

Effects of the peptides endothelin and CGRP on cerebral blood vessels in vitro

Summary of PhD thesis

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2004

Szeged

List of abbreviations

CGRP: calcitonin gene-related peptide

COX: cyclooxygenase

EC: endothelial cell

EDHF: endothelium-derived hyperpolarizing factor

ET: endothelin

MCA: middle cerebral artery

mRNA: messenger RNA

NO: nitric oxide

NOS: nitric oxide synthase

PCR: polymerase chain reaction

PG: prostaglandin

RAMP: receptor activity-modifying protein

RT-PCR: reverse transcription polymerase chain reaction

S6c: sarafotoxin 6c

SMC: smooth muscle cell

SAH: subarachnoid hemorrhage

Summary

In maintaining the vascular tone of the blood vessels different type of vasoactive peptides are involved. Some of them act to induce vasodilation, others in a vasocontractile manner via specific receptors. Endothelial cells play a key role in the local regulation of the vascular smooth muscle tone by producing relaxing and contracting factors (Volpe M and Cosentino F 2000).

Endothelins (ETs) are very potent vasoconstrictor agents of all blood vessels in all species, including humans. In the cerebral vasculature ET is thought to be involved in several pathological conditions, including vasospasm following SAH, ischemic stroke and vascular headaches such as migraine. Endothelial dysfunction may contribute to the development of these cerebrovascular disorders (Volpe M and Cosentino F 2000). Yanagisawa et al. (1988) isolated endothelin, a 21-amino acid peptide (2500 MW) from porcine aorta cultured endothelial cells. Three isoforms are known: ET₁, ET₂ and ET₃. ETs are secreted not only from endothelial cells, but also from neurons, glial cells, choroid plexus cells, and hypothalamus (Ehrenreich et al. 1992, MacCumber et al. 1990). In vitro on isolated cerebral arteries ETs cause contraction. Also in vivo this vasoconstrictive effect has been demonstrated. In mammals two endothelin receptors exist: ET_A and ET_B receptors. An ET_C receptor has only been identified in frog *Xenopus laevis*. The ET_A receptor is characterised by the rank order of affinity ET₁=ET₂>ET₃ (Arai et al. 1990). The ET_B receptor shows equal affinity towards the three isopeptides (ET₁=ET₂=ET₃). In smooth muscle cells predominantly the ET_A receptors are present, whereas in endothelial cells both ET_A and ET_B receptors have been demonstrated. The endothelial ET_B receptors are thought to account for ET-mediated vasodilation (Szok et al. 2001). The ET receptors are G-protein (G_q) coupled receptors. The signal transduction pathway is through phospholipase C (PLC) – inositoltriphosphate (IP₃) – diacylglycerol (DAG) – protein kinase C (PKC) increased sensitivity towards calcium ions (Ca²⁺). Vasodilation caused by endothelins is mediated through and is dependent upon the endothelium. Stimulating the endothelium produces nitric oxide (NO) and prostaglandins leading to vasorelaxation. The stimulation leads to the similar course of events as above, i.e. increased Ca²⁺ concentration, but the main difference is that nitric oxide synthase (NOS) and cyclooxygenase (COX) are activated (Nilsson 1997). The resultant NO and prostacyclin (PGI₂) have a relaxant effect upon vascular smooth muscle cells.

Calcitonin gene-related peptide (CGRP) is a potent endogenous vasodilatory agent which is released from perivascular sensory nerves in the peripheral nervous system (PNS) (Edvinsson et al., 1987; Hanks et al., 1985; Uddman et al., 1985, 1986). CGRP₁ and CGRP₂ receptors have been classified so far (Quirion et al. 1992, Poyner et al. 1995). Human α -CGRP-(8-37) is a relatively selective CGRP₁ receptor antagonist (Dennis et al. 1990), whereas human [Cys(ACM)^{2,7}]-CGRP α and human [Cys(Et)^{2,7}]-CGRP α are considered selective CGRP₂ receptor agonists (Dumont et al. 1997). The presence of mRNAs encoding human CGRP₁ receptors has been demonstrated in lung, heart, trigeminal ganglion and in cranial arteries (Aiyar et al. 1996, Edvinsson et al. 1997, Tajti et al. 1999, Sams and Jansen-Olesen et al. 1998).

Adrenomedullin is also a vasodilatory peptide. Immunoreactivity of adrenomedullin has been demonstrated in cultured vascular endothelial and SMCs. Increased levels of adrenomedullin have been shown during subarachnoid hemorrhage (SAH) and stroke. Release of adrenomedullin and CGRP may represent two independent pathways to counterbalance vasoconstriction. CGRP₁ and adrenomedullin receptors are derived from a common seven transmembrane domain Gs-protein coupled receptor: the calcitonin receptor-like receptor. Functional pharmacological experiments demonstrated that both CGRP and adrenomedullin induce α -CGRP-(8-37) sensitive vasodilation in artery segments of various diameters. Significant negative correlations between artery diameters and maximal responses were seen for adrenomedullin and CGRP. The potency of both peptides tended to increase with decreasing artery diameter.

In our study different effects of these vasoactive peptides have been tested in animal and human cerebral circulation *in vitro*. The vasodilatory effects of adrenomedullin and CGRP have been investigated in human brain arteries of different sizes by *in vitro* pharmacological and molecular methods. Furthermore the very potent ET-1 has been evaluated on its possible vasodilatory effect in rat middle cerebral arteries by pressurized arteriography. The presence of endothelin receptors mRNA has been studied in macro- and microvessels of the human intracranial arteries by PCR.

Both CGRP and adrenomedullin induced vasodilation via CGRP₁ receptors in human cerebral arteries of various diameter. The artery responsiveness to the CGRP receptor agonists increased with smaller artery diameter, whereas the receptor phenotype determining mRNA ratios tended to decrease.

Both intra- and extraluminal application of ET-1 produced contraction on rat MCAs *in vitro*. Mild concentration-dependent vasodilation could be shown with intraluminal administration of a selective ET_B receptor agonist sarafotoxin S6c on precontracted and pretreated with an ET_A receptor blocker cerebral arteries of rats. Inhibition of NOS significantly reduced the ET_B receptor induced vasodilation, suggesting that this type of vasodilation is mediated by NO in rat brain blood vessels.

In endothelial cells of human macro- and microvessels both ET_A and ET_B receptor mRNA were detected. In samples from normal cerebral arteries only ET_A receptor mRNA was detected, whereas in vessel samples from patients with cerebrovascular disease and cerebral tumours additional ET_B receptor mRNA was detected. The ET_A receptor is known to mediate vasoconstriction, and the predominance of ET_A receptor in these arteries in man suggest that the function of the endothelins is to maintain vascular tone. The presence of ET_B receptor mRNA in the brain vessels of the patients of cerebral disorders might be speculated its role of pathophysiology of cerebrovascular diseases.

Introduction

Endothelin (ET) is one of the most potent endogenous vasoconstrictors acting both peripherally and centrally. So far three isopeptides, ET-1, ET-2, and ET-3, have been identified. The amino acid sequences (21 residues) are quite unique in comparison with other mammalian peptides; only the sequence of the rare snake (*Atractaspis Engaddensis*) venom sarafotoxin has been found to be similar to that of ET (Kloog Y et al. 1989). The ETs can exert both constrictor and dilator effects. These opposite actions have been attributed to the existence of different ET-receptors. The discovered receptor subtypes in mammals are ET_A and ET_B receptors. Unlike other receptor systems where often numerous classes and subclasses of a given receptor have been identified and characterized – thus also allowing specific pharmacological targeting – only these two endothelin receptors have so far been identified. For this reason, any attempt at affecting the endothelin system is likely to have widespread effects. The ET-receptors are members of the rhodopsin-like seven transmembrane receptor family, that couple to second messenger systems via the G_q-protein. Typically, the ET_A receptor, located on the vascular smooth muscle cells (SMC), mediates strong and long-lasting contractile effects (Kloog Y et al. 1989), whereas the ET_B receptor, located on the endothelium, induces vasodilatation via the release of nitrogen oxide (NO) and prostaglandins (de Nucci G et al. 1988). This straightforward distribution of contractile and dilatory ET receptors has been confounded by the discovery that ET_A receptors may also be

present on brain capillary endothelial cells (Stanimirovic et al. 1994), as well as the fact that contractile ET_B receptors have been demonstrated in SMC (Fukuroda T et al. 1994, Sudjarwo SA et al. 1993).

ET_B receptors, however, have also been described as being present on the vascular smooth muscle cells of veins inducing contraction (Clozel M et al. 1995). In various parts of the human circulation, mRNA for both receptors has been found expressed in the smooth muscle cells (Davenport AP et al. 1995, Nilsson T et al. 1997).

In the present study we have examined the presence of ET_A and ET_B receptor mRNA in both normal human cerebral arteries as well as cerebral arteries from patients with cerebral disease. Two types of vessels were studied: macroscopic arteries and microvessels obtained through a sensitive separation method. mRNA was detected using reverse transcription polymerase chain reaction (RT-PCR).

The present study was performed to provide a catalogue as to the presence of ET_A and ET_B receptor mRNA in conductance arteries as well as small arterioles and capillaries in the human cerebral circulation. In the case of conductance arteries, the vascular smooth muscle cells and the endothelial cells were studied separately. The vessels were obtained post-mortally from both patients dying from cerebral as well as non-cerebral causes.

Whereas the ET_A receptor is generally believed to mediate vasoconstriction, ET_B receptors seem elusive; both dilative and constrictive responses have been reported. Using the in vitro pressurized arteriograph, a method allowing compartmentalized study of vessel segments, segments of rat middle cerebral artery (MCA) were cannulated with micropipettes, pressurized and lumenally perfused. By this method we have sought to more thoroughly characterize the ET_B receptor in rat middle cerebral arteries and to pinpoint the mediator mediating the vasodilative effect of endothelins between the endothelium and the vascular smooth muscle cells.

CGRP is thought to play a significant role in certain diseases involving cerebral vasospasm. Clinical potentials for CGRP receptor agonism in the treatment of SAH-induced cerebral vasoconstriction have been suggested (Ahmad et al. 1996, Edvinsson et al. 1991, Nozaki et al. 1989). The use of CGRP receptor antagonism in the treatment of migraine have been addresses as well (Ashina et al. 2000, Goadsby and Edvinsson et al. 1993). As CGRP receptors are widely distributed in the human organism, clinical potentials of CGRP receptor antagonism and agonism are limited by systemic side effects. However the identification of potential vascular CGRP receptor heterogeneity could challenge these limitations and could be important for a further understanding of the role of CGRP under pathological conditions.

Aims of study

To study the vasodilatative effect of the very potent vasoconstrictive endothelins (ETs) via its endothelial vasodilatory receptors in rats.

To investigate the presence of ET_A and ET_B receptors mRNA in macrovessels and in microvessels of the human cerebral circulation.

To determine functional and molecular characteristics of receptors for CGRP and adrenomedullin in different diameter groups of human cerebral arteries, and to evaluate the presence of perivascular neuronal sources of CGRP in these arteries.

Materials and methods

Rat cerebral vessels experiments

Male Sprague-Dawley rats (250-300g) were anaesthetized using CO₂ and decapitated. The brain was immediately removed and placed in cold (4 °C) buffer solution of the following composition (mM): NaCl 119, NaHCO₃ 15, KCl 4.6, MgCl 1.2, NaH₂PO₄ 1.2, CaCl₂ 1.5 and glucose 5.5.

In vitro pharmacology

With the aid of a dissecting microscope, both middle cerebral arteries (MCAs) were carefully harvested beginning at the circle of Willis and extending 5-8 mm distally. A section of the MCA (1-2 mm in length) was mounted in an arteriograph (*Fig. 2.*) (Living System, Burlington, VT) (Bryan RM Jr et al. 1996, You J et al. 1997).

Micropipettes were inserted into both ends of each MCA and secured in place with nylon ties. The mounted MCA was bathed in buffer solution of the above composition (37 °C) equilibrated with gas consisting of 5% CO₂ - 95% O₂. The resulting pH of the bath solution was 7.4. Luminal pressure of the MCAs was measured at either side of the vessel using pressure transducers and was maintained at 85 mmHg by raising or lowering reservoirs connected to the micropipettes. Luminal perfusion was adjusted to 100 µL/min by setting the two reservoirs at different heights. The mounted vessels were magnified 600-fold with a microscope coupled to a video camera. The analogue output signal of the camera was converted to a digital image using a custom made digitiser, and the final image was displayed on a computer monitor. Outside diameters of the MCAs were measured directly on the computer monitor. Drugs could be delivered either to the luminal reservoir (luminal application) or to the vessel bath (abluminal application).

After mounting and pressurization, the MCAs developed spontaneous tone (approximately 25% of the initial diameter) over the equilibrating period of 1 to 1.5 hours. Experimental protocols were not initiated until the MCA diameter was stable over a 15-minute period. Any MCA that did not develop spontaneous tone was excluded. The presence of a functional endothelium was tested at the beginning of each experiment by adding adenosine triphosphate (ATP) abluminally, 10 µmol/L. A vasodilatation of 30% or more of the resting diameter was considered indicative of a functional endothelium.

ET-1 (a mixed agonist), or sarafotoxin 6c (S6c, a selective ET_B receptor agonist) were added cumulatively in logarithmic steps to either the abluminal bath or the luminal perfusate in the

concentration range 10^{-13} to 10^{-7} M. For the abluminal administration, the vascular smooth muscle was exposed to each concentration of the agonists for 5 min before next cumulative increase. The endothelium was exposed to each concentration of the receptor agonists for approximately 15 min. The steady-state change in MCA diameter was measured at each concentration. To avoid the risk of tachyphylaxis only one concentration response experiment was conducted for each MCA.

Experiments were conducted both in vessels only having developed spontaneous tone and in vessels additionally contracted by adding the thromboxane A_2 analogue U46619 abluminally ($0.1 \mu\text{mol/L}$).

In experiments performed with S6c as agonist, the selective ET_A receptor antagonist FR 139317 was used at a concentration of $10^{-5.5}$ M to block any unwanted stimulation of the ET_A receptor, and the selective ET_B antagonist IRL 2500 was employed at a concentration of $10^{-6.5}$ M to characterize the response (Adner M et al. 1998, Hansen-Schwartz J et al. 2000, Leseth KH et al. 1999). To elucidate the mechanisms responsible for dilatory effects of ET receptors, L-NAME ((N^{ω} -nitro-L-arginine methylester, a NO synthase inhibitor), indomethacin (a cyclooxygenase inhibitor) and charybdotoxin (a blocker of voltage-sensitive and calcium-activated potassium channels, by definition an inhibitor of the endothelium-derived hyperpolarizing factor, EDHF) were employed at concentrations of 10^{-5} M, 10^{-5} M, and 10^{-8} M, respectively (Malmsjo M et al. 1998). These drugs were added both luminally and extraluminally to the system.

Human brain blood vessels experiments

With the approval of the local ethical committees (Szeged, Hungary and Lund, Sweden), cortical arteries and brain tissue were obtained from patients undergoing neurosurgical operation for brain tumours or angiomas ($n=6$), and cortical arteries as well as larger arteries from the circle of Willis obtained at autopsy done within 12 hours from the time of death of patients who had died from either cerebral ($n=8$) or non-cerebral causes ($n=8$). The mean age of the population was 48.8 years (range 9-88 years) and the ratio of male to female was 1:2.

Large vessel preparation

The arteries obtained were carefully dissected free of connective tissue leaving the vessel with intact intima, media and adventitia. Arteries with macroscopical signs of atherosclerosis were avoided. To separate the endothelium, the vessels were cut open longitudinally and the

internal surface artery was scraped carefully with a scalpel. After preparation the material was snap frozen in liquid nitrogen and subsequently stored at -80°C .

Microvessel preparation

Cortical tissue samples without pial membranes were placed in ice-cold phosphate-buffered saline (PBS) solution (NaCl 8.5 g/L, $\text{Na}_2\text{PO}_4\text{H}$ 1.42 g/L, pH 7.4). One gram of tissue was weighed of and gently homogenized in ice-cold PBS through slow up-and-down strokes in a glass-mortar. The homogenate was spun down at 1.500 g for 10 min and the pellet subsequently washed and resuspended in PBS-buffer followed by another spin down. The tissue was then resuspended in a 15% dextran (MW 40.000) solution and spun down at 3.500 g for 45 min. The resultant pellet, containing the microvessel fraction, was filtered through a nylon-mesh (pore size 150 μm) and after another wash in a forceful flush of cold PBS filtered again though this time using a nylon mesh with a pore diameter of 50 μm and subsequently collected in a PBS-filled test tube and spun down at 10.000 g for 10 min. The content of micro-vessels was verified light microscopically (Garcia-Villalon AL et al. 1991, Sudjarwo SA et al. 1993).

Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA extraction: Blood vessel preparations (either endothelium denuded vessel or endothelium proper) were homogenized in 1 ml TriZol[®] reagent and left for 5 min at room temperature. Chloroform (200 μL in 1000 μL TriZol[®]) was added, spun down at 12.000 g for 30 min (at $+4^{\circ}\text{C}$). The water phase was transferred to new tubes. Isopropanol (500 μL /1000 μL TriZol[®]) was added and mixed. The solution was left for at least 1 hour at -20°C , then centrifugated for 30 min at 15.000 g (at $+4^{\circ}\text{C}$). The resultant pellet was washed in 300 μL 75% ethanol and spun down at 900 g for 5 min (at $+4^{\circ}\text{C}$). The pellet (containing RNA) was then redissolved in 20 μL DEPC-treated water and unless processed immediately stored at -80°C .

The reverse transcription of RNA to cDNA was carried out using the GeneAmp RNA PCR kit (Perkin Elmer, USA) on a DNA Thermal Cycler (Perkin Elmer). cDNA was synthesized from 1 μg total RNA in 20 μL reaction volume using random hexamers as primers. Transcription was completed in one cycle; 25°C for 10 min, then 42°C for 15 min and finally 99°C for 5 min and subsequently cooled to 5°C .

The PCR assay was performed using the following primers:

Human ET_A receptor forward: '5-TGGCCT TTTGATCACAATGACTTT-3' (bases 436-459), reverse: '5-TTTGATGTGGCATTGAGCATAC AGGTT-3' (bases 737-711);

Human ET_B receptor forward: '5-ACTGGCCATTTGGAGCTGAGATGT-3' (bases 497-521), reverse: '5-CTGCATGCCACTTTTCTTTCTCAA-3' (bases 924-901).

For each primer pair a 9- μ L portion from the resultant cDNA was amplified by PCR in a final volume of 50 μ L.

The PCR was carried out with the following amplification profile: after an initial 5 min at 95 °C, the reaction mixture was taken through 30 cycles, each cycle consisting of 1 min at 95 °C, 1 min at 57 °C and 30 sec at 72 °C; the final extension period was carried on for another 7 min. A blank (water) was included in all experiments.

Electrophoretic analysis the of PCR products was carried out by placing 15 μ L aliquots on a 1.5 % agarose-gel stained with ethidiumbromide (1 μ g/mL) in 0.5 x TBE buffer (1x TBE buffer contains 89 mM TRIS-borate, 2 mM EDTA, pH 8.0) and photographed in a UV-box. (For further details including validation see Möller et al. 1997.)

Evaluation of CGRP and adrenomedullin receptor populations in human cerebral arteries

The experiments were performed on human post mortem tissue by permission of the ethical committee.

Lenticulostriate branches of the human middle cerebral artery were chosen as the source of different diameter cerebral arteries. The lenticulostriate arteries were obtained from 4 male patients undergoing autopsy. Arteries were dissected 24-32 hours post mortem and transferred to a physiological salt solution (PSS) described above. All arteries were kept at 0-4 °C and were divided into 3 groups representing different diameter intervals: group I. artery diameter: 300 μ m, group II. 700 μ m, group III. 1400 μ m.

Functional experiments: in vitro pharmacology

From each of the four patients, a total of 12 artery segments representing artery groups I, II and III were used. Artery segments were mounted in PSS on wires or on pins in a Multimyograph (Model 610M, Danish Myotechnology) for recording of the artery tension. The mounted blood vessel segments were allowed to equilibrate for 30 min in PSS that was continuously aerated with 5% CO₂ and 95% O₂ at 36 °C (details of the method from Mulvany and Halpern 1977). The artery segments were challenged twice by 125 mM potassium to verify vasocinstrictive function. Each segment were then precontracted for 30 min by 3x10⁻⁶ M prostaglandin F_{2 α} and subsequently challenged with 10⁻⁵ M acetylcholin. Cummulative

addition of 10^{-10} to 10^{-7} M human α -CGRP or 10^{-8} to 10^{-6} M human adrenomedullin was given to the vessel segments in the presence or in the absence of 10^{-6} M α -CGRP-(8-37).

Molecular experiments: reverse transcriptase polymerase chain reaction (RT-PCR)

Messenger RNA was extracted from each of the frozen artery samples by the use of a RNeasy mini protocol. The amount of total RNA from each sample was estimated by determining A_{260} and A_{280} and RNA was stored at -70 °C. Specific amounts of total RNA obtained from each sample were reverse transcribed in final volumes of 40 μ l by use of a RT-PCR kit. Serial dilutions of cDNAs were prepared from each sample and PCR reactions of templates from each dilution were carried out as previously described (Sams and Jansen-Olesen 1998). From the lowest concentration of cDNA resulting in a distinct band on an ethidium bromide containing agarose gel, an additional set of serial dilutions were subject to PCR reactions.

Immunohistochemistry

From one patient the lenticulostriate branches of the MCA was dissected out, placed in a phosphate buffered saline solution (pH 7.4) and cut into the three diameter groups of interest. The investigations were performed as described earlier (Knyihar-Csillik et al. 1998). CGRP immunoreactivities were detected following three successive incubations separated by washings in PBS. I. Anti-CGRP raised in rabbit, II. biotinylated anti-rabbit IgG, III. peroxidase coupled avidin. CGRP immunoreactivity was finally visualized as a brown peroxidase product of diaminobenzidine in the presence of H_2O_2 . Sections were mounted on slides, dehydrated and coverslipped.

Statistics

Rat cerebral vessels experiments

Data are presented as Mean \pm S.E.M. To compensate for variation amongst vessels, measured diameters in a given experiment were indexed to either the resting diameter (contractile experiments) or the precontracted diameter (dilatory experiments). E_{max} denotes, depending on the experiment, either the maximal contractile, or maximal dilatory effect, and pEC_{50} denotes the negative logarithm to the concentration at which half the maximal effect was obtained. Data analysis was performed using GraphPad Prism from GraphPad Software Inc. Differences were statistically evaluated using Student's t-test considering p values below 0.05 significant.

Evaluation of CGRP and adrenomedullin receptor populations in human cerebral arteries

In the pharmacological experiments, each of the 48 artery segment obtained from the four patients were treated by either CGRP or adrenomedullin both in the presence and in the absence of α -CGRP-(8-37). From each artery segment, values of actual maximal vasodilatory response (E_{max}) were determined and the equivalent agonistic potency (pEC_{50}) were calculated by fitting the data to a sigmoidal dose response relation (GraphPad Prism). Differences in E_{max} and pEC_{50} were evaluated for diameter dependency using linear regression analysis (GraphPad Prism).

Results

Rat cerebral vessels experiments

The mean diameters of the MCAs after initial pressurization and after development of spontaneous tone were $266 \pm 20 \mu m$ and $215 \pm 20 \mu m$ ($n=40$), respectively.

Both luminal and abluminal application of ET-1 produced contraction in a concentration-dependent manner (*fig. 3A*). Abluminally E_{max} was $26.9 \pm 0.7\%$ and pEC_{50} was 9.6 ± 0.1 ; luminally the same values were $24.3 \pm 1.1\%$ and 9.8 ± 0.2 , respectively. The differences were not statistically different. No dilatory effect of ET-1 was observed, not even in the presence of the ET_A antagonist FR 139317.

In the spontaneously contracted artery there was no effect of S6c neither abluminally nor luminally. However, upon precontraction with U46619 (10^{-8} M) and selective blockage of the ET_A receptor with FR 139317 ($10^{-5.5}$ M) abluminally, luminal administration of S6c gave rise to a concentration-dependent dilatation with an E_{max} of $4.9 \pm 0.1\%$ and a pEC_{50} of 10.1 ± 0.1 (*fig. 3B*). Abluminally administered only a negligible response was observed with an E_{max} of $0.3 \pm 0.1\%$ and a pEC_{50} of 10.2 ± 0.1 . Luminal application of the selective ET_B receptor antagonist IRL 2500 ($10^{-6.5}$ M), 15 minutes before the application of a single dose of S6c (10^{-8} M) also administered luminally, significantly attenuated the vasodilative effect of S6c (*fig. 4A*).

The effect of pretreating the precontracted and ET_A blocked perfused MCA both abluminally and luminally with either L-NAME, indomethacin or charybdotoxin on the vasodilative effect of a single dose of S6c administered luminally is shown in *figure 4B*. Only pretreatment with L-NAME had a significantly attenuating effect on the vasodilative effect of S6c; $1.8 \pm 2.7\%$ versus $5.6 \pm 2.5\%$ in the not pretreated vessel ($p < 0.05$). Pretreatment with

charybdotoxin gave rise to a non-significant attenuation whereas indomethacin had no effect (*fig. 4B*).

Following the advent of the arteriograph allowing discrete investigation of luminal and abluminal effects of vasoactive agents, we have investigated the MCA from the rat in terms of responsiveness to ET agonists. The cerebral arteries possess the well-known blood brain barrier (BBB) which in effect comes about through endothelial tight junctions (Edvinsson L et al. 1993, Reese TS et al. 1967). In the present study, only hydrophilic substances were used thus strengthening the two-compartment approach. The effect of ET-1 was independent of administration route. Since ET-1 cannot cross the BBB passively, the clear inference must be, that contractile ET receptors are probably present both on the endothelium as well as on the SMC. In view of the dilative response of the precontracted and ET_A blocked artery to S6c, the contractile effect of lumenally administered ET-1 must be through an ET_A receptor. How the endothelium mediates the contractile response is at present unknown.

The dilative effect of S6c was not very potent ($E_{max} \sim 6\%$) when comparing to application of ATP ($E_{max} \sim 30\%$), used for testing the presence of a functional endothelium. ATP was capable of dilating the vessel at resting tone, whereas precontraction and blockage of the ET_A receptor (despite the ET_B receptor selective nature of S6c) was required to unmask the effect of S6c.

Human brain blood vessels experiments

RT-PCR performed on the macro- and microvessel preparations are presented in *Table 1*. ET_A receptor mRNA was present in all but one preparation, the exception being one of the micro-vessel preparations (*fig. 5*). In 6 out of 8 large-vessel preparations from patients suffering from cerebral disease mRNA for the ET_B receptor was detected whereas it was only detected in 1 out of 8 large-vessel preparations from patients with non-cerebral disease ($p < 0.05$ using Fisher's exact test). In the micro-vessel preparations ET_B receptor mRNA was detected in 2 patients. The result of RT-PCR performed on endothelial cells is shown in *figure 6*; mRNA for both the ET_A and the ET_B was detected.

Evaluation of CGRP and adrenomedullin receptor populations in human cerebral arteries

In vitro pharmacological experiments

CGRP and adrenomedullin induced concentration-dependent dilatation of all the investigated human cerebral arteries. The maximal amount of vasodilatation induced by CGRP and adrenomedullin was not different, however, the potency of CGRP exceeded that of adrenomedullin by approximately two orders of magnitude in all three groups of arteries. In

all groups of artery diameters, the effects of both CGRP and adrenomedullin were inhibited by a selective CGRP1 receptor antagonist. Emax values and potencies of both CGRP and adrenomedullin were consistently higher in smaller as compared to larger arteries. The pEC50 values of CGRP showed significant linear correlation to the artery diameter.

Molecular experiments

Distinct RT-PCR bands were detected in the majority of the 12 artery segments from the 4 patients. The mRNA ratios of CRLR vs. RAMP1 and CRLR vs. RAMP2 tended to increase in larger arteries as compared to smaller arteries. There was no correlation between post mortem age and the amount of mRNA present.

Immunohistochemistry

CGRP immunoreactivity was detected in the longitudinal and transversal sections as perivascular nerve fibres in the adventitia and media of all three artery sizes.

Discussion

Vasoactive effects of endothelins

Our work demonstrates that the vasoactive effects of endothelins partly depend on administration (i.e. intraluminally or extraluminally) of the drug. Previous studies have shown that ET-1 produces a potent and long-lasting contraction of large cerebral arteries *in vitro* (Jansen I et al. 1989). In our studies we used an *in vitro* pressurized arteriography method that permits the separate application (i.e. lumenally or ablumenally) of the drugs tested.

In comparison with previously published characterizations of the ET receptors in cerebral arteries, the arteriographical method of investigation has clearly demonstrated the presence of a contractile ET_A receptor on endothelial cells not hitherto appreciated. In addition, it has allowed the characterization of the ET_B receptor mediated vasodilative response. Further advantages of the method includes both maintained intraluminal pressure as well as the possibility of maintaining shear stress through perfusion of the artery, possibilities not open to the classical *in vitro* pharmacological method described above. Combining the two methods, the classical *in vitro* set-up allows for quantity (many vessel segments may be run simultaneously; with the arteriograph only one vessel segment may be investigated at any one time) and the arteriograph complements these results with a more discrete analysis of the relative contributions of the endothelium and the vascular smooth muscle cells.

So far three major signalling substances of endothelial origin have been defined, NO, prostaglandins (the most notable being prostacyclin, PGI₂) and endothelium-derived hyperpolarizing factor (EDHF) (Luscher TF et al. 1997). Through tests with an inhibitor of NO synthase, an inhibitor of cyclooxygenase and a potassium channel inhibitor (the hypothesized site of action of EDHF), the results revealed that NO seems to be the principal mediator of the ET_B receptor stimulated vasodilatation, though minor role may also be ascribed to EDHF. Cyclooxygenase products do not seem to be of importance in the present set-up.

In the human cerebrovascular bed, ET-1 induces vasoconstriction via activation of ET_A receptors. The function of ET_B receptors is more controversial, but in man we could only observe vasorelaxation following application of the selective ET_B receptor agonist sarafotoxin 6c (Nilsson T et al. 1997). Stimulation of ET_B receptors can induce increases or decreases in vessel diameter depending on experimental conditions. Subdural administration in the rat of the ET_B antagonist BQ-3020 resulted in an increase by 20% in cortical blood flow (Touzani O et al. 1997). Thus, the role of the ET_B receptor is more elusive, both contractile and relaxant

responses have been observed. In some peripheral vessels a contractile ET_B receptor has been observed (Leseth KH et al. 1999, Sand AE et al. 1998), however this was never the case for fresh human brain vessels (Nilsson T et al. 1997). With a model to induce phenotypic change, putatively minimising the events occurring in cerebrovascular disease, we observed in rat that a contractile ET_B receptor appeared, but this did not occur in man (Hansen-Schwartz J et al. 2002). The ET_B receptor on the endothelium in the isolated rat middle cerebral artery responded with relaxation, mediated via nitric oxide release (Szok et al. 2001). This agrees well with our study in man (Hansen-Schwartz J et al. 2000) demonstrating that the vasodilation *in vitro* was mediated via ET_B receptor and abolished by removal of the endothelium.

Presence of ET_A and ET_B receptors mRNA in human cerebral blood vessels

The results confirm a predominant presence of ET_A receptor mRNA in larger cerebral arteries as has also been observed in peripheral arteries. ET_B receptor mRNA was detected in approximately 50% of the larger arteries with a clear predominance of the arteries obtained from patients with cerebral disease. In cerebral microvessels ET_A receptor mRNA also dominates, although ET_B receptor mRNA was found in 2 out of 5 preparations; all microvessel preparations were obtained from patients with cerebral disease. In endothelial preparations both ET_A and ET_B receptor mRNA was detected.

In general the presence of mRNA for a given receptor may be taken as evidence of synthesis of that receptor although no conclusion may be drawn as to the functional status of the receptor. The receptor may be fully functional and inserted into the cell membrane, but it could also be located in a non-functional form in a subcellular fraction.

Regulation of blood flow takes place at the arteriolar and the capillary levels. At higher levels the artery is a conducting vessel, e.g. circle of Willis. The ET_A receptor is only known to mediate vasoconstriction and the predominance of the ET_A receptor in these arteries in man therefore suggests that the function of the endothelins is to maintain vascular tone. Although no *in vitro* contractile effect of the ET_B receptor has been described in cerebral arteries in man, we still find that the detection of ET_B receptor mRNA in some of the vascular preparations is significant, especially since they were predominantly detected in patients with cerebral disease. On these grounds it may be speculated that the ET_B receptor mRNA has appeared as a result of the cerebral disease. A possible pathophysiological significance of this has yet to be elucidated. These findings might have importance in clinical practice treating different types of cerebrovascular diseases.

Functional and molecular characteristics of CGRP and adrenomedullin receptors

CGRP and adrenomedullin act via α -CGRP-(8-37) sensitive CGRP receptors in various sizes of human lenticulostriate arteries and artery responsiveness towards the peptides demonstrated significant negative linear correlation to artery diameter. No heterogeneity of CGRP and adrenomedullin receptor populations in varying sizes of lenticulostriate branches of the human MCA was demonstrated. The pharmacological evidence for CGRP receptors in the investigated tissue is supported by the presence of mRNA encoding calcitonin receptor-like receptor and RAMP1. A possible function of the mRNA encoding RAMP2 and RAMP3 cannot be interpreted from this study and no correlation between mRNA levels and receptor function can be suggested. The investigated arteries are likely to be regulated by CGRP *in vivo*, since CGRP containing nerve fibres surround the arteries.

Conclusions

The endothelin B receptor agonist sarafotoxin S6c administered luminally caused a mild and concentration-dependent dilatation of cerebral arteries (precontracted and pretreated with an ET_A receptor antagonist) in the rat. This vasodilation might be mediated by nitric oxide, since inhibition of nitric oxide synthase significantly reduced the dilation induced by sarafotoxin 6c.

ET_A receptor mRNA was present both in macrovessels and microvessels in human brain. ET_B receptor mRNA was strongly present in macrovessels compared to microvessel preparations from human cerebral arteries. In our work ET_B receptor mRNA was more detected in human cerebral macrovessels from patients with cerebral disease than from those with non-cerebral disease.

Our results confirm a predominant presence of ET_A receptor mRNA in larger cerebral arteries. ET_B receptor mRNA was detected in approximately 50% of the larger arteries with a predominance of arteries obtained from patients with cerebral disease. In cerebral microvessels ET_A receptor mRNA also dominates compared to ET_B receptor mRNA presence. In endothelial preparations both ET_A and ET_B receptor mRNA was detected.

CGRP and adrenomedullin act via α -CGRP-(8-37) sensitive CGRP receptors in various sizes of human lenticulostriate arteries. In addition, arteries were responsiveness towards the peptides demonstrated significant negative linear correlation to artery diameter. No heterogeneity of CGRP and adrenomedullin receptor populations in varying sizes of lenticulostriate branches of the human middle cerebral artery was demonstrated. The pharmacological evidence for CGRP receptors in the investigated tissue is supported by the presence of mRNA encoding calcitonin receptor-like receptor and RAMP1. A possible function of the mRNA encoding RAMP2 and RAMP3 cannot be interpreted from this study and no correlation between mRNA levels and receptor function can be suggested. The investigated arteries are likely to be regulated by CGRP *in vivo*, since CGRP containing nerve fibres surround the arteries.

Acknowledgements

I am grateful to Professor László Vécsei to give me the opportunity doing these studies and for his tolerance.

I feel respectful gratitude to Professor Lars Edvinsson. His enthusiasm helped me a lot to do this work.

Great thanks to my parents for their love.

I do thank the friendship and helpness of my dear colleague JánosTajti.

Many thanks for teaching and liking me by Jacob Hansen-Schwartz and Anette Sams.

Thanks to Hanne Aggergaard for her friendship and to Elisabeth Nilsson for her experience in laboratory work.

Thanks to Professor Robert M Bryan Jr. and Marie L. Steenberg teaching me the pressurized arterography method in Houston.

I am grateful to all of my colleagues in Szeged, in Glostrup and in Lund.

These studies were supported by grants from the Swedish Medical Research Council (no. 05958) and Soros Foundation (no. 222/3/3714), Eötvös Hungarian Governmental Scholarship (MÖB 65.120/98) and Training Program in Neuroscience TEMPUS S-JEP.

The CGRP and adrenomedullin study was supported in large part by the PhD administrating board of the Royal Danish School of Pharmacy. In addition, by the Danish Pharmacist Foundation, The Lundbeck Foundation and The Danish Medical Research Council (9601844 and 9702065), the Hungarian Ministry of Education (no. MKM-FKPP 1077/0997), and the Hungarian Medical Research Council (no. T-022724).

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