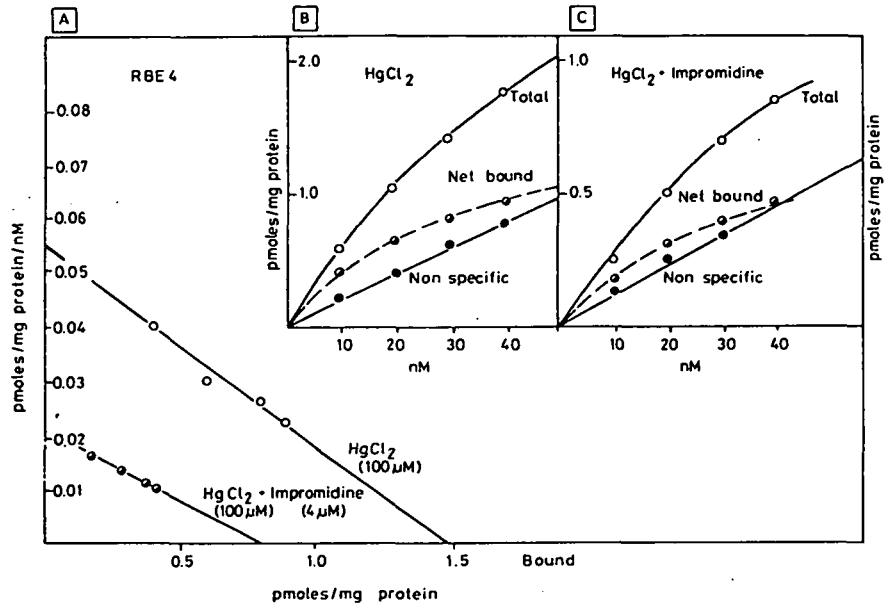

Fig. 9. Kinetics of mercury-stimulated [^{3}H]-histamine bindings in the presence of impromidine (4 µM) in RBE4 cell line. **A:** Scatchard plots of [^{3}H]-histamine binding to uptake carrier in the presence and absence of impromidine. **B:** Saturation curves of mercury-stimulated histamine bindings in equilibrium (30 min incubation at 37°C). Specific binding (broken line ⊖), total binding (○) and nonspecific binding (●) in the presence of $HgCl_2$ (100 µM). **C:** Specific binding (broken line ⊖), total binding (○), and nonspecific binding (●) in the presence of $HgCl_2$ (100 µM) and impromidine (4 µM). Data represent measurements made with one batch of RBE4 cell line, treated as given in Methods. (triplicate determinations). Experiments were replaced twice. Lines were fitted by linear and non-linear regressions.

TABLE I. Summary of the Apparent K_D and B_{max} Values in Control Conditions and in the Presence of 100 µM Mercuric Chloride

	Cultured astroglial cells	Rat brain endothelial cell line (RBE4)
K_D[a]		
Control	26.7 ± 3.9	22.5 ± 3.2
100 µM $HgCl_2$	28.2 ± 4.2	26.5 ± 3.6
B_{max}[b]		
Control	0.63 ± 0.10	0.18 ± 0.03*
100 µM $HgCl_2$	3.32 ± 0.47**	1.20 ± 0.36**

[a](nM).
[b](pmol/mg protein).
B_{max} of rat brain endothelial cell line (RBE4) was compared to that of cultured astroglial cells ($P < 0.01$).
Mercury effects were compared to controls ($P < 0.001$).

binding could not be reduced by freezing and thawing (data not shown); furthermore, histamine binding parameters were about one order of magnitude less than kinetic parameters (K_m and V_{max}) derived from the saturation kinetics of histamine uptake in cultured astroglial cells (Huszti et al., 1994, 1995). On the other hand, it is unlikely that [^{3}H]-histamine was binding to any of the three well-characterized subclasses of histamine receptors while assays were carried out in the presence of tripolidine, cimetidine and thioperamide (40 µM each), the potent antagonists of histamine H_1, H_2, and H_3 receptors. All of these suggest that measurements represent true binding, insensitive to histamine receptor agonists and antagonists.

The sodium dependent binding site of the histamine carrier has noted sensitivity to mercurials in both astroglial cells and RBE4 cell line. Mercuric chloride (100 µM) that had been identified as a stimulator of histamine uptake was effective in enhancing the capacity (B_{max}) of Na^+-dependent histamine binding without affecting the affinity (K_D) of the binding sites for the amine. The analysis of the enhancing effect of mercury revealed a specificity for the histamine uptake carrier. Organic mercurials, such as p-chloromercurisulfonic acid, which was also found to be stimulatory for HA uptake (unpublished results) decreased the affinity of the binding sites for [^{3}H]-mepyramine without affecting the number of binding sites (binding capacity) at histamine H_1 receptors (Yeremian et al., 1985).

In these experiments, impromidine the potent inhibitor of basal and stimulated histamine uptake, was the only

compound to significantly decrease the enhanced binding sites, probable through specific interactions with the histamine carrier. There was about 50% reduction in the enhanced histamine binding capacity at 4 μM of impromidine which was comparable with its apparent K_i (2.8 μM).

The above findings suggest that [^{3}H]-histamine binding measured under conditions used in the present experiments, represent binding to uptake carrier in both astroglial cells and RBE4 cell line. On the other hand, the close similarities in binding characteristics and the closely equal apparent K_D values obtained for the different cell types suggest identical mechanisms for uptake and identical structures of the uptake carrier in astroglial and cerebral endothelial cells. The increased binding capacities (B_{max}) caused by mercurials suggest that the uptake carrier undergoes similar processes in both cell types; possible SH-protein cross linkings (conformational changes) in the structure, stabilizing an activated state of the carrier.

Still additional investigations are needed to clarify the molecular consequences of mercury-exposure, principally to establish the direct binding of mercury to histamine carrier.

ACKNOWLEDGMENTS

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IV

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Inflammation Research

Intracarotid histamine administration results in dose-dependent vasogenic brain oedema formation in new-born pigs

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Introduction

The existence of three pools of brain histamine (in neurons, perivascular mast cells, cerebral endothelium) suggests involvement of histamine released after physiological and pathological stimuli in neuronal transmission, regulation of cerebral blood flow and brain oedema formation [1]. Cerebral endothelial cells have been shown to take up histamine from either side but release it mainly luminally [2, 3]. Indirect evidence implicates histamine in the pathogenesis of neonatal brain oedema since histamine accumulated in brain compartments in asphyxia [3]. Studies with antihistamines suggested that H_2 receptors play a major role in the development of brain oedema in asphyxiated new-born pigs, but H_1 receptors also contribute [4]. The direct effect of histamine in neonates has not yet been reported. Histamine regulates the activity of cerebral endothelial acid phosphatase in vitro, and might influence blood-brain barrier (BBB) permeability by increasing endocytic transport [5]. The present study investigated the effects of intra-carotid histamine administration on brain oedema formation and endothelial acid phosphatase activity in new-born pigs.

Materials and methods

New-born pigs of either sex (n = 30; 4–8 h; 1.07–1.56 kg) were anaesthetised with pentobarbital (30 mg/kg) and one umbilical artery was catheterised to monitor cardiovascular, blood gas and acid-base parameters [6]. The right internal carotid artery was catheterised through the external branch and histamine (10^{-4} mol–10^{-6} mol in 0.5 ml isotonic saline) was given by slow intra-arterial injection. The external carotid artery was then ligated. Experimental procedures were approved by the Ethical Committee on Animal Investigation, Albert Szent-Györgyi Medical University, Szeged, Hungary.

The development of vasogenic brain oedema was measured 1 h after histamine injection, as the extravasation of sodium fluorescein (SF, MW 376) and Evan's Blue-labelled albumin (EBA, MW 67.000), both from Sigma. Dyes were administered intravenously in isotonic saline (2% solutions, 5 ml kg^{-1}) 30 min after histamine. Intravascular dyes were removed by perfusion with 200 ml kg^{-1} isotonic saline. Sera and tissue samples from the right cerebral cortex were homogenized in cold 7.5% trichloroacetic acid (3 ml) and centrifuged (10.000 g for 10 min). Concentrations were measured in supernatants as previously described: the absorbance of EBA at 620 nm, and the fluorescence emission of SF at 525 nm (excitation wavelength 440 nm) [6]. *Extravasation was expressed as brain tissue concentration divided by* serum concentration, ie (μg dye mg^{-1} brain tissue)/(μg dye μl^{-1} serum).

Acid phosphatase (ortho-phosphoric monoester hydrolase, EC 3.1.3.2.) activity was measured by the rate of hydrolysis of p-nitrophenyl-phosphate (p-NPP) in isolated cortical microvessels [5]. Samples were incubated in 1 ml 1.0 M acetate buffer (pH 5.5) containing 2.5 mM p-NPP at 37°C. After 1 h, 0.3 ml of 1 M NaOH was added and absorbance was read at 410 nm. Enzyme activity was expressed as mU mg^{-1} protein.

Groups were compared using Kruskal-Wallis one way analysis of variance (ANOVA) on ranks followed by Dunn's test. Correlation between acid phosphatase activity and BBB permeability was determined by linear regression.

Results and discussion

Dose-dependent BBB opening was found in the cerebral cortex of new-born pigs 1 h after intra-carotid injection of histamine. There were statistically significant ($p < 0.05$) increases in permeability to both SF (Fig. 1A) and albumin (Fig. 1B) at the highest doses. Histamine increased acid phosphatase activity in cortical microvessels. Linear correlations were found between the enzyme activity and the BBB transport of both tracers.

SF transport = 0.0265 × [acid phosphatase activity] + 0.346 ($r = 0.893$; $n = 24$; $p < 0.001$)
EBA transport = 0.0471 × [acid phosphatase activity]− 0.432 ($r = 0.948$; $n = 24$; $p < 0.001$)

Intra-carotid histamine, at levels similar to those measured during asphyxia [3], induced a rapid and dose-dependent increase in BBB permeability for both intravascular tracers and thus may contribute to vasogenic brain oedema formation in new-borns. This neonatal porcine study agrees with results obtained in adult animals [1, 7] and in studies where antihistamines prevented brain oedema formation [1, 4]. In a recent in vitro study, histamine increased the transcellular passage of albumin through the BBB, but not the permeability for sucrose and inulin (paracellular markers) [8]. Increased pinocytotic activity was also seen after intra-

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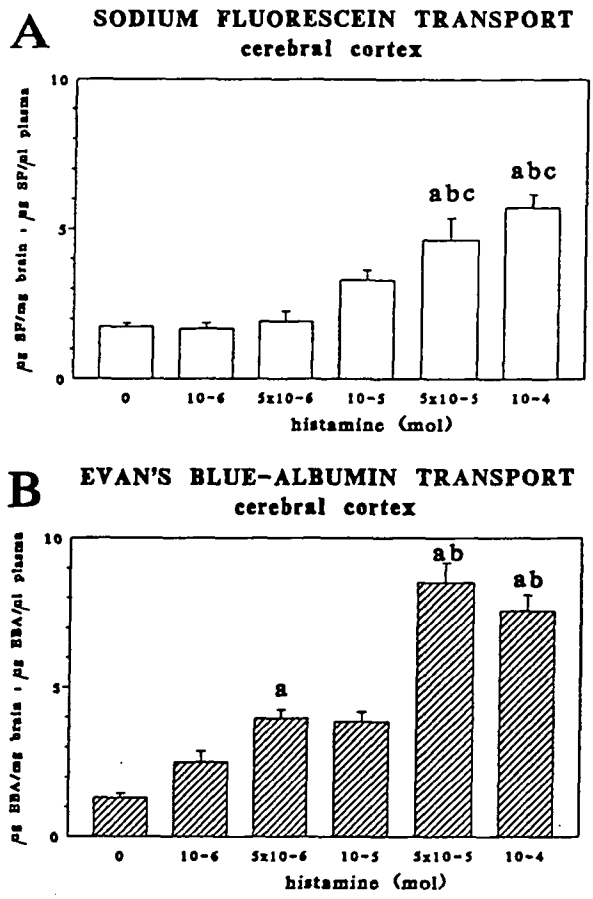

Fig. 1. Development of brain oedema in new-born pigs 1 h after intracarotid histamine challenge. BBB permeability markers were SF (A) and EBA (B). Symbols indicate significant differences ($p < 0.05$) compared to the following treatments: a = 0 mol, b = 10^{-6} mol, c = 5×10^{-6} mol histamine.

carotid injection of histamine in adult rats [3]. Our present results accord with this, because albumin is supposed to pass through the BBB by pinocytosis, while sodium fluorescein is thought to extravasate both para- and transcellularly. This is the first in vivo study suggesting a correlation between endothelial acid phosphatase activity and transendothelial transport after histamine challenge. This enzyme takes part in the endocytic pathway for transport of proteins through the BBB, but does not increase paracellular permeability [5], perhaps explaining our in vitro demonstration of histamine-induced selective albumin permeation [8]. Further studies will investigate the role of acid phosphatase isoenzymes in histamine-induced cerebral oedema formation.

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V

Neurobiology 5(1) pp. 1–16 (1997)

Research report

PRODUCTION OF PURE PRIMARY RAT CEREBRAL ENDOTHELIAL CELL CULTURE: A COMPARISON OF DIFFERENT METHODS

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Summary: To study the blood-brain barrier *in vitro* pure cerebral endothelial cell cultures, without contaminating cells have to be obtained. Most other cell types besides endothelial cells can be pericytes, a few astrocytes, some smooth muscle cells, fibroblasts and meningeal cells. Careful removal of large vessels and meninges during the dissection and the optimal duration of enzymic digestions can reduce the ratio of contaminant cells. In order to further increase the purity of the culture endothelial cells can be subcloned, however, this is not useful for cells of every species. An alternative choice in cultures from rat is to perform a selective cytolysis by complement and monoclonal anti-Thy 1.1 antibody to eliminate pericytes and astrocytes. The presence of growth factors and the type of serum are also important for successful endothelial cell cultures. With the combination of the cytolysis of contaminating cells and the use of plasma-derived serum, the culturing of pure primary cerebral endothelial cells was successful.
Keywords: blood-brain barrier, cerebral endothelial cells, pericyte, complement killing

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INTRODUCTION

The blood-brain barrier (BBB) based in part on specialized characteristics of cerebral endothelial cells (CEC), including the occurrence of tight intercellular junctions, high electrical resistance, few pinocytotic vesicles as well as the presence of specific, polarized, and highly discriminatory membrane transport systems. These features form a selective permeability barrier that restricts the movement of most polar molecules and proteins.

The *in vitro* study of the blood-brain barrier (BBB) started in the 70s when Joó and Karnushina (1973) isolated fractions enriched in microvessels from rat cortex. This method could be used for short-term studies of the BBB. Couple of years later a new method was described for culture of CEC (Panula et al., 1978). These new model systems provided a better insight into the morphology, biochemistry and physiology of the CEC and new means to study the transport processes of nutrients and drugs across the BBB. In order to obtain CEC cultures porcine, murine (Tontsch and Bauer, 1989), bovine (Méresse et al., 1989), human (Vinters et al., 1987, Nagy and Vastag 1994) and rat brains (Bowman et al., 1981, Abbott et al., 1992) are used most frequently in different laboratories. Syngeneic co-cultures of rat cerebral endothelial cells (RCEC) and other cell types like astrocytes, smooth muscle cells, pericytes, neurons is an advantage of the rat model. For molecular biological studies rodents are widely used, this is also favours choosing rat CECs. Almost all methods use gray matter of brain and enzymic digestion step(s) for dissociation of microvessels. The capillary fragments are separated by Percoll gradient (Bowman et al., 1981) or by series of centrifugation steps (Tontsch and Bauer, 1989).

For characterisation of the primary culture of CEC it is necessary to check the purity and the expression of specific markers of endothelial cells. Cultures of RCEC can be easily characterised, since several antibodies are available for this species. The primary cultures are usually not pure, pericytes, astrocytes, fibroblasts, smooth muscle or leptomingeal cells may appear among endothelial cells. There are different methods to increase the purity of brain endothelial cell cultures like the subcloning of endothelial cell islands by microtrypsinisation as described by Méresse et al., (1989) and the complement mediated specific cytolysis (Risau et al., 1990).

The growth and differentiation of endothelial and the other contaminant cell types depend on the presence of different mitogenic factors. The endothelial cell growth factor (ECGF) (Folkman and Haudenschild, 1982) or the acidic and the 10-30 times more potent basic fibroblast growth factors (FGF) (Gospodarowicz et al., 1986) were found to be necessary for the

continuous growth and the expression of the phenotypic features of the endothelial cells *in vitro*. The quality of serum is also very important. There is a variability in the growth rate stimulating effect of different types or batches of serum. The fetal calf serum (FCS) different amount of platelet-derived growth factor (PDGF) which stimulates proliferation of contaminating cells as well. The plasma derived serum is free from PDGF.

The thymus-derived lymphocytes, the mesangial cells, the cerebral pericytes and astrocytes express the cell surface differentiation antigen Thy 1.1 while endothelial cells do not. Thy 1.1 antigen can be used to reduce the non-endothelial contamination of CECs (produce optimal purity of CEC cultures) by selectively lysing by antibody/complement treatment the pericytes and astrocytes (Risau et al, 1990).

In order to find an optimal culture protocol for RCEC in our laboratory we compared different techniques.

MATERIALS AND METHODS

Chemicals

Materials used in the experiments were the following origin: collagenase type II, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium/ nutrient mixture F-12 Ham (DMEM/F-12), fetal calf serum (FCS), basic fibroblast growth factor (bFGF), heparin, monoclonal anti-mouse Thy 1.1 antibody, complement serum HLA-ABC (rabbit), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium, *Bandeiraea simplicifolia* isolectin B_4, 3,3'-diaminobenzidine (DAB), fluorescein isothiocyanate-dextran (FITC-dextran) (Sigma), collagenase-dispase (Boehringer), Percoll (Pharmacia), L-glutamine, penicillin-streptomycin (Gibco), plasma derived derum (PDS) (Advanced Protein Products LTD).

Preparation of primary cultures

Two-week-old Sprague-Dawley CFY rats of either sex were obtained from our Institute's animal house. Rats were anesthesized with ether. After thorough rinse with 70% ethanol, than with iodine in 70% ethanol, heads were cut, and placed into a sterile glass Petri dish. In the laminar flow box forebrains without the cerebellum were removed from the skulls with sterile microdissecting forceps and scissors, and collected in cold sterile phosphate

buffered saline (PBS, without calcium and magnesium, pH: 7.4). Meninges were removed on sterile filter paper (Whatman 3M) from each brain hemisphere while at the same time white matter was "peeled off" with the aid of fine curved forceps. Gray matter was carefully collected from the filter paper (meninges stuck to it) and minced to approximately 1 mm^3 pieces by sterile disposable scalpels in the incubation medium (270 U/ml collagenase, 1 mg/ml dispase, DMEM-F12 containing antibiotics) in a sterile glass Petri dish. Incubation media for enzymic digestion was always prepared freshly from lyophilised enzymes, then sterilised by filtration. Their pH was adjusted to 7.4.

The minced tissue was transferred into a centrifuge tube with the rest of the collagenase-dispase solution (total: 2 ml/brains) and triturated with a pipette (10 up and down), than incubated at 37 °C for 1.5 h in shaking waterbath. After this incubation, cold DMEM-F12 was added to the homogenate and centrifuged at 1000 *g* for 8 min. The supernatant was aspirated and 15% BSA/DMEM-F12 (2 ml/brain) was added to the homogenate, mixed well by trituration and centrifuged at 1000 *g* for 15 min. The myelin layer and the supernatant was aspirated, the pellet washed once in DMEM-F12 (700 *g* for 5 min) then further digested in waterbath for an other, 1 h in the incubation medium 1ml/brain. The cell suspension was centrifuged (700 *g* for 5 min). The pellet was suspended in 2 ml DMEM-F12 and carefully layered on a continuous 33% Percoll gradient and centrifuged at 1000 *g* for 10 min. For the gradient 10 ml Percoll, 18 ml PBS, 1 ml FCS and 1 ml 10¥ concentrated PBS were mixed, sterile filtered and centrifuged at 4 °C, 30000 *g* for 1 h.

The band of the endothelial cell clusters (clearly visible as a white-grayish layer above the red blood cells) was aspirated, washed twice in DMEM-F12 (at first 1000 *g*, 8 min, then 700 *g*, 5 min). The cells were suspended in culture medium (DMEM-F012 containing 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamicin, 2 mM glutamine, 20 % heat inactivated FCS or PDS and from the second day 1 ng/ml bFGF) and were seeded onto rat tail collagen-coated 35 mm plastic dishes or Falcon cell culture inserts (pore size 4 mm, d=25 mm). Starting culture from 10 brains, we could obtain confluent primary culture of RCECs in 10 pieces of 35 mm tissue culture dish, equivalent approximately to 100 cm^2 surface area. The medium was changed on the next day, later on every third day.

Characterisation of the cultures

FVIII-related antigen (FVIII) immunohistochemistry. After a brief washing in PBS and fixing in ethanol at 4°C for 15 min, cells were treated with 1% H_2O_2 in PBS for 10 min, followed by washing in PBS. Non-specific binding sites were blocked by incubation in 3% normal goat serum in PBS at room temperature for 20 min. Anti-FVIII rabbit immunoglobulin (Dako) was used as primary antibody and biotin-labelled anti-rabbit IgG (Dako) as secondary antibody, both applied for 30 min. After a 30 min incubation with avidin-biotin-horseradish peroxidase (HRP) complex (ABC kit, Vector Lab, CA, USA), DAB was used as HRP substrate, followed by hematoxylin-eosin (HE) counterstaining.

Lectin-binding. RCECs were washed in PBS, fixed in 4% formalin and 70% ethanol in PBS for 15 min, treated with 1% H_2O_2 in PBS for 10 min, washed again in PBS, then incubated in 15 mg/ml HRP-conjugated BS-I-B_4 in 0.1% BSA-PBS for 90 min. DAB was used as HRP substrate. The preparations were counterstained by HE.

Complement killing

RBEC cultures were washed twice with serum free DMEM-F12, then incubated with monoclonal anti-mouse Thy 1.1 antibody (antibody: DMEM-F12 = 1:500) for 1 h at 37 °C. After washing twice with DMEM-F12, the cultures were incubated in the presence of rabbit complement (complement : DMEM-F12 = 1:3) for 2 h at 37 °C, which mediated the cytolysis of those cells which expressed on their surface the antigen Thy 1.1. After washing in DMEM-F12 the cells were cultured in the previously described complete medium.

RESULTS AND DISCUSSION

Isolation and characterisation of endothelial cells

The method we were using was based on two enzymic digestion steps to free microvessels from the surrounding nervous tissue and eliminate possible contaminating cells, followed by a Percoll gradient centrifugation to isolate the capillary fragments. The small vessel fragments obtained at the end of

the isolation procedure attached rapidly to collagen coated surfaces, and in 2-3 days, colonies of RCECs emerged and formed a non-overlapping continuous monolayer with some swirling patterns at the end of the first week. RCECs displayed a so-called "fibroblast-like" morphology: cell-shape is fusiform with an oval nucleus in the center, neighbouring cells tightly attached to each other in such a way that no intercellular space could be observed. The endothelial cells gave specific immunohistochemical staining with anti-FVIII antibody (Fig. 1 A, C), bound the galactose-specific BS-I-B_4 isolectin (Fig. 1 B) and showed positive histochemical staining for alkaline phosphatase enzyme.

For RCEC monolayers grown on Falcon 25 mm insert, 120 W¥cm^2 transendothelial electrical resistance was obtained. The passage of 70 kDa FITC-dextran was restricted through RCEC monolayer: 99.12 ± 8.79 mg/cm^2/h *vs.* 655.80 ± 12.37 mg/cm^2/h (n=6) in the case of cell-free filter (Deli et al., in press).

Effect of different sera and growth factors

Serum from fetal calf contains PDGF which stimulates fibroblast and smooth muscle cell proliferation in addition to promoting endothelial cell growth (Abbott et al., 1992). In FCS containing medium CECs loose the elongated, spindle-shape phenotype, the cells move away from each other (Fig. 2 A) and pericytes become the dominant cell type. Pericytes appear as large spreading cells with highly irregular edges which do not express FVIII (Fig. 1 C) (Shepro and Morel, 1993).

When the cultures were fed with bovine PDS which does not contain PDGF the endothelial cells showed healthy, uniform phase-bright appearance. RCECs were tightly packed against each other without intercellular gaps (Fig. 2 B). Less contaminating cells could be found than in those cultures which received FCS. There was also a difference in RCECs number between the cultures which were treated with different sera. In the presence of PDS we counted 962 ± 65 cells/mm^2 while in FCS containing medium the cells grew more sparsely: 807 ± 53 cells/mm^2.

From the second day on, basic FGF (1ng/ml) was added to the cultures, which greatly improved the growth rate of the endothelial cells.

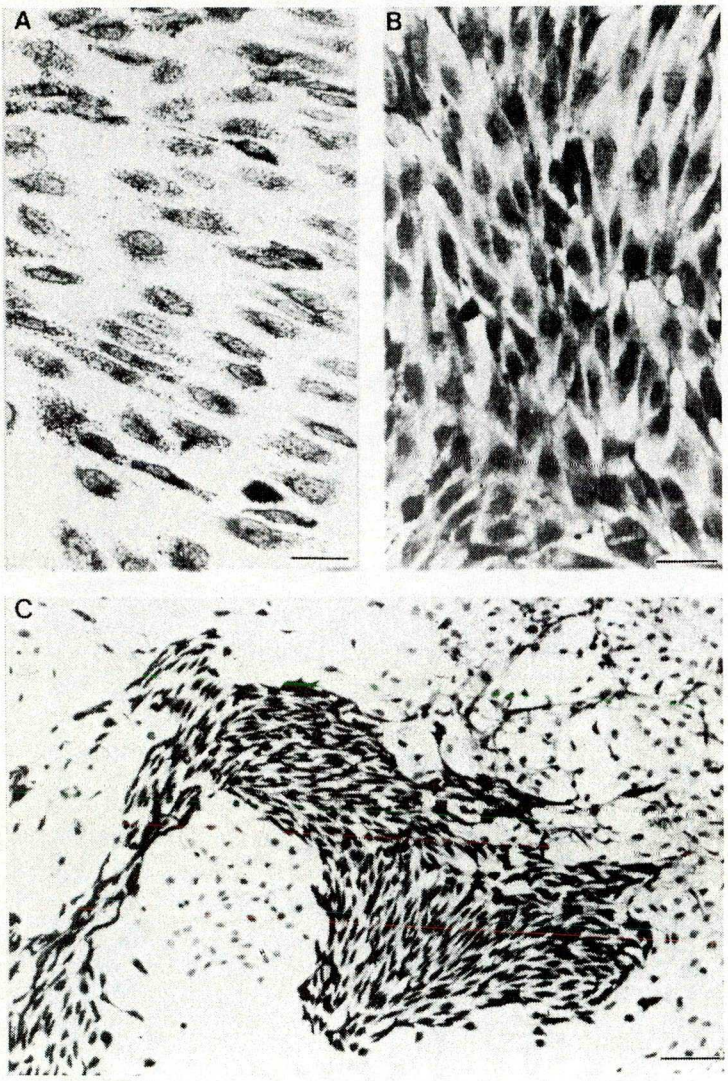

Fig. 1. (A) Antibody against FVIII related antigen gives a punctuate staining in the perinuclear zone in 5 day-old pure RCEC culture. Bar, 50 mm. (B) *Bandeiraea simplicifolia* isolectin B_4 binding gives a perinuclear homogenous staining on endothelial cells. HE counterstaining. Bar, 50 mm. (C) In a 7 day-old RCEC culture FVIII-related antigen positive endothelial cell colony is surrounded by not stained contaminating polygonal cells with processes which are probably pericytes. Bar, 250 mm

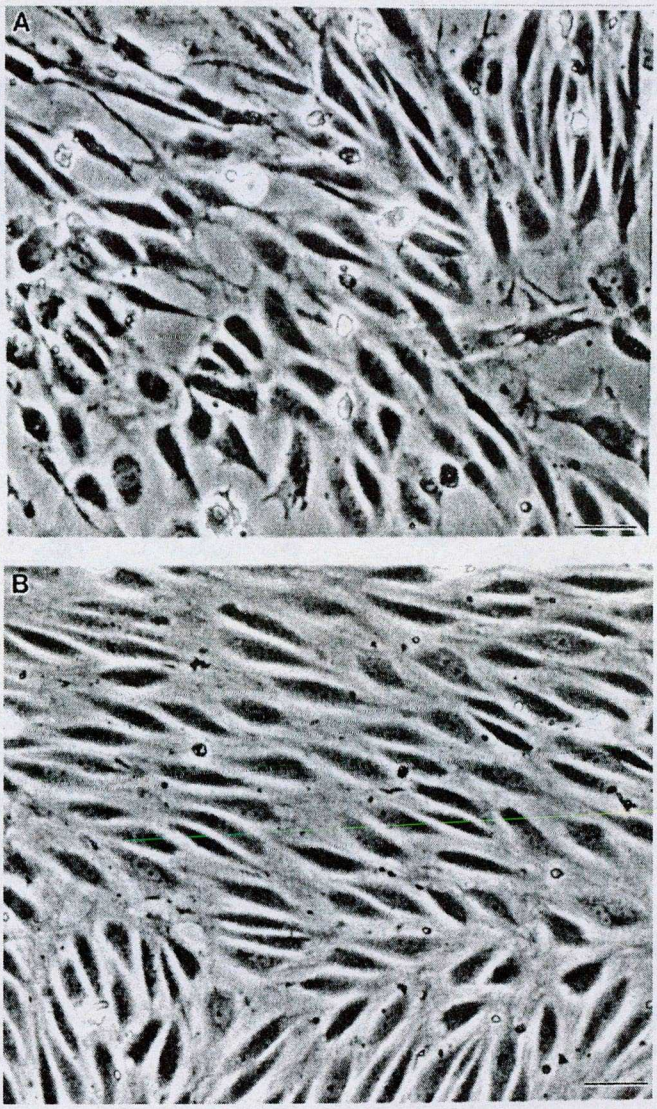

Fig. 2. (A) Primary RCEC in medium containing 20% FCS 6 days after plating. There are gaps between the elongated endothelial cells that might be caused by contaminating cells, probably pericytes. Bar, 50 mm. (B) Uniform RCEC monolayer without any other cell types at 6th day *in vitro* in medium containing 20% PDS. The cells show the fusiform, elongated morphology without intercellular gaps. Bar, 50 mm

Contaminating non-endothelial cells / Complement killing

During the production of purified RCEC cultures contamination from pericytes is the most frequent problem. Pericytes are elongated, polymorphic, multibranched periendothelial cells of mesodermal origin (similarly to the endothelium). *In vivo* they are integrated in the capillary wall and wrapped by the basement membrane of the endothelium. They are related to smooth muscle cells due to their content in contractile elements. Pericytes play a role in the proliferation and differentiation of endothelial cells, secrete vasoactive agents, release structural components of the basement membrane. The endothelium and pericytes form a morphological and functional unit (Shepro and Morel, 1993). Pericytes inhibit adrenal capillary endothelial cell proliferation *in vitro* in a contact-dependent manner and they became the dominant cell type in the culture. The activated transforming growth factor b (TGFb) is the mediator of EC growth inhibition. The co-cultures produce active form of TGFb while the separated EC and pericyte cultures secrete latent TGFb which is activated by acidic pH (Olridge et al., 1989). We also observed that pericytes tend to overgrow RCECs in long-term culture especially if the serum is not free from PDGF.

The optimal duration of enzymic digestion can keep the ratio of pericytes and astrocytes to a minimum level. If the cells are digested for a longer period or the enzyme solution is more concentrated, pericyte contamination is decreased but single endothelial cells will appear instead of capillary fragments. It turned out that single cells do not attach to the surface and do not grow well in culture, possibly because they lack 'survival factors' from their neighbours (Abbott et al., 1994). In contrast, if the basement membrane is left intact by shorter digestion time pericytes can contaminate the culture.

Some of the contaminating non-endothelial cells, pericytes and astrocytes, express Thy 1.1 antigen, whereas endothelial cells do not therefore, these cell types can be removed by selective cytolysis using anti-Thy 1.1 antibody and complement (Risau et al., 1990). We could considerably reduce the number of contaminating cells (Fig. 3) resulting in more than 95% pure RCEC cultures (Fig. 4 A). If non-endothelial cells were not removed in time, they could overgrow the RCECs in a couple of days (Fig. 4 B). It is important to find the optimal time-point for complement killing at each culture. This depends on the ratio of CECs and pericytes and the absolute number of the endothelium. If this treatment is done too early, we can kill the young and weak endothelial cells, while later we cannot remove the pericytes. We found that the optimal time is about 2-3 days after the plating of the cells.

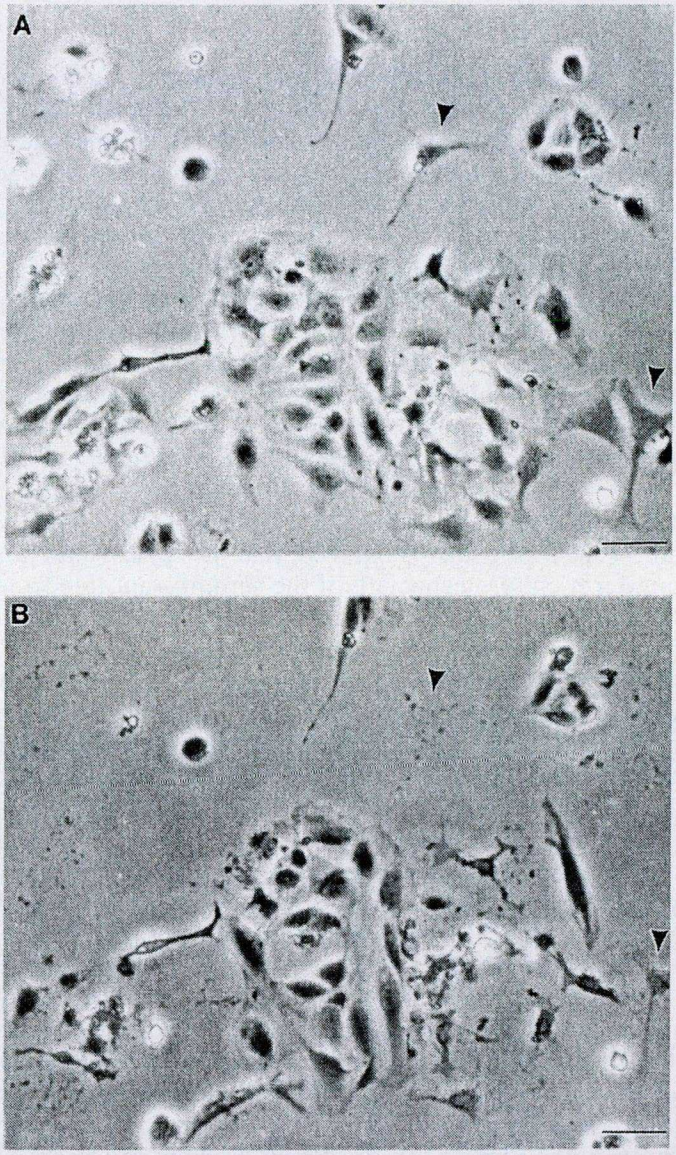

Fig. 3. Complement killing. (A) Before the cytolysis the contamining cells look as multipolar or round flat cells around or on the top of the endothelial cell clusters. (B) Immediately after the killing we can see only the shadows or the nucleus of the lysed pericytes and astrocytes, sometimes the whole cell disappears during the process (Fig. 3 B)

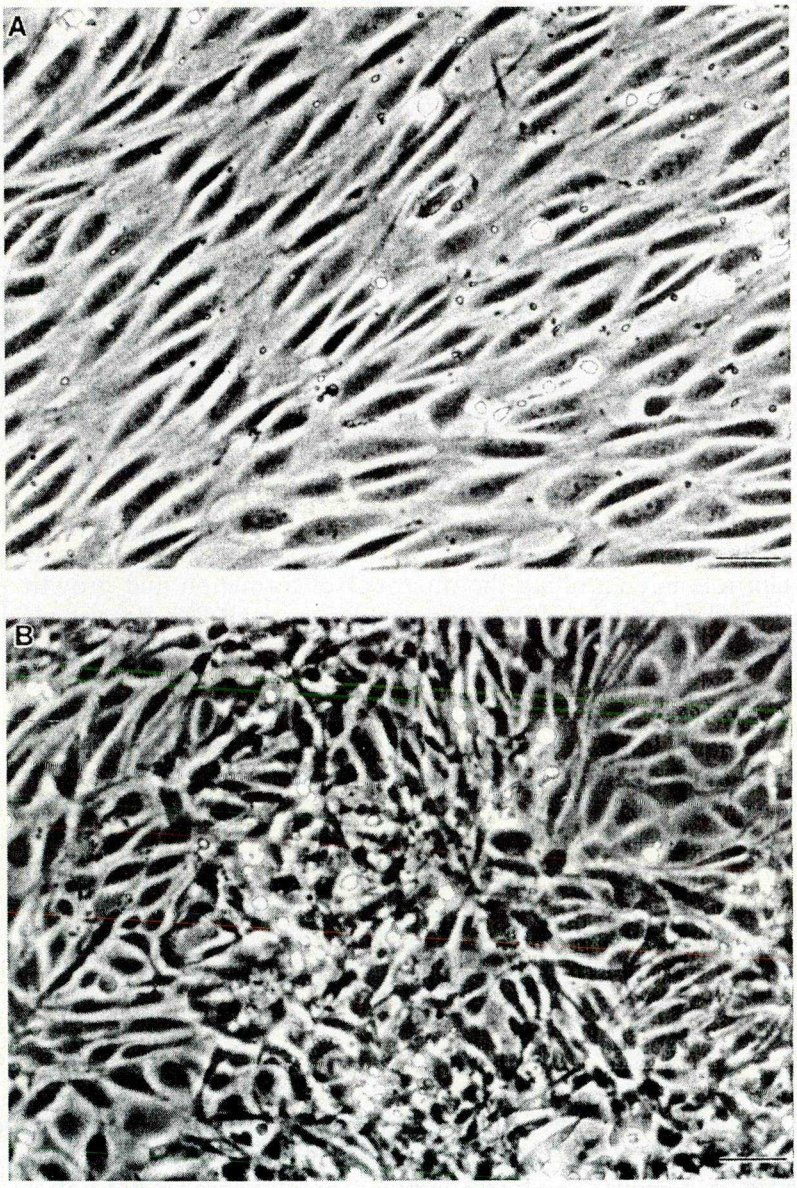

Fig. 4. (A) RCEC monolayer 4 days after the complement killing. Bar, 50 mm. (B) RCEC culture from the same preparation as A, but without any treatment at the same time-point. Contaminating cells growing on the surface of the monolayer can be observed. Bar, 50 mm

Several laboratories (Tontsch and Bauer, 1989, Dehouck et al., 1990, Nagy and Vastag, 1994) described the cloning of endothelial cells to remove the contaminating cell types and enrich the culture. Although this method is suitable for porcine, murine, bovine and human brain microvascular endothelial cells cannot be used for rat CECs. Primary RCECs cannot be subcultured because the subclones are not viable and are not able to form spontaneusly growing cell lines.

Reduction of the percentage of smooth muscle cells and fibroblasts in the cell culture can be attained by careful removal of large vessels, meninges and choroid plexus tissue during the dissection. These contaminanting cells, like pericytes, grow faster then the endothelial cells do and can overgrow the endothelium.

Angiogenesis

In some cases capillary-like structures may appear in the primary CEC cultures. Experimental evidence suggests that perivascular astrocytes play an important role in controlling the microvessel formation and growth in CNS (Laterra and Goldstein 1993). Ling and Stone (1988) described that the retinal vascularisation is coordinated by migrated astrocytes into the retina from optic nerve. The role of special cell and matrix components (collagens, laminin and fibronectin) interaction as well as some diffusible factors such as TGFb, FGF has been recently identified in modulating angiogenesis. In routine culture conditions the spindle-shaped endothelial cells form confluent monolayers in the first 7-10 days *in vitro*. When the endothelial cells differentiate into capillary-like structures they have real lumens, ramify and may develop a network in the culture dish (Robinson et al., 1990) There size, shape is similar to the capillaries seen *in vivo* and they secrete basement membrane proteins (Laterra and Goldstein, 1993). In some primary cultures of RCECs we could also observe capillary-like structures (Fig. 5 A, B).

Concluding remarks

The CECs lose the expression of differentiated functions during the culture period. Adaptation to cell culture environment might cause changes in certain properties of the cells. The tight junctions may be present but not assembled into complex structures in cell culture. On the 'filter model' - CEC monolayers grown on porous membranes - they inhibit the passage of

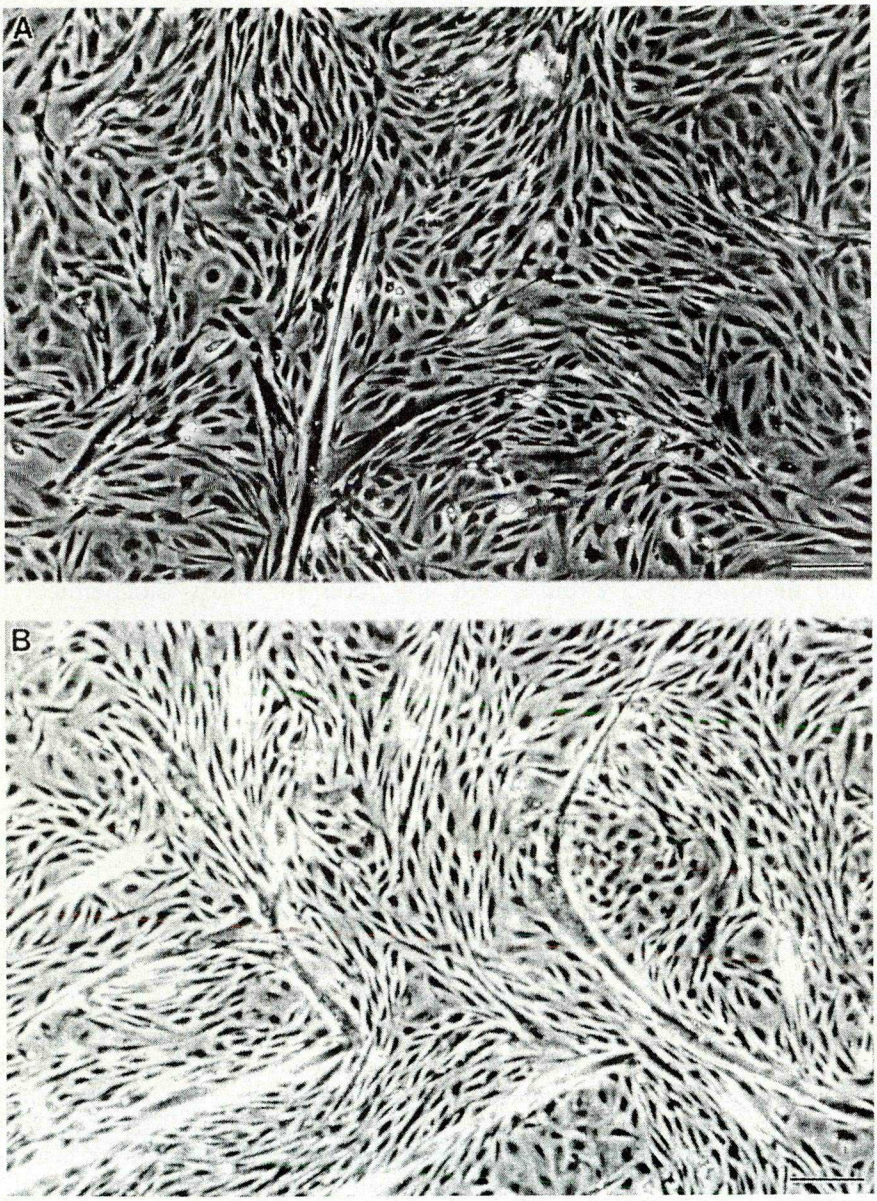

Fig. 5. (A, B) Capillary-like structures in 12 day-old primary RCEC cultures. Bar, 250 mm

proteins but do not restrict the movement of smaller molecules and ions (Bowman et al., 1983). The endocytotic capacity is elevated *in vitro* (Rupnick et al., 1988). It has been observed by Goetz et al., (1985) that g glutamyl transpeptidase and alkaline phosphatase activity may be lost with increasing time in culture. The reason of these changes might be the absence of associated cells (e.g., pericytes, astroglia, neurons). Many of the barrier features lost during culture can be reinduced by co-culture with astrocytes or by addition of astrocyte-conditioned medium (Joó, 1992) or neuronal membrane fraction (Tontsch and Bauer, 1991). Intracellular cAMP elevating drugs can further increase the tightness of the intercellular junctions and decrease the permeability of the CEC monolayers for small molecular weight substances (Rubin et al., 1991, Deli et al., 1995)

The yield of rat CECs is relatively low as compared to that obtained from bovine or porcine brain, but sufficient amounts of endothelial cells can be obtained for Western-blot or PCR techniques (Deli et al., 1993, Krizbai et al., 1995). Using the above described protocol, feeding the cells with special serum and performing the complement killing of contaminating cells, we succeded to obtain routinely the primary RCEC cultures in our laboratory that are more then 95% pure, and are good for most biochemical and molecular biological experiments

To study the structural, biochemical and physiological features of the BBB, the primary CEC culture remains one of the best objects.

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VI

HISTAMINE-INDUCED VASOGENIC BRAIN OEDEMA FORMATION IN NEWBORN PIGS: A ROLE FOR ENDOTHELIAL ACID PHOSPHATASE?

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1. INTRODUCTION

It is well-known that some specific morphological characteristics of cerebral endothelium, such as the presence of tight intercellular junctions, paucity of pinocytotic vesicles and the absence of fenestrations, contribute to the maintaining of the blood-brain barrier properties. Cerebral endothelial cells have relatively few lysosomes and the number of these compartments seems to parallel the presence of intracellular vesicles in different endothelia (1). On the other side, there is a growing evidence that endothelial lysosomes play a role in regulating entry of internalized macromolecules into the central nervous system despite limited expression and the significance of lysosomes is increased during pathological conditions (1,2). Specific lysosomal enzyme systems have been identified in cerebral endothelium, such as acid phosphatase, trimethaphosphatase, phosphoprotein phosphatase, β-galactosidase and aryl sulphatase (1). These enzymes may participate in the alteration of the blood-brain barrier permeability and the pathogenesis of brain oedema during different diseases. Particularly, increased acid phosphatase activity was shown to be involved in the enhancement of transendothelial transport of macromolecules through the blood-brain barrier in stroke-prone spontaneously hypertensive rats (2). We have recently published that histamine regulated the activity of acid phosphatase enzyme in cultured cerebral endothelial cells and suggested that increased activity might have effect on blood-brain barrier permeability (3).
The role of histamine in cerebral oedema formation is well established (4). In order to confirm the possible role of acid phosphatase enzyme in the development of vasogenic brain oedema, we investigated the effect of intracarotid histamine administration both on the acid phosphatase activity in isolated cortical microvessels and the blood-brain barrier permeability changes in the newborn pig.

2. MATERIALS AND METHODS

2.1. Animal Study

Newborn pigs of either sex (age: 4-8 h, weight: 1,130-1,600 g) were included in the study. The animals were given intramuscular pentobarbital anesthesia (30 mg/kg), then an umbilical artery was catheterized and physiological parameters were monitored (5). The right internal carotid artery of the animals was catheterized through the external branch and histamine was given in slow intraarterial injection (0.5 ml isotonic saline) in the following doses: 0 mol; 10^{-6} mol; $5*10^{-6}$ mol; 10^{-5} mol; $5*10^{-5}$ mol; and 10^{-4} mol (6 groups, n=4 in each). Then the catheter was removed and the external carotid artery was ligated. The experimental procedures were approved by the Ethical Committee on Animal Investigation, Albert Szent-Györgyi Medical University, Szeged, Hungary.

2.2. Acid Phosphatase Measurements

Acid phosphatase enzyme (ortho-phosphoric monoester hydrolase, EC 3.1.3.2.) activity was measured by the rate of hydrolysis of *p*-nitrophenylphosphate both in homogenized brain tissue and isolated cortical microvessels (6). Microvessels were prepared using the

method of Tontsch and Bauer (7). Different samples 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 with or without L-(+)tartrate (10 mM). After incubation, 45 μl of 1 M sodium hydroxide was added to stop the reaction and the absorbance was read at 405 nm by an ELISA reader (Labsystems Multiskan Biochromatic type 348). Enzyme activity, expressed in mU/mg protein, was determined from a calibration curve using increasing concentrations of purified acid phosphatase (AcP Lintrol, Sigma) of known activity, as it was previously described (3).

2.3. Blood-Brain Barrier Permeability Measurements

The development of vasogenic brain oedema, as the extravasation of two intravascular tracers: sodium fluorescein (mw: 376, Sigma) and Evan's blue labelled albumin (mw: 67,000, Sigma), was also measured 1 h after the intracarotid histamine injection. The animals were given a solution of both dyes (2%, 5 ml/kg) in an intravenous injection 30 min before the end of the experiments. Then blood samples were taken and pigs were perfused with 200 ml/kg isotonic saline. Serum as well as tissue samples from right cerebral cortex were homogenized in 3.0 ml of cold 7.5% trichloroacetic acid and centrifuged with 10,000 *g* for 10 min. The concentration of tracers was measured in supernatants by a Hitachi fluorimeter, the absorbance of Evan's blue albumin at 620 nm, while the emission of sodium fluorescein at 525 nm after excitation at 440 nm, and the extravasation was expressed as brain tissue concentration divided by serum concentration, as described earlier in detail (8).

2.4. Statistical Analysis

The values measured in different groups were compared using the Kruskal-Wallis one way analysis of variance on ranks followed by the Dunn's test. Correlation between doses of histamine and either acid phosphatase activity or BBB permeability was determined by linear regression analysis.

3. RESULTS

3.1. Total and Tartrate-Resistant Acid Phosphatase Enzyme Activity

Intracarotid histamine administration resulted in a dose-dependent increase in acid phosphatase activity in homogenized isolated microvessels, but not in cortical tissue samples. Total acid phosphatase activity was significantly ($P < 0.05$) higher in the cerebral capillary endothelium of 10^{-4} mol histamine-treated animals than that in vessels of control (0 mol histamine-treated) pigs. There was a linear correlation between the dose of histamine and total enzyme activity (Fig. 1A.). Each dose of histamine significantly ($P < 0.05$) increased the tartrate-resistant acid phosphatase activity in cortical microvessels of newborn pigs compared to that measured in control animals. Fig. 1B. shows that the dose of vasogenic amine injected and tartrate-resistant acid phosphatase activity are also correlated. We could detect neither a dose-dependent change in the enzyme activity between different groups nor any correlation between treatments and activities in brain tissue homogenates (Fig. 1C. and 1D.).

FIGURE 1. Total (A, C) and tartrate-resistant (B, D) acid phosphatase activity in homogenized cerebral microvessels (A,B) and cerebral cortex (C,D) 1 h after intracarotid administration of 0, 10^{-6}, $5*10^{-6}$, 10^{-5}, $5*10^{-5}$ and 10^{-4} mol of histamine in the newborn pig.

3.2. Blood-Brain Barrier Permeability

A significantly ($P < 0.05$) increased transport of both tracers was also found in cerebral cortex of newborn pigs 1 h after histamine injection in $5*10^{-5}$ mol and 10^{-4} mol histamine-treated groups compared to that in control group. Significant linear correlations were found between dose of histamine and extravasation of both sodium fluorescein (Fig. 2A.) and albumin (Fig. 2B.).

FIGURE 2. Permeability of sodium fluorescein (SF) (A) and Evan's blue labelled albumin

(EBA) (B) through the blood-brain barrier 1 h after different doses of intracarotically injected histamine in cerebral cortex of the newborn pig.

4. DISCUSSION

In the present study, intracarotid histamine administration resulted in an activation of acid phosphatase enzyme in cortical capillaries concomitantly with a dose-dependent increase in the blood-brain barrier permeability, but no change was found in the enzyme activity in the whole cortical tissue. Isolated microvessels contain endothelial cells in vast majority as well as a few percents of pericytes, while neurons and astrocytes are present only in trace. It seems probable for us that cerebral microvascular endothelial cells are responsible for the increased activity of acid phosphatase. Tagami and colleagues (3) described acid phosphatase positive transendothelial channels in cerebral capillary endothelium of stroke-prone rats, while interendothelial junctions were well-preserved. They suggested that endothelial lysosomes fusing with these structures might play an important role in the transport of macromolecules (3). We have recently published that histamine administration induced a selective albumin permeation without affecting the transport of tight junction markers through monolayers of bovine cerebral endothelial cells co-cultured with rat astrocytes (9). Previous studies revealed that both H_2-receptor-dependent adenylate cyclase-mediated and H_1-receptor-dependent phosphoinositol-mediated mechanisms participate in the histamine-induced brain oedema formation (4). H_2-receptor antagonists could reduce the tartrate-resistant, while H_1-receptor antagonists decreased the tartrate-sensitive increase in acid phosphatase activity after histamine treatment in an immortalized rat brain endothelial cell line (3). The role of different isoforms of acid phosphatase enzyme in the process of histamine-induced brain oedema remains to be elucidated. Our present study strengthens the views that acid phosphatase enzyme system, similarly to other lysosomal enzymes present in cerebral endothelium, may play an important role in the regulation of macromolecular transport during physiological and pathological conditions.

5. ACKNOWLEDGEMENTS

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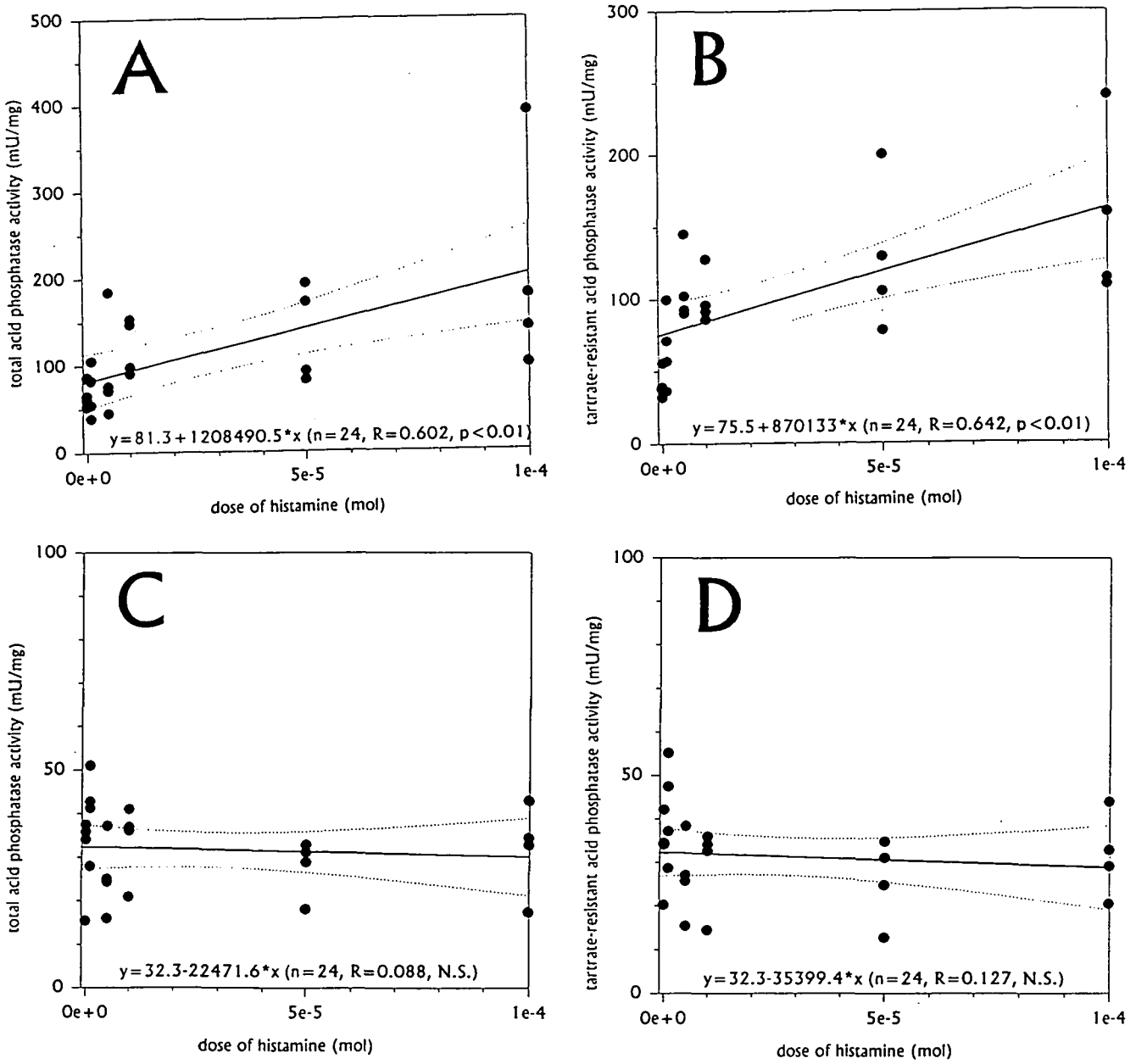
A
total acid phosphatase activity (mU/mg)
500
400
300
200
100
0
y=81.3+1208490.5*x (n=24, R=0.602, p<0.01)
0e+0
5e-5
1e-4
dose of histamine (mol)
B
tartrate-resistant acid phosphatase activity (mU/mg)
300
200
100
0
y=75.5+870133*x (n=24, R=0.642, p<0.01)
0e+0
5e-5
1e-4
dose of histamine (mol)
C
total acid phosphatase activity (mU/mg)
100
50
0
y=32.3-22471.6*x (n=24, R=0.088, N.S.)
0e+0
5e-5
1e-4
dose of histamine (mol)
D
tartrate-resistant acid phosphatase activity (mU/mg)
100
50
0
y=32.3-35399.4*x (n=24, R=0.127, N.S.)
0e+0
5e-5
1e-4
dose of histamine (mol)

Fig. 1.

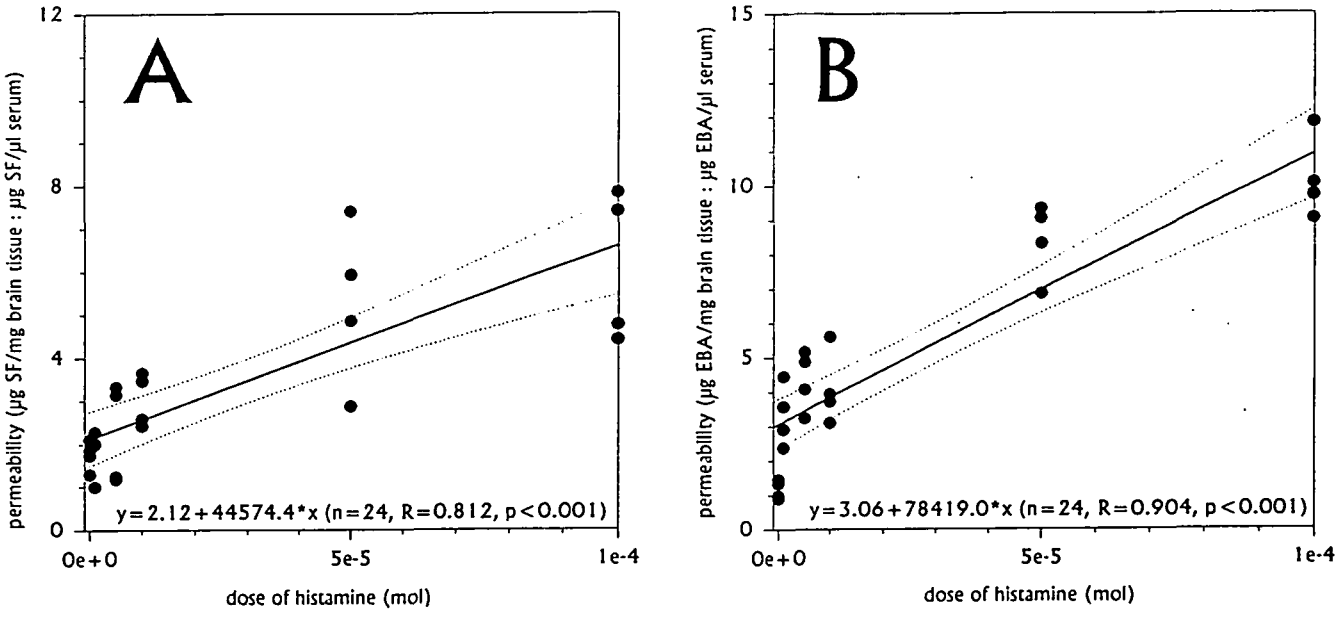
A
B
permeability (µg SF/mg brain tissue : µg SF/µl serum)
permeability (µg EBA/mg brain tissue : µg EBA/µl serum)
y=2.12+44574.4*x (n=24, R=0.812, p<0.001)
y=3.06+78419.0*x (n=24, R=0.904, p<0.001)
dose of histamine (mol)
dose of histamine (mol)
0
4
8
12
0
5
10
15
0e+0
5e-5
1e-4
0e+0
5e-5
1e-4

Fig. 2.

VII

CEREBRAL ISCHEMIA-REPERFUSION INDUCED VASOGENIC BRAIN EDEMA FORMATION IN RATS: EFFECT OF AN INTRACELLULAR HISTAMINE RECEPTOR ANTAGONIST

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SUMMARY:

Resuscitation in pediatric emergency and some neurosurgical interventions may result in ischemia-reperfusion induced cerebral injuries. Histamine is one of the well established mediators of cerebral swelling and H1- and H2-receptor antagonists could prevent the development of ischemic brain edema. In the present study, time-dependent changes in the blood-brain barrier (BBB) permeability were investigated in the cerebral cortex of male Wistar rats 1, 2, 4, 8, and 16 h after the beginning of postischemic reperfusion. Cerebral ischemia-reperfusion evoked by the 4-vessel occlusion model resulted in significant ($p < 0.05$) elevations in BBB permeability for albumin, but not for sodium fluorescein. Pretreatment with a new intracellular histamine receptor antagonist could not prevent ischemic brain edema formation in that model. We conclude that experimental studies could help us to reveal the therapeutical role of histamine receptor antagonists during ischemic brain edema.

KEY WORDS:

Brain edema, Cerebral ischemia-reperfusion, Histamine, Intracellular histamine receptor

INTRODUCTION

Cerebral ischemia-reperfusion occurs during resuscitation, stroke, brain trauma and may develop during certain neurosurgical interventions in pediatric practice. Better understanding of the postischemic brain edema formation would help the prevention and treatment of this life-threatening complication. Rat model of 4-vessel-occlusion (vertebral and common carotid arteries on both sides) described by Pulsinelli and Brierley (11) is appropriate for investigating ischemia-reperfusion in experimental conditions and for studying the underlying pathophysiological processes.

Histamine is one of the well established mediators of brain edema formation (5,7). It is known that there are three cerebral histamine pools (in neurons, in perivascular mast cells, and in cerebral endothelium) and histamine released after physiological and pathological stimuli may also play a role in neuronal transmission, and regulation of cerebral blood flow (5,12). Cerebral endothelial cells possess both H1- and H2-histamine receptors and have been shown to take up and release histamine in different pathological conditions (6,8). We have recently confirmed that intracarotid histamine injection results in vasogenic brain edema formation in newborn pigs (9). Joó and his coworkers described the beneficial effects of H1- and H2-antagonists in the prevention of ischemic brain edema formation (7). However, there is no data available about the occurrence of H3-receptor and a novel intracellular affinity site for histamine (Hic) on brain capillaries. N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine HCl (DPPE) is a well-known compound that antagonizes histamine binding at the Hic site in the micromolar range (2). Several studies suggested that, in higher doses, DPPE might have acute neurological side-effects in humans (2), and potentiated drug-induced seizures,

and produced abnormal behaviour in mice (13).

The aim of this study was to reveal the time-dependent changes in the blood-brain barrier (BBB) permeability after cerebral ischemia-reperfusion in rats. On the other hand, the possible effect of DPPE on ischemic brain edema formation was also investigated.

MATERIALS AND METHODS

Animal Study

Cerebral ischemia was induced according to the 4-vessel occlusion model of Pulsinelli and Brierley (11) (**Figure 1**). Male Wistar rats were anesthesized 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 was identified on both sides and the neurovascular branch was coagulated by a bipolar diathermical forceps. During this part of the procedure we used a Zeiss operating microscope. The wound was closed by 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.* (14) was implanted around the vessels (**Figure 2A**). 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 (**Figure 2B**). The occlusion can be released with the help of the thicker wire and the reperfusion of vessels can begin (**Figure 2C**). 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 ischemia 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 ischemic animals were given intravenous injection of 5 mg/kg DPPE diluted in 0.5 ml isotonic saline and permeability changes were determined 2, 4 and 8 h after the injection (n=6 in each group). Animal experiments were performed according to the NIH Guidelines and accepted by the local ethical committee.

Blood-Brain Barrier Permeability Measurements

The development of vasogenic brain edema, as the extravasation of two intravascular tracers: sodium fluorescein (SF, mw: 376, Sigma) and Evan's blue labelled albumin (EBA, mw: 67,000, Sigma), was measured as it was described in details (1). The animals were given a solution of both dyes (2%, 5 ml/kg) in an intravenous injection 30 min before the end of the experiments. Then blood samples were taken and rats were perfused with 200 ml/kg isotonic saline. Serum as well as tissue samples from cerebral cortex were homogenized in 3.0 ml of cold 15% trichloroacetic acid and centrifuged with 10,000 *g* for 10 min. The concentration of tracers was measured in supernatants by a Hitachi fluorimeter, the absorbance of Evan's blue/albumin at 620 nm, while the emission of sodium fluorescein at 525 nm after excitation at 440 nm, and the extravasation was expressed as brain tissue concentration divided by serum concentration.

Statistical Analysis

The values measured in groups with different duration of reperfusion were compared using the Kruskal-Wallis one way analysis of variance on ranks followed by multiple comparison by the Student-Newman-Keuls test. Comparisons between DPPE-treated and non-treated ischemic rats were performed by the Mann-Whitney rank sum test. $P < 0.05$ values were regarded as significant differences.

RESULTS

Ischemia-reperfusion did not modified significantly the BBB permeability for SF, a low molecular weight tracer in the cortex of rats (**Figure 3A**). Albumin extravasation from cerebral vessels was significantly elevated ($P < 0.05$) since 2 h reperfusion compared to control, the tendency was increasing with a peak at 8 h (**Figure 3B**). A single bolus of DPPE given to ischemic rats at the beginning of the reperfusion period could not change the SF and albumin permeability at any time-point detected (**Figure 4**).

DISCUSSION

According to our data, cerebral ischemia-reperfusion resulted in vasogenic brain edema formation in rats. Interestingly, the transport of albumin, a high molecular weight intravascular tracer, was increased several-fold during reperfusion period, while BBB permeability for the low molecular weight tracer did not change significantly. An explanation

for this paradoxical finding is that increased cerebrovascular permeability may occur through two main mechanisms: (i) paracellularly by the means of the opening of tight interendothelial junctions, or (ii) transcellularly through vesicular structures or temporary transendothelial channels, and serum proteins cross the BBB by the second mechanism. Similar changes were found after intracarotid histamine administration: high pinocytotic activity was seen in adult rats (4) and increased permeability for albumin, but not for sodium fluorescein, was detected in newborn pigs (9). In a recent *in vitro* study, histamine increased the transcellular passage of albumin, but the BBB permeability for sucrose and inulin, known as markers of paracellular route, was unchanged (3).

It is well known, that histamine plays an important physiological role in the central nervous system (12). The concentration of histamine in different brain compartments is elevated during cerebral injuries (8,10). It was suggested in previous animal studies that both H1 and H2 receptor antagonists were beneficial in the prevention of ischemic brain edema (5,7). In contrast to these antagonists, Hic receptor antagonist DPPE could not prevent the development of postischemic vasogenic brain edema in the present animal model. However, further studies are warranted to reveal the role of intracellular histamine receptor in the regulation of BBB permeability.

Acknowledgements:

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LEGEND TO FIGURES

Figure 1. Schematic drawing of (**A**) the vertebral arteries with the foramens; and (**B**) the device with threads placed around the common carotid arteries

Figure 2. Photos of the occluding device: (**A**) around the common carotid arteries; (**B**) the moment of the occlusion; and (**C**) the position after the release during reperfusion.

Figure 3. Blood-brain barrier permeability for sodium fluorescein (**A**) and Evan's blue-albumin (**B**) in the cerebral cortex of rats before cerebral ischemia and after 1, 2, 4, 8, or 16 h reperfusion period. Significant ($P < 0.05$) differences are seen compared to values measured: [a]in the control group at 0 h; as well as [b]2 h, and [c]4 h after the beginning of reperfusion in postischemic animals.

Figure 4. Blood-brain barrier permeability for sodium fluorescein (**A**) and Evan's blue-albumin (**B**) in the cerebral cortex of postischemic rats 2, 4, and 8 h after the beginning of reperfusion. Experimental groups were as follows: rats underwent ischemia-reperfusion (I-R); and postischemic rats receiving DPPE (I-R + DPPE), no significant difference was found.

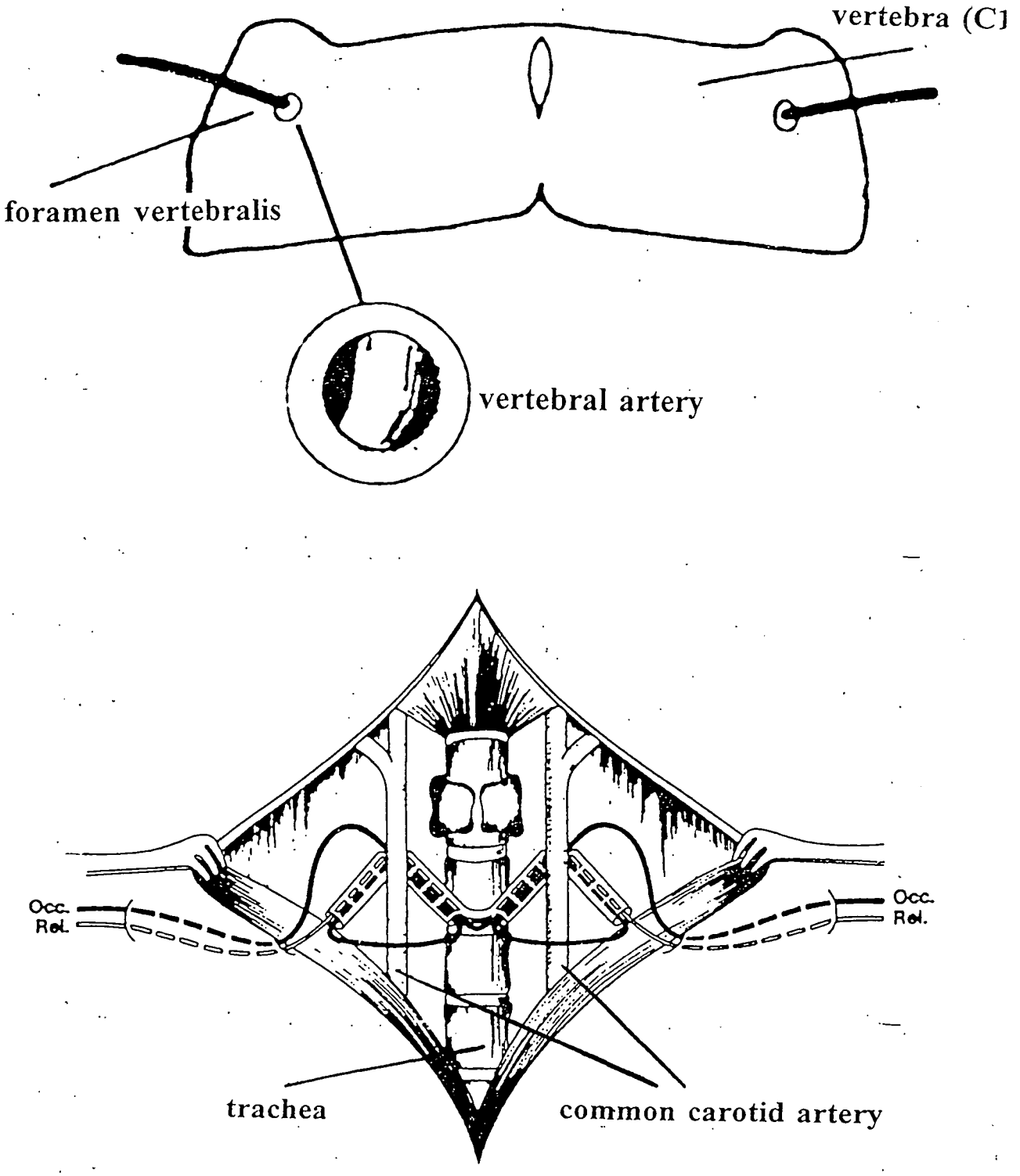
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Fig. 1.

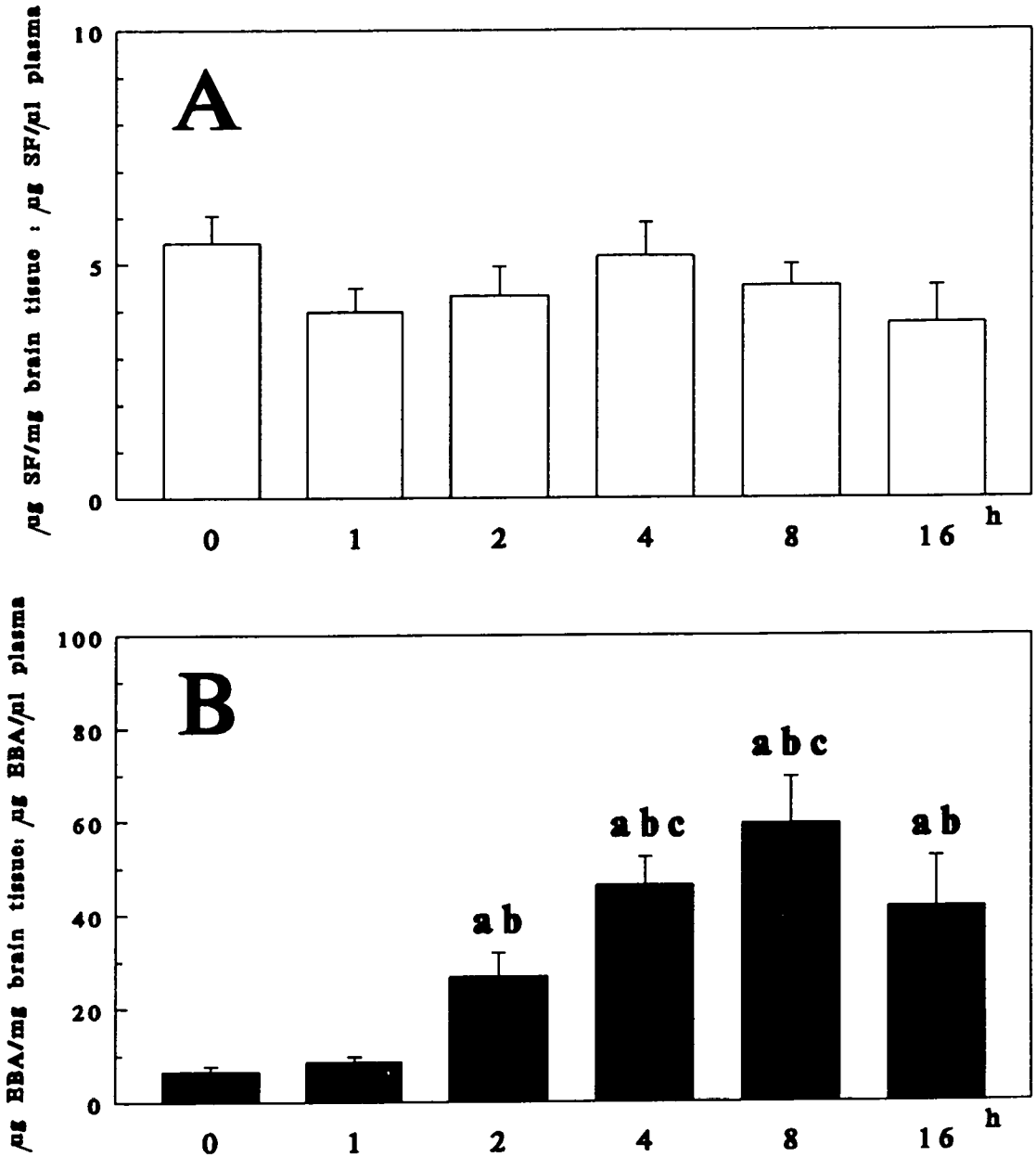
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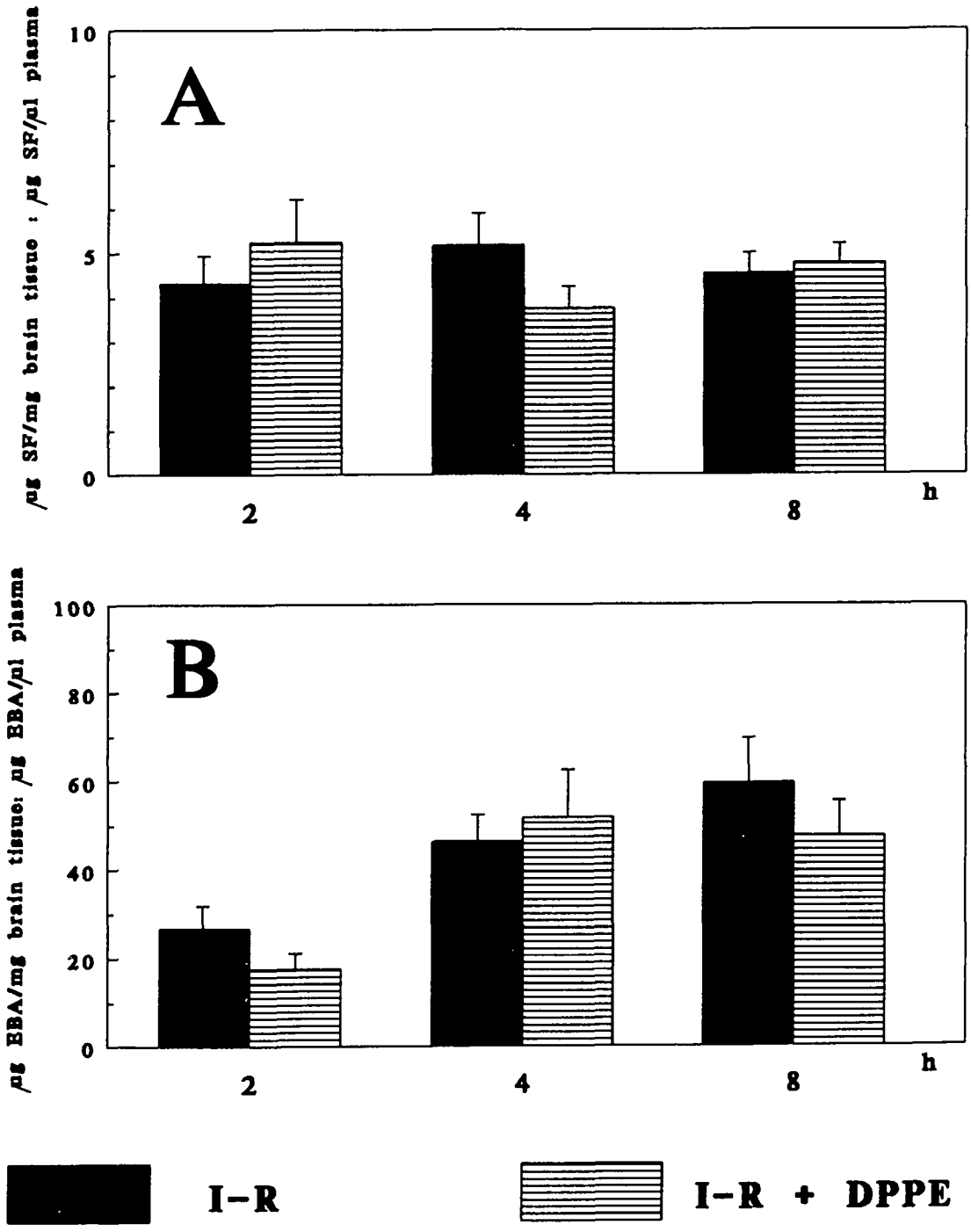
Fig. 3.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I-R
I-R + DPPE

Fig. 4.

VIII

VASOACTIVE SUBSTANCES PRODUCED BY CULTURED RAT BRAIN ENDOTHELIAL CELLS: A COMPARISON OF PRIMARY CELLS AND IMMORTALIZED CELL LINES

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Summary

The vasoactive substance synthesized by primary cultures of rat brain endothelial cells (RBECs) was investigated and compared to that of two, immortalized cell lines, RBE4 and GP8. The vasoactivity of endothelium-derived substances was measured on isolated canine coronary artery. The vascular tone was significantly decreased by both primary and GP8, but not by RBE4 cells. Indomethacin pretreatment of primary and GP8 cells turned vasorelaxation into contraction while N^{ω}-nitro-L-arginine pretreatment decreased the vasorelaxation induced by primary, but not by GP8 cells. The eicosanoid production was determined after incubation with [^{14}C]-arachidonic acid. The predominant vasoactive eicosanoid was prostaglandin E_2 both in primary and GP8 cells. RBE4 cells synthetized mainly prostaglandin $F_{2\alpha}$ and thromboxane B_2 and significantly less prostaglandin E_2 compared to either primary or GP8 cells. The capacity of cerebral endothelium to regulate vascular tone by production of dilator and constrictor substances can be preserved under certain circumstances in immortalized cell lines.

Key Words: blood-brain barrier, prostaglandins, endothelial cell, vasorelaxation

Cerebral endothelial cells form the blood-brain barrier (BBB) which is important in maintaining the homeostasis of central nervous system (CNS) by controlling the traffic of molecules and cells between blood and brain. The brain microvascular endothelium, like other endothelial cells, is also capable of producing several substances mediating endothelium-dependent vasorelaxation and contraction (1). The released vasoactive agents can modulate the endothelial second messenger systems, regulate the BBB permeability (2) and play a key role in the regulation of the vascular tone of cerebral vessels.

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An important molecule in the determination of vascular tone synthetized by endothelial cells is the endothelium-derived relaxing factor (EDRF), which has been identified as nitric oxide (NO), or a closely related compound (3). In different cell types, a variety of stimuli are known to activate phospholipase A_2 which leads to the release of free fatty acids, including arachidonic acid (AA), the precursor of the family of eicosanoids. Prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) are known to mediate vascular relaxation, while tromboxane A_2 (TxA_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are implicated in the vasoconstriction in different vessels (4, 5, 6). Previous studies revealed the eicosanoid profile of isolated cerebral microvessels (7, 8, 9) and it was also suggested that pure cultures of rodent cerebrovascular endothelial cells produced predominantly the vasodilatory PGE_2 and PGI_2 (10, 11). The eicosanoids released in cerebral microvessels play also a role in the changes of BBB permeability (12), cerebral edema formation, and inflammatory reactions in the CNS (13). Significant differences in the type, amount, and ratio of vasoactive eicosanoids released under basal condition or upon chemical or physical stimulation have been demonstrated, depending on the localization of the endothelium within the circulatory system.

In the present experiment we compared the production of vasoactive agents of three different rat cerebral endothelial cell types: primary RBECs and two immortalized rat brain endothelial cell lines, RBE4 and GP8. These cell lines display a non-transformed, well differentiated endothelial phenotype and express several structural and pharmacological characteristics of the BBB (14, 15, 16). A bioassay system based on the effect of cultured endothelial cell suspensions on isolated coronary artery from dogs was constructed in order to detect the vasoactive action of endothelium-derived substances. The effect of the cyclooxygenase inhibitor, indomethacin, and the NO synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine (NOLA), on the vasoactive substance producing activity was determined, too. The profile of eicosanoid synthesis was also investigated.

Methods

Materials used in the experiments were of the following origin: collagenase type II, bovine serum albumin (BSA), Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12), fetal calf serum, basic fibroblast growth factor (bFGF), heparin, monoclonal anti-mouse Thy 1.1 antibody, rabbit complement serum (HLA-ABC), prostaglandin $F_{2\alpha}$, indomethacin, acetylcholine chloride, N^{ω}-nitro-L-arginine, arachidonic acid (grade I), 12-L-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) standards, Medium 199, (Sigma, Germany), collagenase-dispase (Boehringer, Germany), Percoll (Pharmacia, Sweden), L-glutamine, penicillin-streptomycin, geneticine (Gibco, UK), plasma derived serum (PDS) (First Link LTD, UK), 1-[^{14}C]-arachidonic acid (spec. act. 2035 MBq/mM) (Amersham, UK), silica gel thin-layer plates (0.25 mm) (Merck AG, Germany), PGE_2, PGD_2, TxA_2, TxB_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$,(Upjohn Co, USA).

Primary RBEC culture.
Primary cultures of cerebral endothelial cells were prepared and characterized as previously described (17). Cerebral cortex from two week old CFY rats were finely minced, than incubated in collagenase-dispase solution (270 U/ml collagenase, 1 mg/ml dispase, DMEM-F12 containing antibiotics) at 37 °C for 1.5 h in shaking waterbath. After this incubation 20% BSA/DMEM-F12 (2 ml/brain) was added to the homogenate and centrifuged at 1000 *g* for 15 min. The pellet containing the microvessels was washed once in DMEM-F12 then further digested for an other 1 h with the above mentioned enzymes at 37 °C. The cell suspension was carefully layered on a continuous 33% Percoll gradient and centrifuged at 1000 *g* for 10 min. The band of the endothelial cell clusters was aspirated, washed twice in DMEM-F12. The cells were seeded onto

rat tail collagen-coated 35 mm plastic dishes in culture medium (DMEM-F12 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, 2 mM glutamine, 20 % heat inactivated PDS and from the second day 1 ng/ml bFGF). Primary cultures at DIV2 were treated with monoclonal anti-mouse Thy 1.1 antibody followed by rabbit complement serum to eliminate the few contaminating non-endothelial cells, mainly pericytes, by selective cytolysis (18). The cultures became confluent on DIV7 and consisted of more than 98 % of endothelial cells, verified by positive Factor VIII-related antigen, negative GFAP and neurofilament immunohistochemistry.

Cell lines.
RBE4 cells are derived from rat brain microvessel endothelium immortalized with the plasmid pE1A-neo and characterized in respect to BBB properties (14, 15). The cells were passaged twice a week in DMEM/F12, supplemented with 10% heat-inactivated fetal calf serum, 2mM glutamine, 1 ng/ml bFGF and 300 μg/ml G418 onto rat tail collagen coated dishes and used between passages 30 and 50. GP8 is a temperature sensitive SV40 large T immortalized rat brain capillary cell line described by Greenwood et al. (16). These cells, used between passages 10 and 20, were cultured in 20% PDS, 2mM glutamine, 1ng/ml bFGF and 200 μg/ml G418 in DMEM/F12.

Bioassay for the vasoactive metabolites of brain endothelial cells.
Mongrel dogs of either sex weighing 9-15 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.) and heparinized (1000 I.U./kg i.v.). The heart was excised and placed into a Krebs-Henseleit (KH) solution of the following composition (in mM): NaCl 120, KCl 4.2, $CaCl_2$ 1.5, $NaHCO_3$ 20, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 11. Rings (1.1-1.9 mm o.d., 5 mm widths) from the circumflex branches of left coronary artery were isolated. Endothelium was removed by gently rubbing the endothelial surface with a stainless steel wire covered with a cotton swab. Rings were mounted in water-jacketed baths containing 2 ml of KH solution bubbled with 95 % O_2 and 5% CO_2 gas mixture at 37 °C. The isometric tension was recorded with a force-displacement transducer (Hugo Sachs Elektronic, Type F30, Germany). Rings were stretched up to 10 mN and allowed to stabilize for 45 min. This tension was readjusted to 10 mN during equilibration. The arterial rings were exposed to 25 μM $PGF_{2\alpha}$ and at the maximal amplitude of contraction 1 μM acetylcholine was applied. Only those arterial preparations were used for the experiments that responded with contraction after addition of the endothelium dependent vasodilator, acetylcholine. This protocol served as an evidence for functionally deendothelialized arterial preparations. Confluent monolayers of cultured RBECs were suspended in KH solution resulting in 5×10^6 cells/ml and 5×10^5 cells/ml. The cell suspensions were divided into two parts: one was treated with solvents (control), the other either with 100 μM NOLA or with 10 μM indomethacin for 30 min at 37 °C. Final concentration of indomethacin at the highest cell number in the organ bath was 0,3 μM. When $PGF_{2\alpha}$ induced contraction of coronary rings had reached the steady-state amplitude the control or treated endothelial suspensions were added cumulatively. Animal experiments were performed according to the NIH Guidelines and approved by the ethical committee of the Biological Research Center.

Assay of prostaglandin synthesis in RBEC.
Confluent cultures of primary, RBE4 or GP8 cells in petri dishes (35 mm diameter, approx. 3×10^5 cells/dish) were incubated at 37 °C with the tracer substrate, 1-[^{14}C]-AA (0.172 pmol, 3.7 kBq) in 1 ml serum free DMEM-F12. Thirty minutes later the incubation medium of the cell culture was removed and acidified to pH 3 with formic acid. According to our preliminary experiments a period of 30 min was appropriate for labelling *in vitro* the rat brain capillary endothelial cells. The arachidonate metabolites were immediately extracted with ethyl acetate

(2×3 ml) from the samples, and the organic phases were pooled and evaporated to dryness under nitrogen. The residues were reconstituted in 150 µl ethyl acetate and quantitatively applied to silica gel thin-layer plates. The plates were developed to a distance of 15 cm in an organic phase of ethyl acetate : acetic acid : 2,2,4-trimethylpentane : water (110:20:30:100) by means of overpressure thin-layer chromatography. The radiolabeled products of arachidonic acid were identified with unlabeled authentic standards, which were detected with anisaldehyde reagent. Each 3 mm band of the chromatograms was scraped off and the radioactivity was determined in a Packard Tri-Carb 2100TR liquid scintillation analyser, using 5 ml toluene containing 0.44% w/v 2,5-diphenyloxazole, 0.02% w/v 1,4-di-[2-(5-phenyl)-oxazole]benzene and 10% v/v ethanol.

Statistical analysis.
Enhancement or reduction of arterial tone was calculated as percent of maximum increase (+) or decrease (-) of contractile force compared to the pre-drug values. Results are expressed as mean ± S.E.M. and n refers to the number of experiments. One way analysis of variance (ANOVA) followed by Newman-Keuls multiple range test or followed by the Tukey-B multiple comparison *post hoc* test was used to determine the significance of differences between the corresponding mean values. $P<0.05$ was taken as statistically significant.

Results

Bioassay.
In the first part of the experiments the vasoactive action of endothelial cell culture suspensions was determined. Fig.1. demonstrates the effect of primary RBEC on the tone of isolated coronary artery precontracted with $PGF_{2\alpha}$. The primary cells decreased the vascular tone which correlated with the cell number and the maximal relaxation was 32.5 % after administration of 2×10^5 cells/ml. RBE4 cells caused a small but not significant contraction (Fig.2.) Enhancement of arterial tone by these cells showed no cell-response relationship between 0.2-6.2×10^5 cells/ml with an average of 15-18 % contraction. GP8 cells, like primary cells decreased markedly the tone of precontracted arterial rings (Fig.3.) which depended on the number of cells with a maximum of 33.2 ±8.2% (n=7) decrease of $PGF_{2\alpha}$ induced tone.

In the second part of the bioassay experiments the possible involvement of NO as an endothelium dependent vasodilator in the changes of coronary tone was investigated. After 30 min incubation of primary endothelial cells with the inhibitor of nitric oxide synthesis, NOLA, the RBEC induced vasorelaxation was decreased (Fig.1.). On the contrary no significant change in the vasoactivity of the two immortalized cell lines was achieved by NOLA (Fig.2, 3.). This suggests the absence or the very low level of basal NO production by constitutive endothelial NOS enzyme in the cell lines. .

In the third series of bioassay experiments the cells were pretreated with the cyclooxygenase enzyme inhibitor, indomethacin, for assessing the role of PGs in the vasoactive potency of cerebral microvascular endothelium. The largest concentration of indomethacin (0.3 µM) by itself caused a small but significant increase of the acceptor vascular tone (from zero to +9.6 ± 1.4%, n=6, $p<0.05$). This increase reflects the known basal release of vasodilator prostaglandins from the smooth muscle of the acceptor coronary artery (19). The small enhancement of tone by indomethacin was deduced from the effect of the endothelial suspension in Figure 2. Indomethacin did not influence the contraction induced by RBE4 cells (Fig.2.) but turned the primary RBEC (Fig.1.) and GP8 cells (Fig.3.) induced vasorelaxation into contraction. These results suggest a higher activity of cyclooxygenase enzyme in primary RBEC and GP8 cells.

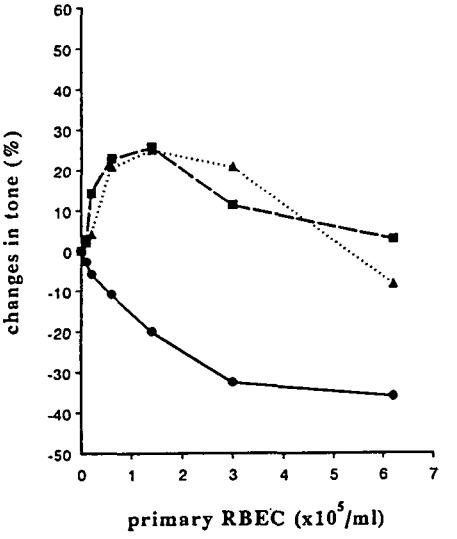

Fig. 1.
Effect of primary RBECs on the tone of endothelium denuded coronary artery isolated from dogs after the steady-state contraction by $PGF_{2\alpha}$. In control condition (——) primary cells decreased $PGF_{2\alpha}$ induced tone that was dependent on the number of cells. Pretreatment of the cells with 100 μM NOLA (-----) or 10 μM indomethacin (·····) for 30 min turned the vasorelaxation into contraction. (n=1-3)

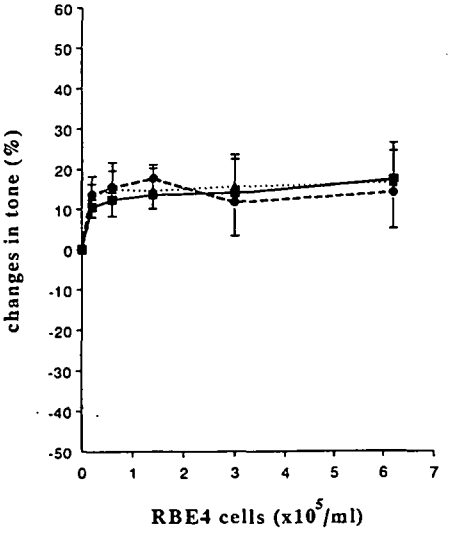

Fig. 2.
Effect of RBE4 cell line on the tone of endothelium denuded coronary artery isolated from dogs after the steady-state contraction by $PGF_{2\alpha}$. RBE4 cells (——) caused a small contraction of the coronary artery which was cell number independent. A 30 min preincubation neither with 100 μM NOLA (---) nor with 10 μM indomethacin (·····) induced any change in the tone of coronary artery. Each value represents the mean ± S.E.M. (n=6)

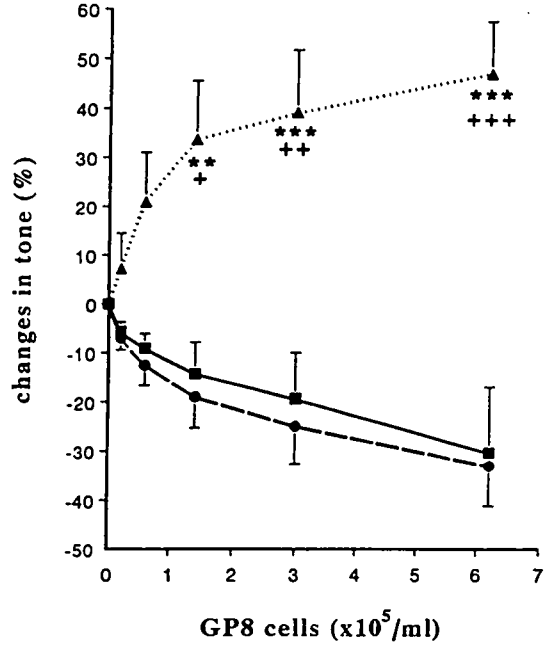

Fig. 3.

Effect of GP8 cell line on the tone of endothelium denuded coronary artery isolated from dogs. In control condition (——) GP8 cells decreased $PGF_{2\alpha}$ induced tone that was dependent on the number of cells in the organ bath. After 30 min pretreatment of endothelial cells with 100 μM NOLA (-----) the vascular tone was not altered significantly as compared to the control. The vasorelaxation by GP8 cells was turned to contraction after inhibition of endothelial cyclooxygenase enzyme by 10 μM indomethacin (·····). Each value represents the mean ± S.E.M. (n=7). Significant differences between the control and NOLA treated cells are: *$p<0.05$, **$p<0.01$, ***$p<0.001$; between the NOLA and indometacin treated cells are: $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$.

Production of AA metabolites in cerebral endothelial cells.

The cyclooxygenase pathway of arachidonate cascade, 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2), PGD_2, PGE_2, $PGF_{2\alpha}$, TxB_2 (the stable metabolite of TxA_2) and 12-HHT were identified in the incubation medium of three different types of nonstimulated RBEC cultures. The profile of eicosanoid production by primary RBEC, RBE4 and GP8 cells is shown in Table I.

Each type of RBECs was found to be capable of synthesizing prostaglandins. Significant ($p < 0.05$) differences in total cyclooxygenase metabolite release were found, the synthesis rate in increasing order was as follows: RBE4 < primary cells < GP8 cell line. The predominant vasodilator substance was PGE_2 both in primary cells and GP8 cell line, 28.51 and 42.56 % of total eicosanoid production, respectively. PGE_2 release (9.38 % of total), from RBE4 cells was significantly ($p < 0.05$) lower than that from the other cells, and the main metabolites formed by RBE4 cells were $PGF_{2\alpha}$ and TxB_2, (25.43 and 24.39 %), respectively. While primary and GP8 cells released more than three times more PGE_2 than PGI_2, the ratio of PGE_2:PGI_2 produced by RBE4 cell line was approximately 1:2. The percentage distribution of TxB_2 and 12-HHT was in similar range in all three cell types.

TABLE I.

The Profile of Eicosanoid Production by Primary RBEC, RBE4 and GP8 Cells.

	primary RBEC		RBE4		GP8	
	in DPM/ 3×10^5 cells/30 min and in percent of the total production					
	DPM	%	DPM	%	DPM	%
6-keto-$PGF_{1\alpha}$	427 ± 67	8,1	481 ± 12	16,6	635 ± 17	8,4
PGE_2	1500 ± 17	28,5	271 ± 41‡	9,3	3186 ± 41†*	42,5
PGD_2	734 ± 15	13,9	368 ± 16	12,7	408 ± 73	5,4
$PGF_{2\alpha}$	626 ± 84	11,9	735 ± 74	25,4	711 ± 15	9,5
TxB_2	1166 ± 28	22,1	705 ± 74	24,3	1476 ± 14*	19,7
12-HHT	889 ± 13	16,9	338 ± 12	11,7	1086 ± 31	14,5
TOTAL	5261 ± 60	100	2890 ± 33‡	100	7486 ± 91†*	100

The prostanoid production was measured from the incubation solution (1ml DMEM/F-12) of 3×10^5 cells at 37 °C 30 min after the administration of 1-[^{14}C]-arachidonic acid (0.172 pmol, 3.7 kBq). Each value represents the mean ± S.E.M. (n=6). Significant difference between primary RBEC and RBE4 is indicated by ‡ ($p<0.05$), between primary RBEC and GP8 by † ($p<0.05$) and between RBE4 and GP8 by * ($p<0.05$). Abbreviations: 6-keto-$PGF_{1\alpha}$ - 6-keto-prostaglandin $F_{1\alpha}$ (the stable metabolite of PGI_2), PGE_2 - prostaglandin E_2, PGD_2 - prostaglandin D_2, $PGF_{2\alpha}$ - prostaglandin $F_{2\alpha}$, TxB_2 - thromboxane B_2 (the stable metabolite of TxA_2), 12-HHT - 12-L-hydroxy-5,8,10-heptadecatrienoic acid, TOTAL - amount of all prostanoids measured.

Discussion

Primary cultures of cerebral microvessels, as an in vitro BBB model system, are widely used for morphological, functional and pharmacological studies (for review see Joó 20, 21). Immortalized cell lines present several advantages: the time consuming and expensive procedure of preparing primary cultures can be avoided, and it is easy to produce large amount of cells for genetic, immunological or biochemical experiments. Besides using in basic research a new area of application of cerebral endothelial cell lines can be gene therapy. These cells are good candidates for gene delivery to the nervous system because of their normal localization at the blood-parenchyma interface and their ability to proliferate *in vivo* and *in vitro*. It has been recently reported that RBE4 cells modified to express β-galactosidase reporter can be stably engrafted to growing gliomas in rats (22). Altered RBE4 cells expressing both β-galactosidase and human fibroblast growth factor-1 gene survived following implantation to neonatal and adult rat brain (23).

The in vitro vasoactive properties of three types of RBECs, such as primary cells as well as two immortalized cell lines, RBE4 and GP8, were investigated. In order to analyse the composition of the paracrin signal derived from these cells in the absence of chemical stimulation, a bioassay system was constructed. The effect of vasoactive substances released by RBECs was investigated on the isometric tone of an endothelium-denuded canine coronary arterial in vitro preparation. The smooth muscle of this blood vessel is known to be very sensitive to PGs (24), and also has an active soluble guanylate cyclase enzyme for detecting NO in vitro (4, 25). PGE_2 and PGI_2 have been found to be the main cyclooxygenase metabolites released by RBECs in vitro (10, 11). Our findings obtained both in the bioassay experiments and determination of AA metabolites are in accordance with the previous observations. Vasorelaxation effect of primary cells and GP8 cell line corresponds well to the larger amount of PGE_2 release. Furthermore, the abundance of the dilator type of PGs (PGE_2 and PGI_2) released by primary RBECs and GP8 cells in relation to the amount of the vasoconstrictor type of cyclooxygenase metabolites, such as $PGF_{2\alpha}$ (26), PGD_2 (19) and TxA_2 (27), also explains the vasorelaxant effect. The release of vasoactive eicosanoids from RBE4 was limited in agreement with the low-profile functional effect induced by these cells in bioassay.

The presence of functionally relevant quantity of AA metabolites released from primary and GP8 cells suggests that eicosanoids of cerebral endothelium may have physiological and/or pathophysiological significance in vivo. It has been recently demonstrated that vasodilator prostanoids derived from small cerebral blood vessels play roles in the regulation of cerebral circulation (28, 29). Endogenous vasodilator PGs (PGE_2 and PGI_2) have been found to inhibit cerebral vasoconstriction induced by experimental hematoma (30), while exogenous PGI_2 was succesfully used to protect against endothelial dysfunction during chronic cerebral vasospasm (31). Cultured brain microvascular endothelium was shown to produce less vasodilator PGs than the endothelium of a large vessel (29). It is interesting that the higher PGE_2: PGI_2 ratio found in primary cells and GP8 cell line is similar to that seen in the endothelium of microvessels, while the predominance of PGI_2 measured in RBE4 cell line resembles to the eicosanoid profile of larger vessels (32). In our experiments, endothelial cell-induced vasodilation on precontracted canine coronary artery was turned to vasoconstriction when primary and GP8 endothelial cells were preincubated with indomethacin. We do not know which mediators are responsible for the in vitro contractile effect, but indomethacin may shift the arachidonate cascade to the lipoxygenase pathway releasing vasoconstrictor leukotriene (LT) C_4, LTD_4 and LTE_4 (33, 30). In addition indomethacin could enhance the vasoconstrictor effects of endothelin-1 on porcine pial arterioles in vivo (30).

Both primary cells and the GP8 cell line significantly decreased the coronary vascular tone, while the RBE4 cell line was ineffective. In a previous study only the inducible type of NOS was detectable in RBE4 cells (14). In accordance with these findings, our recent preliminary results indicated the expression of mRNA of constitutive, endothelial NOS in cultured primary RBECs and GP8 cell line, which was missing in RBE4 cells (unpublished data, Krizbai et al.). However, the NOS inhibitor, NOLA, applied into the organ bath decreased the vasorelaxation of canine coronary artery caused by primary cells, but failed to do so in GP8 cells. The ineffectiveness of NOLA on GP8 cell line might be explaned by that NO plays only a minor role in the vasorelaxation observed compared to the eicosanoids. We can not exclude the ineffective basal release of NO in GP8 cells, at least up to the concentration of 1.2×10^6 cells in 2 ml volume. It is possible that this number of endothelial cells released insufficient amount of NO for producing relaxation. The volume of KH solution was four order of magnitude larger than the greatest estimated endothelial cell volume (approx. 150 μm^3/cell), and NO molecules produced with a half-life of shorter than a second, could lose activity before reaching the effector cells.

In the present study we used primary cerebral endothelial cells as well as RBE4 and GP8 immortalized cell lines in order to determine their bioactivity and the production of vasoactive eicosanoids. We conclude that the eicosanoid profile of GP8 cell line is closer to that of primary cerebral endothelial cells than that of RBE4 cells, which was also reflected by the results of the bioassay. Nevertheless both cell lines may be useful tools for biotechnological and therapeutic applications in the near future (22, 23).

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Abbreviations

6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$
12-HHT	12-L-hydroxy-5,8,10-heptadecatrienoic acid
AA	arachidonic acid
APase	acid phosphatase
B_{max}	binding capacity
BBB	blood-brain barrier
BCCAL	bilateral common carotid artery ligation
bFGF	basic fibroblast growth factors
CEC	cerebral endothelial cell
CNS	central nervous system
DMEM	Dulbecco's modified Eagle's medium
DPPE	N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine
DTT	dithiothreitol
EBA	Evan's blue labelled albumin
ECGF	endothelial cell growth factor
EDRF	endothelium-derived relaxing factor
FBS	fetal bovine serum
HRP	horseradish peroxidase
ICMV	isolated cortical microvessels
K_D	dissociation constant
KH	Krebs-Henseleit
LT	leukotrien
NO	nitric oxide
NOLA	N^{ω}-nitro-L-arginine
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDS	plasma derived serum
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin, prostaglandin I_2
RBEC	rat brain endothelial cell
SF	sodium fluorescein
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2

12 HHT ? není správně
12 hydroxy hepta-deka trienoic acid