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2005

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Citation for published version (APA):

Stenman, E. (2005). *Vascular receptor changes in ischemic stroke*. [Doctoral Thesis (compilation), Medicine/Emergency Medicine, Lund]. Department of Clinical Sciences, Lund University.

Total number of authors:

1

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Vascular Receptor Changes in Ischemic Stroke

Emelie Stenman, MSc

Doctoral thesis



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The public defence of this thesis for the degree Doctor of Philosophy in
Medicine will, with due permission from the Faculty of Medicine, Lund
University, take place in Segerfalksalen, Wallenberg Neuroscience Centre,
Lund, Sweden on Saturday the 10th of December 2005 at 10 am.

Faculty opponent: Per Wester
Department of Public Health and Clinical Medicine, Umeå University,
Umeå, Sweden

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PAPER I-VI

1. ORIGINAL ARTICLES

This thesis is based on the following papers:

- I. Stenman E, Malmjö M, Uddman E, Gidö G, Wieloch T, Edvinsson L. Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. *Stroke*. 2002;33:2311-2316
- II. Henriksson M, Stenman E, Edvinsson L. Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat. *Stroke*. 2003;34:1479-1483
- III. Stenman E, Edvinsson L. Cerebral ischemia enhances the vascular angiotensin AT₁ receptor mediated contraction in rat. *Stroke*. 2004;35:970-974
- IV. Xu CB, Stenman E, Edvinsson L. Reduction of bFGF-induced smooth muscle cell proliferation and endothelin receptor mRNA expression by mevastatin and atorvastatin. *Biochem Pharmacol*. 2002;64:497-505
- V. Stenman E, Henriksson M, Vikman P, Edvinsson L. Impact of cytokines and growth factors on contractile endothelin responses in rat cerebral arteries. *Submitted manuscript*.
- VI. Stenman E, Henriksson M, Edvinsson L. Low dose inhibition of AT₁ receptors decreases ischemic brain damage in rat. *Submitted manuscript*.

2. ABBREVIATIONS

| | | | |
|------------------|---|------------------|---|
| ACE | angiotensin converting enzyme | IP ₃ | inositol triphosphate |
| Ang II | angiotensin II | K ⁺ | potassium ion |
| ANOVA | analysis of variance | kDa | kilo Dalton |
| AP-1 | activator protein-1 | L | leucine |
| ARAP1 | type 1 angiotensin II receptor associated protein 1 | M | mol/liter |
| ATBP50 | AT ₂ receptor binding protein of 50 kDa | MAP | mean arterial blood pressure |
| ATP | adenosine triphosphate | MAP kinase | mitogen-activated protein kinase |
| ATRAP | angiotensin II type 1 receptor-associated protein | MCA | middle cerebral artery |
| bFGF | basic fibroblast growth factor | mRNA | messenger ribonucleic acid |
| BSA | bovine serum albumin | MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide |
| Ca ²⁺ | calcium ion | N | asparagine |
| cAMP | 3',5'-cyclic adenosine monophosphate | Na ⁺ | sodium ion |
| cDNA | complementary deoxyribonucleic acid | NF-κB | nuclear factor- κB |
| C/EBP | CCAAT/enhancer-binding protein | NO | nitric oxide |
| cGMP | 3',5'-cyclic guanosine monophosphate | N ₂ O | nitrous oxide (laughing gas) |
| Cl ⁻ | chloride ion | N-terminal | amino group terminal |
| CO ₂ | carbon dioxide | O ₂ | oxygen |
| C-terminal | carboxyl group terminal | P | proline |
| DAG | diacylglycerol | PBS | phosphate-buffered saline |
| DMEM | Dulbecco's modified Eagle's Medium | pCO ₂ | partial pressure of carbon dioxide |
| ECE | endothelin converting enzyme | PCR | polymerase chain reaction |
| EDTA | ethylenediaminetetraacetic acid | PDGF | platelet-derived growth factor |
| EGF | epidermal growth factor | PKC | protein kinase C |
| ELISA | enzyme-linked immunoabsorbant assay | PLA ₂ | phospholipase A ₂ |
| ERK 1/2 | extra-cellular signal regulated kinases 1 and 2 | PLC | phospholipase C |
| ET-1 | endothelin-1 | PLD | phospholipase D |
| F | phenylalanine | pO ₂ | partial pressure of oxygen |
| FBS | fetal bovine serum | R | arginine |
| FITC | fluorescein isothiocyanate | RNA | ribonucleic acid |
| G-protein | guanine nucleotide binding protein | S6c | sarafotoxin 6c |
| H | histidine | SAH | subarachnoid hemorrhage |
| I | isoleucine | SD | standard deviation |
| Il-1β | interleukin-1β | SEM | standard error of the mean |
| | | TNF-α | tumor necrosis factor- α |
| | | TTC | 2,3,5-triphenyltetrazolium chloride |
| | | U | units |
| | | V | valine |
| | | VEGF | vascular endothelial growth factor |
| | | WHO | World Health Organization |
| | | Y | tyrosine |

3. INTRODUCTION

Stroke is a disease that results from obstruction of the blood flow to a brain area due to intracranial vascular events. According to the WHO criteria, it involves a rapid onset of neurological symptoms lasting more than 24 hours (unless interrupted by death or surgery).¹ The condition is serious and a leading cause of death worldwide. However, despite extensive research with many promising approaches, few therapies have been proven effective in the clinic so far.

There are three types of stroke. The most common type is ischemic stroke due to a moving embolus or narrowing of a cerebral blood vessel. This type accounts for about 88 % of all stroke cases. The other two types of stroke are intracerebral hemorrhage (9 %) and subarachnoid hemorrhage (SAH; 3 %).² The present thesis aimed to examine how the ischemic type of stroke affects the vascular endothelin and angiotensin systems in cerebral arteries. The subject is interesting, since there is accumulating evidence suggesting that these systems are involved in the pathophysiology of cerebral ischemia.

3.1. Pathophysiology of ischemic stroke

3.1.1. Ischemic stroke and the penumbra

Ischemic stroke arises from a permanent or transient obstruction of a cerebral artery, which causes insufficient blood supply to a part of the brain. Since the brain has a relatively high consumption of oxygen and glucose and is highly dependent on oxidative phosphorylation, it is extremely vulnerable to a reduced blood supply.³ Already within 15 - 90 seconds after commencement of ischemia the neuronal membrane potential begins to change.⁴ Due to decreased oxygen and glucose levels, the cells are unable to produce adenosine triphosphate (ATP). This disturbed energy metabolism results in three major events which threatens cell survival. Firstly, the loss of energy stimulates anaerobic glycolysis causing intra- and extracellular acidosis. Secondly, the ion homeostasis is disturbed, causing an excessive influx of Na^+ , Ca^{2+} and Cl^- into the cells, with osmotic water uptake and subsequent edema as a consequence. The uptake of Ca^{2+} -ions will also trigger many detrimental events, like

activation of proteases, lipases and DNAses and degradation of the cytoskeleton.⁵ Thirdly, the structural integrity of the cells is disturbed.⁶ These events lead eventually to cell death by a combination of necrosis and apoptosis.⁷ The area affected by ischemia is considered to consist of two parts: The central ischemic core where the neurons have no chance to survive without rapid reperfusion, and a perifocal area with less severe ischemia, the so called penumbra, which is potentially salvageable.⁶

3.1.2. Vascular pathophysiology of ischemic stroke

The vascular pathophysiology of ischemic stroke can be divided into three separate phases. The acute phase (hours) includes a disturbed vascular tonus⁸ along with disruption of the blood-brain barrier, partly due to generation of oxygen radicals⁹ and an excessive production of ET-1.¹⁰⁻¹² In the subacute phase of ischemic stroke (hours to days), proinflammatory genes like tumor necrosis factor (TNF)- α and interleukin (IL)-1 β and transcription factors like nuclear factor (NF)- κ B are activated, products that can stimulate expression of adhesion molecules and thereby disturb the endothelial integrity.⁸ Moreover, factors with angiogenic properties like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) are excessively expressed in the subacute phase.¹³⁻¹⁵ Finally, in the chronic phase (days to months), genes involved in the regulation of apoptosis and angiogenesis are induced.⁸ The studies in this thesis examined vascular events mainly in the subacute phase of ischemic stroke. Therefore the impact of cytokines and growth factors on arterial receptor regulation was of particular interest and two of the included studies are dealing with this subject.

3.2. Endothelin

3.2.1. Endothelin-1; homologous peptides and production

Endothelin is a 21 amino acid long peptide. It was originally discovered in 1985 by Hickey and colleagues who described a polypeptide vasoconstrictor produced by endothelial cells.¹⁶ The peptide was isolated and given the name endothelin a few years later.¹⁷ In mammals, three isopeptides of endothelin have been described, ET-1, ET-2 and ET-3, all with vasoconstrictor and pressor properties.¹⁸ ET-1 is the most well-known, and the endothelin

studies included in the present thesis is based on this isoform. In addition to its homology to ET-2 and ET-3, the amino acid sequence of ET-1 show high homology to a peptide family called sarafotoxins. These peptides are found in the venom of the snake *Atractaspis engaddensis* (burrowing asp) and display a strong cardiotoxic activity.¹⁹ The isoform sarafotoxin 6c (S6c) has been used in three of the included studies, since it is a specific agonist to the endothelin ET_B receptor (the receptors are further described in the section below).

The mRNA product of the gene encoding ET-1 is a precursor of ET-1 called preproendothelin (212 amino acids).²⁰ This is further converted in two steps to bigET-1, which possesses some biological activity.²¹ BigET-1 is finally cleaved to ET-1 by endothelin converting enzymes (ECEs).^{22, 23} ET-1 has later been shown to be produced not only by endothelial cells, but by various cell types, for example by macrophages,²⁴ vascular adventitial fibroblasts,²⁵ epithelial cells²⁶ and neurons,²⁷ and the production of ET-1 can be regulated by a number of factors such as shear stress,²⁸ thrombin²⁹ and angiotensin II (Ang II)³⁰ (interestingly, ET-1 has the ability to stimulate angiotensin converting enzyme (ACE), which produces Ang II,³¹ suggesting a reciprocal influence between the endothelin and angiotensin systems). In addition, increased levels of ET-1 have been reported in both plasma and cerebrospinal fluid after ischemic stroke^{12, 32} as well as in focal ischemic tissue.³³

3.2.2. Endothelin receptors

Two endothelin receptor subtypes have been described in mammals, the endothelin ET_A and ET_B receptors.³⁴ In addition the existence of a combined endothelin and angiotensin receptor in rat brain has been suggested.³⁵ However, this receptor will not be further discussed in the present thesis. The ET_A and ET_B receptors belong to the seven transmembrane G-protein coupled receptor family,^{36, 37} and both receptors can connect to the G_q-protein.³⁸ Thereby they activate phospholipase C (PLC) which releases inositol triphosphate (IP₃) with a subsequent increase of intracellular calcium. PLC also releases diacylglycerol (DAG), which in turn can activate protein kinase C (PKC).^{39, 40} In addition, ET_A receptors are suggested to stimulate production of cAMP via interaction with G_s-proteins, while ET_B receptors are believed to inhibit cAMP accumulation via G_i-proteins.⁴¹ ET-1 binds to both the ET_A and ET_B receptors with high affinity,⁴² although the pharmacological effects differ between the receptors. Both receptor subtypes are expressed in vascular smooth muscle cells as well as in endothelial

cells,^{43, 44} though the ET_A receptors are normally mediating the contractile effect of ET-1 on the arterial side of the circulation via release of intracellular calcium and activation of PKC,^{39, 45-47} while ET_B receptors located on arterial endothelial cells mediate vasodilatation via release of nitric oxide (NO).⁴⁸ However, an upregulation of contractile ET_B receptors has previously been reported in pathological conditions like atherosclerosis⁴⁹ and experimental SAH.⁵⁰ In the present thesis, we show that experimental ischemic stroke induces an ET_B-receptor mediated contraction as well (paper I). An established model for studying ET_A and ET_B receptor regulation is organ culture (described in the methodology chapter). Organ culture of human cerebral arteries has been shown to increase the ET_A receptor mediated contraction,⁵¹ while an upregulation of contractile ET_B receptors after organ culture has been demonstrated in many other arterial systems like human omental,⁵² rat mesenteric⁵³ and rat basilar arteries.⁵⁴ A number of factors have been suggested to affect the endothelin receptor regulation. Glucocorticoids can for example decrease the expression of ET_A receptors.⁵⁵ Some cytokines have in turn been shown to increase the ET_B receptor mediated contraction⁵⁶ and shear stress can increase the production of ET_B receptor mRNA.⁵⁷ In addition, autocrine ET-1 has the ability to downregulate its own receptors,⁵⁸ which may reflect a negative feedback system in the cell. Intracellular factors involved in the expression of endothelin receptors are among others, PKC and extra-cellular signal regulated kinases 1 and 2 (ERK1/2) which are crucial for the upregulation of ET_B receptors in organ culture models.^{59, 60} Although much work has been performed on the subject of endothelin receptor regulation, the phenomenon remains elusive and requires further research.

3.3. Angiotensin

3.3.1. Angiotensin II, production

The discovery of the renin-angiotensin system took place already in the 19th century, when Tigerstedt and Bergman discovered a pressor substance in renal tissue of the rabbit, which they called renin.^{61, 62} The renin-product angiotensin was in turn discovered in the midst of the 20th century.⁶² Ang II, the active component of the renin-angiotensin system, is a water-/sodium-conserving and vasoconstrictor octa-peptide. It is a multifunctional molecule regulating a number of systems like blood pressure, sympathetic activity, thirst, cell growth, inflammation and apoptosis.^{63, 64} Ang II is produced both systemically and locally by the same

route of mechanisms; angiotensinogen is cleaved by the enzyme renin to form a decapeptide called angiotensin I (Ang I). Ang I is in turn converted to Ang II by ACE (Figure 3.1).⁶⁵ However, there are alternative pathways for the synthesis of Ang II. Ang I can also be converted to Ang II by a chymotrypsin-like serine protease (chymase)⁶³ and chymostatine-sensitive Ang II-generating enzyme (CAGE).⁶⁶ Such alternative pathways are believed to cause the phenomenon “escape”, which can moderate the physiological effects of ACE-inhibitors.⁶⁷ A local production of Ang II is believed to occur throughout the body. Tissue ACE has been found in several major organs, such as heart,⁶⁸ brain,⁶⁹ blood vessels⁷⁰ and kidneys,⁷¹ and previous studies suggest that locally produced Ang II is of great importance for the arterial contraction, possibly even greater than circulating Ang II.^{72, 73} Factors known to affect the local renin-angiotensin system activity are among others oxidative stress, which activates tissue ACE,⁷⁴ hypercholesterolemia, which increases the expression of ACE and the angiotensin AT₁ and AT₂ receptors⁷⁵ and shear stress, which reduces the local ACE activity.⁷⁶ As can be seen in the present thesis, paper III, experimental focal ischemia increased the vascular ACE mRNA levels locally, which may suggest an increased production of Ang II after ischemic stroke.

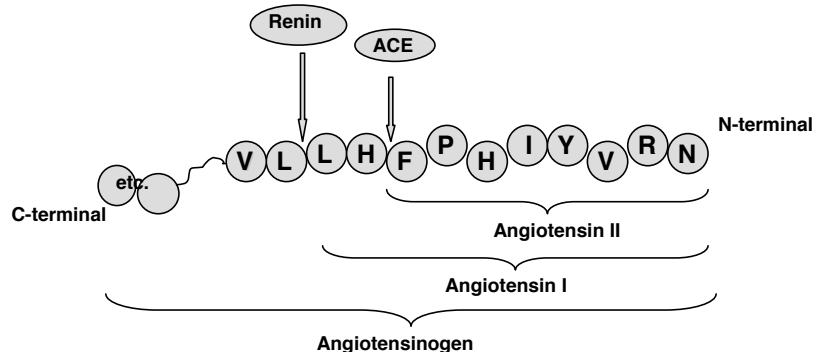


Figure 3.1 Formation of Ang II.⁷⁷

3.3.2. Angiotensin receptors

The effects of Ang II are mainly mediated by two different membrane-bound receptors in mammals, the angiotensin AT₁ and AT₂ receptors.⁷⁸ Two additional receptors have been described, AT₃ and AT₄. The AT₃ receptor recognizes angiotensin II and has so far only been observed in cell lines and AT₄ binds the angiotensin II-derived fragment angiotensin IV.^{79, 80} The AT₃ and AT₄ subtypes will not be further discussed in this thesis. In humans a single AT₁

receptor type has been found, whereas two subtypes have been described in rodents, AT_{1a} and AT_{1b}, with more than 95 % homology and similar pharmacological properties.⁸¹ Regarding the AT₂ receptor, no subtypes have been described.⁶⁵ AT₁ and AT₂ are, like the endothelin receptors, of the seven transmembrane G-protein coupled receptor type^{82, 83} exhibiting almost opposing effects. The AT₁ receptor mediates vasoconstriction and cell proliferation, stimulates drinking behaviour and induces vasopressin release, while the AT₂ receptor mediates vasodilatation and apoptosis, inhibits cell growth and prevents thirst.⁸⁴⁻⁸⁷ Thus, the AT₁ receptor is mediating the “classical” actions of angiotensin II⁸⁸ and stimulates a number of intracellular pathways. Via interaction with G_q-proteins it activates PLC with subsequent release of IP₃, which mobilizes calcium from intracellular stores. This increase in intracellular calcium can result in vascular smooth muscle cell contraction. PLC also stimulates DAG, which in turn activates PKC.^{64, 89} In addition, AT₁ stimulates the phospholipases PLD and PLA₂, which results in activation of PKC, release of arachidonic acid, which is metabolized to eicosanoids, and activation of mitogen-activated protein (MAP) kinases.^{64, 90} Furthermore, AT₁ activates receptor tyrosine kinases and small G-proteins.⁶⁴ While the AT₂ receptor is the dominating angiotensin receptor type in fetal tissues, its expression in adults is limited to certain cells and tissues like vascular endothelial cells and specialized nuclei in the brain.^{88, 91} The intracellular signalling pathways of the AT₂ receptors are in many ways different from the AT₁-induced pathways.⁸⁸ The phosphorylation cascades induced by factors like Ang II via AT₁ are counteracted by phosphatase activation mediated by AT₂, and inactivation of the MAP kinases ERK1/2 seems to be one key event in the AT₂ signaling.^{88, 92} AT₂ is also believed to signal via activation of the NO/cGMP system and via stimulation of PLA₂.⁹³ The expression of angiotensin receptors can be affected by a number of factors. Ang II has been shown to exert negative feedback by downregulating AT₁ receptor mRNA and protein in vascular smooth muscle cells.⁹⁴ Furthermore, an increased amount of AT₁ receptors has been observed in the neointima formed after arterial injury,⁹⁵ and elevated mRNA levels for vascular AT₁ and AT₂ receptors have been demonstrated in spontaneously hypertensive rats as compared to normotensive animals.⁹⁶ In addition, endothelin seems to be involved in the increase in AT₁ receptor mRNA seen in experimental cardiac heart failure.⁹⁷

3.3.3. Angiotensin receptor associated proteins

After activation, the AT₁ receptors are internalized by the angiotensin II type 1 receptor-associated protein (ATRAP).⁹⁸ The receptors are thereafter recycled by another protein called

type 1 angiotensin II receptor associated protein 1 (ARAP1).⁹⁹ Recently, a corresponding AT₂ receptor associated protein, ATBP50 (for AT₂ receptor binding protein of 50 kDa), was discovered and shown to be necessary for AT₂ cell surface expression.¹⁰⁰ In an ongoing study we are examining how experimental ischemic stroke affects the mRNA levels for the two AT₁ receptor associated proteins and the preliminary results will be described in the results and comments section below.

4. AIMS OF THE THESIS

- To examine how experimental ischemic stroke affects expression and function of vascular endothelin and angiotensin receptors in rat cerebral arteries.
- To study the time course of endothelin receptor regulation in rat cerebral arteries after organ culture.
- To examine the impact of growth factors and cytokines on endothelin receptor regulation in cultured rat cerebral arteries and vascular smooth muscle cells from rat brain.
- To examine the effect of AT₁ receptor inhibition in the acute phase of experimental ischemic stroke.

5. GENERAL METHODS

In paper I, III and VI, transient focal cerebral ischemia was induced in rats. Organ culture of rat middle cerebral arteries (MCAs) was used in paper II and V as a model of vascular receptor changes, and in paper IV, vascular smooth muscle cells were cultured for molecular examination of endothelin receptors. Real time PCR has been employed in all studies for examination of relative mRNA levels, and functional myograph studies of rat MCAs have been performed in four of the studies. Finally, in paper V, receptor protein density was examined by immunohistochemistry. All experimental procedures were approved by the Lund University Animal Ethics Committee.

5.1. Induction of transient focal cerebral ischemia in rat

Transient focal cerebral ischemia was induced in rats by an intraluminal filament technique, originally described by Koizumi *et al.*,¹⁰¹ and later modified by Memezawa *et al.*¹⁰² In the model, the entrance of the right MCA is occluded by a filament as described below. Male Wistar rats were housed under controlled temperature and humidity with free access to water and food. In paper I and II, the animals were fasted overnight with access to water immediately before operation. Anesthesia was induced using 4.5 % halothane in N₂O:O₂ (70 %:30 %). Thereafter, the rats were kept anesthetized by artificial inhalation (paper I) or through a mask (paper II and VI) with 1.5 % halothane. A polyethylene catheter was inserted into a tail artery for measurements of mean arterial blood pressure (MAP), pH, pO₂, pCO₂, and plasma glucose. A rectal temperature probe connected to a homeothermal blanket was inserted for maintenance of a body temperature about 37° C during the operational procedure. A skin incision was made in the midline of the neck and the right common, external and internal carotid arteries were exposed. The common and external carotid arteries were permanently ligated by sutures. A filament was inserted into the internal carotid artery via an incision in the common carotid artery, and further advanced until the rounded tip reached the entrance of the right MCA. In paper VI, the MCA occlusion was verified by laser-Doppler flowmetry. In this case, a probe was fixed on the skull (1 mm posterior to the bregma and 6 mm from the midline on the right side), measuring regional blood flow in an area supplied by the right MCA (Perimed, Sweden). Then the resulting occlusion was made visible by a

computer software program as an abrupt reduction of cerebral blood flow. Finally, the filament was fixed by a suture, and the rats were allowed to awaken.

Two hours after MCA occlusion, the rats were reanesthetized to allow for withdrawal of the filament and thereby achieve reperfusion. A proper reperfusion was confirmed by laser-Doppler flowmetry in paper VI. Rectal temperature was measured half an hour before and 1 hour after reperfusion in the awoken animal. In paper VI, candesartan or the corresponding volume vehicle was administered intraperitoneally immediately after MCA occlusion and in awoken animals 24 hours later. At 24 or 48 hours after MCA occlusion, the rats were anesthetized and decapitated. In paper VI, the rats were examined neurologically as described below and MAP was measured before they were sacrificed. The brains were removed and the right and left MCAs were dissected out for examination. In the first paper the basilar artery was studied as well. The brain damage was analysed as described below.

5.2. Neurological evaluation

The animals were examined neurologically by an established scoring system described in the table below.^{103, 104}

| Score | Interpretation |
|-------|--|
| 0 | No visible deficits |
| 1 | Contralateral forelimb flexion, when hold by tail |
| 2 | Decreased grip of contralateral forelimb |
| 3 | Spontaneous movement in all directions, but contralateral circling if pulled by tail |
| 4 | Spontaneous contralateral circling |
| 5 | Death |

5.3. Morphological evaluation of brain damage

Coronal slices of the brains were stained with 1 % 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in physiological buffer solution to confirm a proper brain damage.¹⁰⁵ In paper VI,

the slices were photographed and the size of the ischemic damage was analyzed by the software program Brain Damage Calculator 1.1.. The size of ischemic damage was expressed as percent of the brain volume.

5.4. Organ culture procedure

The use of organ culture for studying arterial receptor changes has previously been described by Adner and colleagues.¹⁰⁶ Male Wistar rats were anesthetized with CO₂, and decapitated. The brains were removed and immediately chilled in cold bicarbonate buffer solution. The right and left MCAs were removed and incubated in Dulbecco's modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 µl/ml) and amphotericin B (25 µg/ml) at 37° C in humidified 5 % CO₂ in air. In experiments with cytokines, growth factors or inhibitors of intracellular factors, these substances were added to the medium in the beginning of the culture.

5.5. Cell culture procedure

Male Sprague-Dawley rats were anesthetized with CO₂, and decapitated. To extract cerebral vascular smooth muscle cells, the brains were removed and homogenized in phosphate-buffered saline (PBS). Cerebral vessels were isolated by 15 % dextran density centrifugation. They were incubated with collagenase/dispase for one hour after which the collagenase/dispase was removed and the remaining vessel segments were explanted into DMEM. The DMEM was supplemented with 10 % fetal bovine serum (FBS), 2 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml epidermal growth factor (EGF), 100 U/ml penicillin and 100 µg/ml streptomycin. Subcultures were obtained using 0.25 % trypsin-EDTA. Aortic smooth muscle cells were extracted by scraping of the endothelium from rat aorta with a surgical blade whereupon the medial layer was removed and cut into segments. The segments were cultured in DMEM as described above.

Smooth muscle cells were identified with a fluorescence microscope, where a > 95 % positive reaction when using monoclonal antibodies against α -smooth muscle actin and a secondary

FITC-labeled antibody was considered acceptable. In addition a typical “hill and valley” growth pattern was observed. Cell viability was confirmed by trypan blue exclusion (> 95 %) and smooth muscle cells from passage 5 to 15 were used for the experiments.

To assay smooth muscle cell proliferation, an MTT proliferation kit was used. Briefly, the cells were seeded in a 96-well plate at a density of about 4000 cells in 100 μ l DMEM with 10 % FBS and incubated at 37° C for 24 hours. Thereafter, the medium was exchanged for serum free DMEM and incubated for another 24 hours to arrest cell growth. After this serum starvation, cells were incubated for 24-48 hours with or without bFGF. In statin experiments, statins were added 4 hours before bFGF. The statins were dissolved in ethanol (25 mg/ml) and further diluted by PBS containing 0.1 % bovine serum albumin (BSA). Control cultures received the corresponding volume of the solvent. Four hours before incubation was discontinued, an MTT labeled reagent was added. Dissolved purple formazan (derived from the cleavage of the tetrazolium ring of MTT) was read in an ELISA reader.

5.6. Functional studies by myographs

Mulvany-Halpern myographs were used for measuring contractile properties of cerebral arteries.^{107, 108} The arteries were cut into cylindrical segments and the endothelium was removed mechanically by rubbing the luminal side with a thread. Two thin wires were then inserted into the segments for mounting in temperature controlled (37° C) myographs containing a bicarbonate buffer solution. One wire was connected to a force transducer attached to an analogue-digital converter unit. The other wire was attached to a movable displacement device to allow for fine adjustments. The experiments were recorded on a computer by the software program ChartTM. The arterial segments were given an initial tension of 1.2 mN, and were allowed to stabilize at this tension for one hour. Thereafter, the contractile capacity was determined by exposure to a 63.5 mM K⁺ buffer solution, which causes smooth muscle cell contraction via membrane depolarization and subsequent Ca²⁺ entry via voltage-operated Ca²⁺-channels.¹⁰⁹ This K⁺-evoked contraction was used as reference in the myograph experiments.

Concentration-response curves were obtained by addition of cumulative concentrations of the agonists studied. In order to measure the ET_A receptor mediated response specifically, the response to ET-1 was recorded 30 minutes after the response to S6c in the same segments. The segments were rinsed thoroughly between the substances. In this way S6c desensitized the ET_B receptors and the following contraction towards ET-1 was mediated entirely by ET_A receptors.¹¹⁰ The vascular contractile responses are expressed as percentage of the K⁺-elicited response. E_{max} represents the maximum contraction induced by an agonist and the pEC₅₀ value refers to the negative logarithm of the concentration eliciting half the maximal response.

5.7. Extraction of total RNA, reverse transcription and real time PCR

Two different kits have been used for extraction of total cellular RNA; TRIzol and the FastRNA Pro Green Kit, in both cases following the suppliers' instructions. The extracted RNA was used as template for producing cDNA by reverse transcription using random hexamers as primers.

Real time PCR was performed in a GeneAmp 5700 Sequence Detection System using SYBR Green Master Mix with the cDNA synthesized above as template. By this method the cDNA amplification in each sample is detected via a fluorescent dye that binds to double-stranded cDNA. Specific primers were designed by use of the software program Primer Express and no-template controls for each primer pair were included in all experiments. The amount of mRNA for each gene product studied was compared to the amount of mRNA for one or more endogenous standards, house-keeping genes, which are continuously expressed in the cells.

The amount of mRNA in each sample was calculated relative to the amount of mRNA for the endogenous standard in the same sample by the formula $X_0/R_0 = 2^{CtR-CtX}$, where X₀ is the original amount of target mRNA, R₀ is the original amount of mRNA for the endogenous standard, CtX is the C_T value for the target and CtR is the C_T value for the endogenous standard. The C_T values refer to the number of PCR cycles performed for each PCR product in a sample at a specific point of time.

5.8. Immunohistochemistry

Arteries were placed in Tissue TEK, frozen at -80° C and subsequently sectioned into 10 µm thick slices. Primary antibodies directed against endothelin and angiotensin receptors were used, which were subsequently detected by fluorescent secondary antibodies. Fluorescence intensity was measured at the appropriate wavelength in a confocal microscope. As control only secondary antibodies were used.

5.9. Statistics

In paper I-IV, data were analyzed parametrically with one-way ANOVA or Student's t-test, whereas in paper V and VI, data were analysed both parametrically and non-parametrically (Kruskal-Wallis or Mann Whitney tests). Finally in paper VI, the real time PCR results were analyzed by two-way ANOVA.

6. RESULTS AND COMMENTS

6.1. The endothelin system

The aim of paper I was to examine if experimental ischemic stroke affects the vascular endothelin receptor regulation. The hypothesis was partly based on previous findings that cardiovascular conditions like atherosclerosis and congestive heart failure can increase the binding of ET-1 to ET_B receptors.^{49, 111} In addition, an upregulation of contractile ET_B receptors has been reported after organ culture.¹¹² Interestingly, we observed a similar upregulation in ischemic stroke. In fresh rat MCAs, there is no contractile response to the specific ET_B receptor agonist S6c (paper II). That was also the case for MCAs from sham-operated rats and the contralateral, left MCAs from stroke-operated rats in paper I. However, ischemic stroke induced an ET_B receptor mediated contractile response in the ipsilateral, occluded MCA 48 hours after MCA occlusion, as shown by accumulative application of S6c (Figure 6.1). The phenomenon was not observed in the basilar artery, suggesting a local induction.

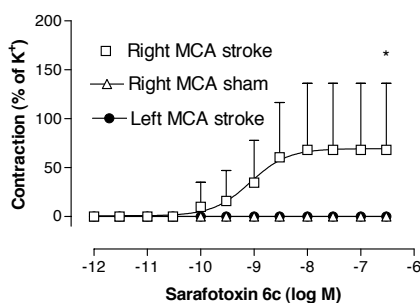


Figure 6.1 Ischemic stroke induced a contractile ET_B receptor mediated response in the occluded MCA. Data are expressed as mean \pm SD.

The ET_A receptor mediated response was not altered at this point of time. However, the mRNA levels for both the ET_A and ET_B receptors were significantly increased in the right occluded MCA as compared to the contralateral, left MCA (Figure 6.2).

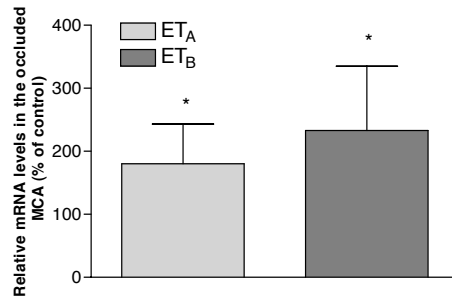


Figure 6.2 The ET_A and ET_B receptor mRNA levels were increased in the right occluded MCA as compared to the left MCA (control) 48 hours after onset of ischemic stroke. Data are expressed as mean \pm SD.

To further analyze the endothelin receptor regulation in ischemic stroke, we have recently started to examine the vascular endothelin receptor regulation after a reperfusion period of 24 hours. Preliminary data suggest that an upregulated contractile ET_B receptor response is present already 24 hours after stroke onset. Interestingly, the ET_A receptor mediated response seems to be increased after this reperfusion period as well, which may explain the beneficial effects of ET_A receptor blockade in experimental ischemic stroke.¹¹³ In addition, we are presently examining endothelin receptor protein expression in MCAs from stroke operated rats by immunohistochemistry. Preliminary results suggest that there is indeed an increased ET_B receptor expression in the media layer of the right occluded MCA as compared to the left non-occluded MCA 48 hours after occlusion (Figure 6.3; unpublished).

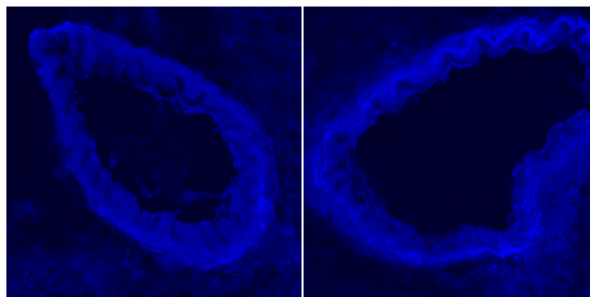


Figure 6.3 ET_B receptor expression (blue) in cross sections of the left non-occluded (left) and right occluded (right) MCA 48 hours after MCA occlusion.

Increased circulating and tissue ET-1 levels have previously been reported after ischemic stroke.^{12, 32, 33} These findings together with the present results suggest that ischemic stroke induces a general activation of the endothelin system. The question whether an upregulation of ET_B receptors after stroke is protective or detrimental remains. Our hypothesis suggests that the induction of a contractile ET_B receptor mediated response after ischemic stroke may be harmful if it implies a reduced perfusion of the ischemic hemisphere due to increased contraction. However, by blocking ET_B receptors after stroke, the arterial dilatation may be impaired due to inhibition of endothelial ET_B receptors. In addition, ET_B receptors are believed to be responsible for clearance of ET-1,¹¹⁴ and by blocking them, an increased amount of ET-1 will be able to activate contractile ET_A receptors. Previous studies concerning endothelin receptor blockade after ischemic stroke are indeed contradictory. However, an increased endothelin mediated vascular tone after focal ischemia, which could be reversed by a combined ET_A and ET_B receptor antagonist has been shown,¹¹⁵ and a combined ET_A and ET_B receptor inhibitor was previously demonstrated to decrease the brain damage in a model of ischemic stroke.¹¹⁶ These studies support our results and suggest that inhibition of ET_B receptors in ischemic stroke may be beneficial.

To further analyze the manner of ET_B receptor upregulation in rat cerebral arteries, we employed the organ culture model in paper II. The aim was to study the time course of endothelin receptor regulation *in vitro* and to examine intracellular mechanisms responsible for the regulation. As mentioned above, the ET_B receptor agonist S6c did not evoke any contractile responses in fresh MCAs. However, already after 6 hours organ culture, a slight ET_B receptor mediated contraction was detected, and after 12 hours culture S6c induced a potent contraction. After 48 hours culture the dose-response curve was further shifted to the left with a significantly increased pEC₅₀ value (Figure 6.4). The E_{max} for ET-1 was not significantly affected by the organ culture, although the pEC₅₀ value was increased after 48 hours culture as compared to fresh arteries. A similar leftward shift for the ET-1 dose-response curve after 48 hours culture, has previously been described in the rat basilar artery, although the shift was even more pronounced.¹¹⁷

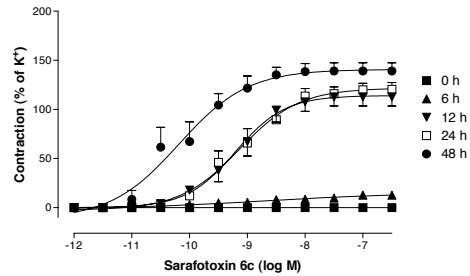


Figure 6.4 Contractile responses to S6c after different incubation periods.

Real time PCR revealed a significantly increased amount of ET_B receptor mRNA in MCAs cultured 24 hours as compared to fresh MCAs, whereas there was no difference in the mRNA levels for ET_A (Figure 6.5).

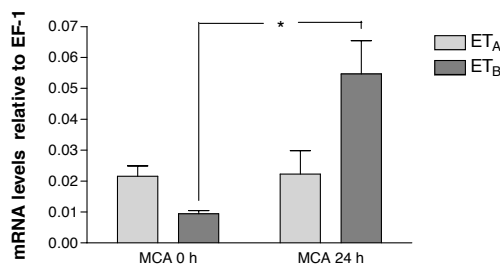


Figure 6.5 The ET_B receptor mRNA levels were increased after 24 hours organ culture.

Organ culture induced upregulation of endothelin receptors has previously been shown to depend on increased transcription,^{51, 118} which is in accordance with the results in the paper II. Organ culture for 24 hours with the transcriptional inhibitor actinomycin D or the translational inhibitor cycloheximide prevented a functional ET_B receptor mediated response, suggesting that the response depends on production of new ET_B receptors from the gene level. To find possible intracellular mechanisms responsible for the transcriptional upregulation of ET_B , we hypothesized that PKC may be involved. It turned out that the PKC inhibitor Ro-31-8220 significantly attenuated the organ culture induced contraction to S6c compared to control (Figure 6.6). In addition, PKC inhibition prevented the organ culture induced increase in ET_B receptor mRNA levels, suggesting an involvement of PKC up-stream from the gene level.

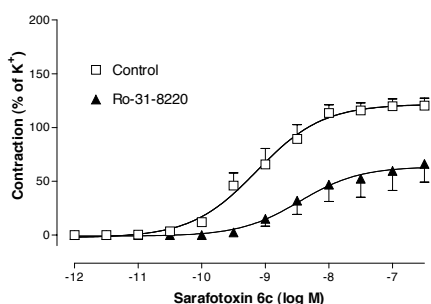


Figure 6.6 Incubation with the PKC inhibitor Ro-31-8220 attenuated the ET_B receptor mediated contraction.

Transcription of the ET_B receptor gene has previously been found to be regulated by the transcription factors activator protein (AP)-1, CCAAT/enhancer-binding protein (C/EBP) and GATA-2 in rat.¹¹⁹ Interestingly, Ang II has been shown to activate AP-1 by a PKC-dependent signaling mechanism in rat vascular smooth muscle cells.¹²⁰ Since there may be enhanced production of Ang II after cerebral ischemia (paper III), this pathway appears to be an attractive possible explanation for the induction of ET_B receptors after ischemic stroke. However, a positive relation between PKC and GATA-2 has also been observed in a human cell line,¹²¹ while PKC has been shown to inhibit C/EBP.¹²²

In paper IV, we aimed to analyze the impact of the growth factor bFGF and lipid-lowering statins on cell proliferation and endothelin receptor regulation in rat vascular smooth muscle cells. The main purpose was to elucidate possible mechanisms involved in atherosclerosis. However, the subject is also interesting regarding stroke, considering our results showing stroke-induced expression of endothelin receptors, along with the facts that focal ischemia can induce bFGF,¹⁵ and that statin treatment in stroke has been shown to be neuroprotective.¹²³ It was demonstrated in paper IV that bFGF induced a time and concentration dependent increase in proliferation in both aortic and cerebral smooth muscle cells, which could be inhibited by statins. This is in accordance with a previous study, which demonstrated that statins can inhibit bFGF-induced DNA synthesis.¹²⁴ Furthermore, bFGF increased the mRNA levels for both the ET_A and ET_B receptors, however with a slight difference in time profiles: In aortic as well as cerebral smooth muscle cells, the ET_A receptor mRNA expression peaked after 3 hours incubation with bFGF, while the expression of ET_B receptor mRNA had a peak as early as after 1 hours incubation with the growth factor (Figure 6.7). A difference between the cell lines was that bFGF affected the ET_A receptor mRNA levels more pronounced in aortic

smooth muscle cells than in cerebral, whereas the effect on the ET_B receptors was the opposite (Figure 7).

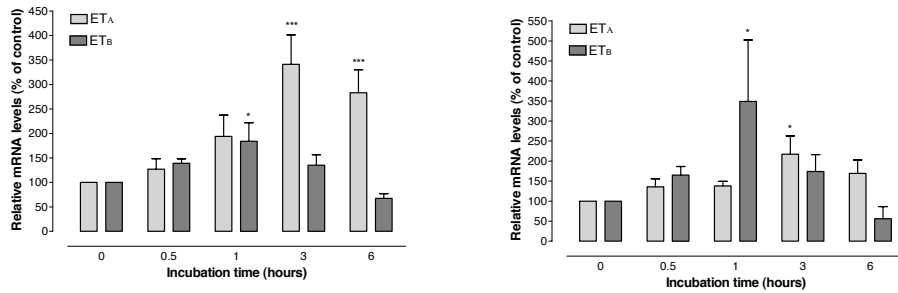


Figure 6.7 Incubation with bFGF increased the mRNA levels for ET_A and ET_B in aortic (left) and cerebral (right) smooth muscle cells.

The increase in endothelin receptor mRNA was attenuated by statins. However the effect did not reach significance in cerebral smooth muscle cells. In addition to the effect of bFGF on endothelin receptor mRNA expression, the endothelin receptors turned out to be involved in bFGF-induced smooth muscle cell proliferation. Both the ET_A receptor antagonist FR139317 and the ET_B receptor antagonist BQ788 attenuated bFGF-induced proliferation significantly, which supports previous findings that endothelin receptors can be involved in vascular growth (Figure 6.8).^{125, 126}

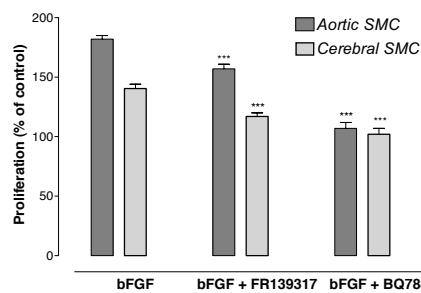


Figure 6.8 Both the ET_A receptor antagonist FR139317 and the ET_B receptor antagonist BQ788 attenuated bFGF-induced proliferation (SMC; smooth muscle cells).

The results of paper IV regarding bFGF-induced endothelin receptor expression, together with previous findings that inflammatory cytokines^{54, 56} can increase the contractile ET_B receptor mediated response aroused our curiosity about the impact of cytokines and growth factors on

endothelin receptor regulation in the rat MCA. Since both inflammation and angiogenesis are common features of cerebral ischemia,^{3, 127} we hoped to find possible mechanisms for the ischemia-induced increase in endothelin receptor expression and function seen in paper I. The aim of paper V was therefore to study the impact of two cytokines; TNF- α and Il-1 β and three growth factors; PDGF, epidermal growth factor (EGF) and bFGF on endothelin receptor expression and function. The study was made *in vitro* with organ culture and the MCAs were incubated for 24 hours based on the results in paper II. It was shown that incubation with TNF- α potentiated the ET_B receptor mediated contractile response in rat MCA (Figure 6.9), whereas the ET_B receptor mRNA levels were not significantly affected by the cytokine. There was a slight increase in ET_B receptor protein density (32 %), which may explain the potentiating effects of TNF- α . However, even though organ culture-induced ET_B receptor upregulation is due to *de novo* production of ET_B receptors, the additional influence of TNF- α may depend on other factors. TNF- α has for example been shown to increase the contraction in tracheal smooth muscle cells via a potentiated release of intracellular calcium.^{128, 129} Similar findings have been shown in rat mesenteric artery, where TNF- α can increase the ET_B receptor mediated contraction, without a change in the ET_A/ET_B mRNA ratio.⁵⁶ Since Il-1 β has been shown to increase the ET_B receptor mediated response in rat mesenteric and basilar arteries,^{54, 56} we chose to examine its effect in the rat MCA as well. However, Il-1 β failed to affect the endothelin receptor mediated response in rat MCA.

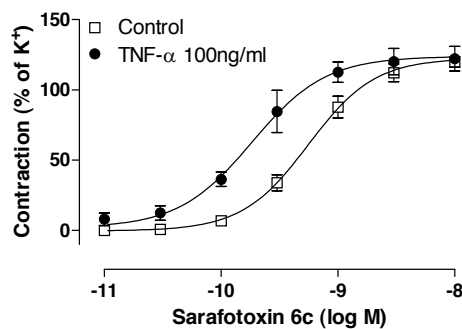


Figure 6.9 Organ culture with TNF- α significantly potentiated the ET_B receptor mediated contractile response in the rat MCA.

Incubation with EGF yielded similar results as incubation with TNF- α . EGF

potentiated the ET_B receptor mediated contraction significantly (Figure 6.10) without a concomitant increase in ET_B receptor mRNA levels or protein density. Increased release of intracellular calcium is a possible mechanism in this case as well since EGF has been shown to exert such impact.¹³⁰ However, this possibility evokes the question: If the potentiated ET_B receptor mediated contraction after incubation with TNF- α or EGF is due to increased release of intracellular calcium, then why is not the ET_A receptor mediated contraction affected by these factors? One possible explanation is that the endothelin receptor subtypes may differ in their dependence on intracellular calcium release. Preliminary results from our research group actually suggest that the ET_A receptor mediated response in rat basilar artery is more dependent on extracellular calcium than the ET_B receptor mediated response.

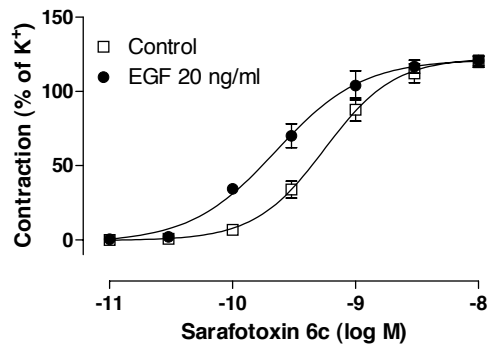


Figure 6.10 Organ culture with EGF significantly potentiated the ET_B receptor mediated contraction in the rat MCA.

Incubation with bFGF affected the endothelin responses in a somewhat complicated manner. The maximal contraction towards ET-1, which was mediated by ET_A receptors due to ET_B receptor desensitization, was slightly enhanced by bFGF (Figure 6.11).

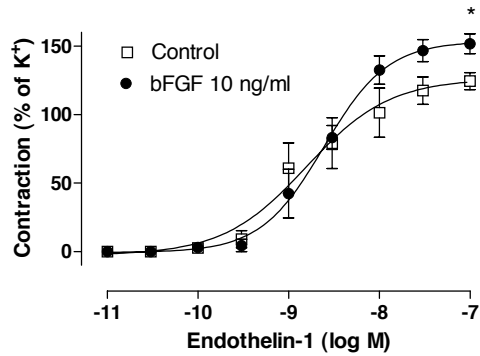


Figure 6.11 Organ culture with bFGF increased the maximal contraction mediated by ET_A receptors in the rat MCA.

Interestingly, the ET_A receptor mRNA levels were not affected by bFGF, whereas the ET_B receptor mRNA levels and protein expression were increased (Figure 6.12).

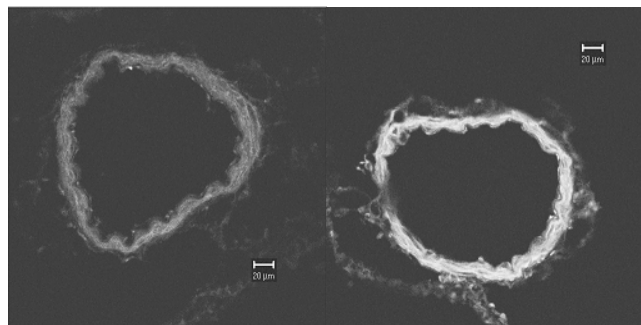


Figure 6.12 Immunohistochemistry photographs showing ET_B receptors in cross sections of middle cerebral arteries. Left: MCA control, right: MCA incubated with bFGF.

The bFGF results are ambiguous, but they do actually remind of the situation in experimental SAH: 48 hours after induction of SAH in rat, the contractile response to ET-1 was significantly potentiated in the MCAs and the basilar artery, an effect which could be prevented by either an ET_A or ET_B receptor inhibitor. In addition, ET_B receptor mRNA and protein levels were increased in SAH operated rats as compared to sham, although no contractile response to S6c could be detected.^{50, 131} Organ culture with PDGF did not affect the endothelin receptor mediated response in rat MCA.

Treatment with growth factors in cerebral ischemia is generally believed to be protective, which is confirmed by many studies.¹³²⁻¹³⁴ However, contradictory data exist. For example a recent experimental stroke study demonstrated a significantly increased brain damage after infusion of EGF/bFGF.¹³⁵

Since growth factors and cytokines are released in the subacute phase of cerebral ischemia,^{8, 13-15} it is possible that they contribute to the induction of contractile ET_B receptors seen 48 hours after MCA occlusion. Thus, let us continue to draw the picture over possible mechanisms for induction of contractile ET_B receptors after ischemic stroke: In paper II, we demonstrated that the organ culture induced upregulation of ET_B receptors was due to *de novo* transcription of the receptors, and that PKC contributed to the upregulation. It is likely that a *de novo* production of ET_B receptors occurs in ischemic stroke as well (paper I), considering the increased mRNA levels. As stated above, the transcription of ET_B receptors is controlled by the transcription factors AP-1, C/EBP and GATA-2.¹¹⁹ Ang II can activate AP-1 by a PKC-dependent signaling mechanism in rat vascular smooth muscle cells,¹²⁰ and this is interesting since ischemic stroke increased the ACE mRNA levels in the ipsilateral MCA, which may involve an enhanced production of Ang II (paper III). Ang II has in turn the ability to stimulate the expression of bFGF,¹³⁶ a growth factor which can increase the endothelin receptor mRNA levels as demonstrated in paper IV and V. In addition, TNF- α , which is induced after stroke¹³⁷ and EGF, which is excessively expressed after vascular injury,¹³⁸ may contribute to the ET_B receptor mediated contraction seen after ischemic stroke, according to paper V, possibly via a potentiated release of intracellular calcium. It is appealing to try to describe the reality in this way. However, it is important to remember that organ and cell culture can never be directly compared to the *in vivo* situation, but should be regarded as models for studying possible interactions between signaling pathways.

6.2. The angiotensin system

In paper III, we aimed to examine the impact of experimental ischemic stroke on the cerebrovascular angiotensin system. The subject is of current interest since recent experimental and clinical studies have demonstrated beneficial effects of AT₁ receptor

blockade in ischemic stroke.^{139, 140} In addition, a major clinical trial examining the effect of AT₁ receptor blockade on survival and disability after stroke has recently been initiated.¹⁴¹ To study vascular changes after ischemic stroke, transient focal ischemia was induced in rats as described above. 24 hours after MCA occlusion Ang II only elicited a negligible contraction, and there was no difference between the right and left MCA. However, 48 hours after occlusion, a potent contractile response to Ang II was observed in the right, occluded MCA, which differed significantly from the response in the left MCA and MCAs from sham-operated rats (Figure 6.13).

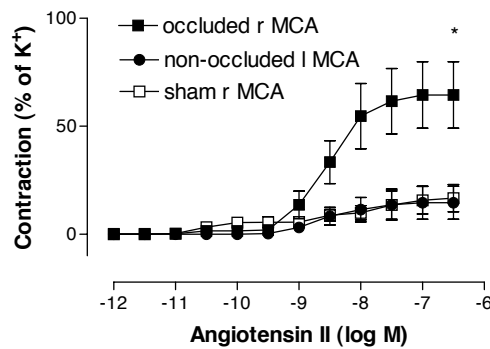


Figure 6.13 Ischemic stroke induced an increased contraction to Ang II in the right (r) occluded MCA 48 hours after MCA occlusion, but not in the left (l) non-occluded MCA or in sham.

The contractile response could be completely blocked *in vitro* by the selective AT₁ receptor inhibitors candesartan and losartan, while the AT₂ receptor inhibitor PD123319 had no such effect. This suggests that AT₁ receptors were mediating the increased responses to Ang II (Figure 6.14).

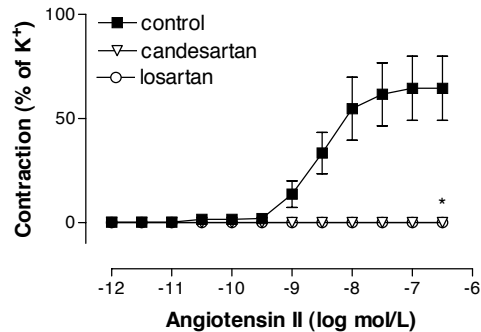


Figure 6.14 The increased contraction to Ang II was completely abolished by the AT₁ receptor inhibitors candesartan and losartan.

The mRNA levels for AT₁, AT₂, ACE and the transcription factor NF-κB were studied by real time PCR. The results showed that 24 hours after MCA occlusion there was no difference between the right and left MCA in the amount of mRNA for the genes studied. Surprisingly, 48 hours after occlusion the mRNA levels for AT₁ were significantly lower in the right occluded MCA as compared to the left (Figure 15). However, similar results have been demonstrated in renal afferent arterioles, where oxidative stress enhanced the contraction to Ang II with a concomitant downregulation of AT₁ mRNA.¹⁴² The downregulation of AT₁ receptor mRNA after ischemic stroke may reflect an increased production of AT₁ receptors, without a corresponding increase in transcription of the AT₁ receptor gene. It may also be due to a negative feedback mechanism, in which Ang II inhibits expression of its own receptor.⁹⁴ Supporting that idea is the fact that the mRNA levels for ACE were increased in the right occluded MCA as compared to the left 48 hours after operation, which may suggest an increased production of Ang II after ischemic stroke (Figure 6.15).

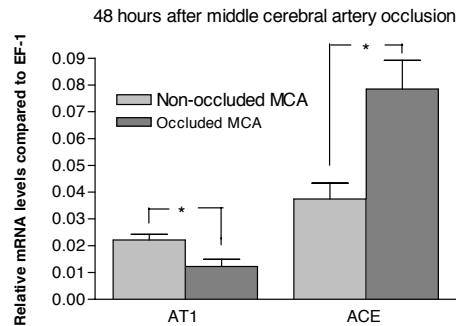


Figure 6.15 mRNA levels for AT₁ and ACE 48 hours after MCA occlusion.

To further analyze the AT₁ receptor regulation after ischemic stroke, we have recently started to examine the protein expression of AT₁, using immunohistochemistry. Ongoing studies suggest that there are more AT₁ receptors in the right occluded MCA as compared to the left MCA 48 hours after MCA occlusion (Figure 6.16; unpublished), a fact that supports our results in paper III.

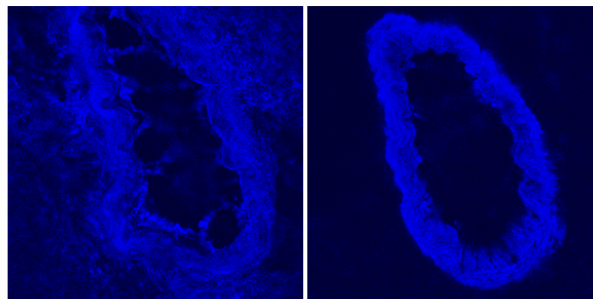


Figure 6.16 AT₁ receptor expression (blue) in cross sections of the left non-occluded (left) and right occluded (right) MCA 48 hours after MCA occlusion.

We have also studied the mRNA levels for the AT₁ receptor associated proteins ATRAP and ARAP1, which are responsible for internalization and recycling of the AT₁ receptors. The results showed that both the ATRAP and ARAP1 mRNA levels were lower in the right occluded MCA as compared to the left non-occluded MCA 48 hours after MCA occlusion; in the case of ARAP1 this difference was significant (unpublished data; Figure 6.17). The fact that the mRNA profiles of the two AT₁ receptor associated proteins were similar to the

mRNA profile of the AT₁ receptors may suggest a coordinated expression, which is a well known phenomenon between interrelated genes.¹⁴³

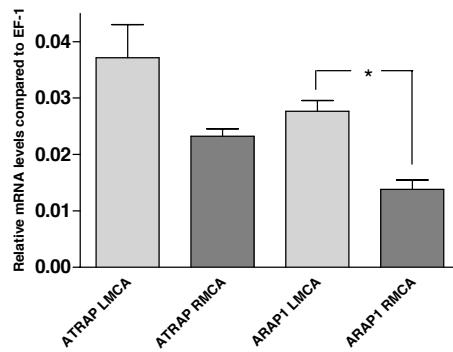


Figure 6.17 The mRNA levels for ATRAP and ARAP1 in MCA 48 hours after induction of ischemic stroke.

In the final study, presented in paper VI, we examined the impact of AT₁ receptor blockade in the acute phase of ischemic stroke. To avoid affecting the blood pressure too much, we used a low dose ($0.05 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) of the AT₁ receptor blocker candesartan. The size of brain damage and the neurological status were evaluated 48 hours after the operation. Since the cytokines TNF- α and IL-1 β and the transcription factor NF- κ B are activated during the subacute phase after ischemic stroke, and can be activated by Ang II,^{8, 144-146} we also chose to examine the mRNA levels for these factors. It turned out that the candesartan treatment decreased the size of brain damage significantly (Figure 6.18).

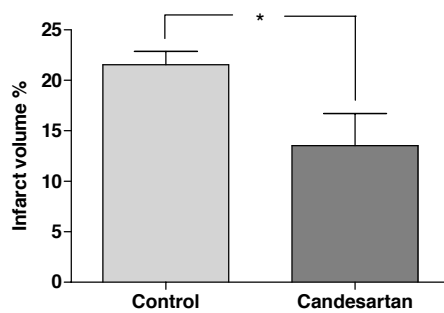


Figure 6.18 Size of brain damage in the candesartan group compared to vehicle treated rats (control).

In addition, there was a tendency towards improved neurological scores in the candesartan treated group (Figure 6.19).

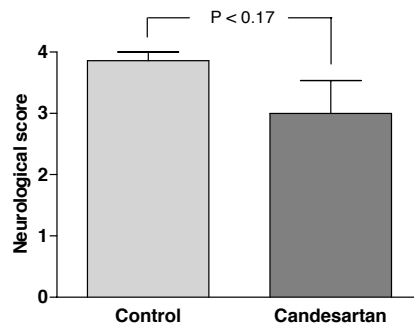


Figure 6.19 Neurological scores. Comparison between the candesartan group and vehicle treated rats (control).

The physiological parameters did not differ between the groups (table in paper VI). The mean arterial blood pressure (MAP) was measured in anesthetized animals before they were sacrificed, and it was not different to the MAP measured during operation suggesting that the candesartan dose used did not have any long-term effect on MAP. Beneficial effects of candesartan treatment in the acute phase of ischemic stroke has been shown before.¹³⁹ However, in the present study the dose was 10 times lower and given intraperitoneally instead of intravenously. PCR-experiments revealed significantly increased mRNA levels for TNF- α and Il-1 β in the right occluded MCA after ischemic stroke compared to the left MCA. However, the candesartan dose used did not significantly affect the increased mRNA levels. There are many possible explanations for the beneficial effects of candesartan in ischemic stroke. According to the results in paper III, our suggestion is an attenuation of the ischemia-induced increase in AT₁ receptor mediated contraction. However, the MAP was not affected in the present study, which may point towards involvement of other mechanisms. Blockade of central AT₁ receptors in ischemic stroke has been shown to prevent expression of the transcription factors c-Fos and c-Jun, which are implicated in neuronal apoptosis.¹⁴⁷ Other possible mechanisms are an enhanced stimulation of protective brain AT₂ receptors¹⁴⁸ or reduced production of free radicals.¹⁴⁹ It is not impossible that the beneficial effects of AT₁ receptor blockade in cerebral ischemia are due to a combination of many factors. However, it will be interesting to see the results of the clinical SCAST study,¹⁴¹ in which the effects of AT₁ receptor blockade in the acute phase of cerebral ischemia will be thoroughly analyzed.

6.3. Additional comment

I have many times been asked if I think that ischemic stroke induces some kind of general increase in the contractile machinery, considering the enhanced ET_B and AT₁ receptor mediated responses. However, the contraction elicited by 63.5 mM K⁺ do not differ between the right and left MCA after ischemic stroke. In addition, the contractions to 5-hydroxytryptamine (5-HT) or 5-carboxamidotryptamine (5-CT) are not increased by the ischemia (unpublished data). Therefore, in my opinion the number of cerebrovascular contractile mediators affected by ischemic stroke seems to be restricted.

7. MAJOR CONCLUSIONS

- Focal cerebral ischemia in rat induces an enhanced contractile ET_B receptor mediated response in the ipsilateral MCA 48 hours after MCA occlusion. The phenomenon is accompanied by increased ET_B receptor mRNA levels. Together with preliminary data showing increased ET_B receptor protein expression as well, these data suggest an upregulation of contractile ET_B receptors.
- Organ culture of rat MCA induces a time-dependent upregulation of contractile ET_B receptors. The upregulation is due to *de novo* transcription of the receptors and is partly dependent on PKC.
- The growth factor bFGF increases the proliferation of rat aortic and cerebral smooth muscle cells in a time- and concentration dependent manner, which can be blocked by lipid-lowering statins. bFGF also has the ability to increase the mRNA expression of ET_A and ET_B receptors in both cell types. Statins can attenuate this effect in aortic smooth muscle cells. In addition, the bFGF-induced cell proliferation can be blocked by specific ET_A or ET_B receptor inhibitors suggesting a reciprocal effect between the growth factor and the endothelin receptors.
- The cytokine TNF- α and the growth factor EGF can potentiate the ET_B receptor mediated contraction in rat MCAs. Neither of the compounds increases mRNA or protein levels for ET_B significantly, suggesting that the effect may be due to other factors like potentiated release of intracellular calcium. Incubation with bFGF enhances the maximal ET_A receptor mediated contraction and increases the mRNA and protein levels for the ET_B receptors in rat MCAs.
- Focal cerebral ischemia in rat increases the contractile response to angiotensin II in the ipsilateral MCA. This phenomenon occurs between 24 and 48 hours after the MCA occlusion, and is mediated by AT₁ receptors. The increased AT₁ receptor mediated contraction is accompanied by increased AT₁ receptor protein density, but not mRNA levels, suggesting an enhanced production of receptors without a concomitant increase in transcription. It may also reflect a negative feedback-system, in which angiotensin II prevents expression of its own receptors. Supporting this idea is the fact that the

mRNA levels for ACE are increased in the ipsilateral MCA after focal cerebral ischemia, which may suggest an increased production of angiotensin II.

- A low dose of the AT₁ receptor blocker candesartan decreases the brain damage after focal cerebral ischemia in rat. In addition, the vascular TNF- α and IL-1 β mRNA levels are increased in the ipsilateral MCA.

8. SVENSK SAMMANFATTNING (SWEDISH SUMMARY)

Stroke är en vanlig och mycket allvarlig form av hjärt-kärlsjukdom som uppkommer på grund av försämrat blodflöde i en del av hjärnan. Eftersom hjärnan är extremt beroende av kontinuerlig tillförsel av syre och näringsämnen leder tillståndet snabbt till hjärnskada. Det finns tre typer av stroke; hjärninfarkt, hjärnblödning och subaraknoidalblödning (blödning under en hjärnhinna), varav hjärninfarkt står för omkring 88 % av fallen. Trots intensiv forskning finns det ännu mycket få behandlingsalternativ som fungerar på människor, varför ämnet är angeläget. Avhandlingen du håller i din hand fokuserar på hjärninfarkt, och hur sjukdomen påverkar hjärnans blodkärl. Vår hypotes är att hjärninfarkt ökar blodkärlens förmåga att dra ihop sig (kontrahera) och att detta kan orsaka en större hjärnskada om det innebär ett försämrat blodflöde i den redan strokedrabbade hjärnan. Vi har koncentrerat de inkluderade studierna till två cirkulerande ämnen, endotelin och angiotensin, som båda kan orsaka kraftig kontraktion av blodkärl då de binder till speciella receptorer i blodkärlsväggarna. För att studera blodkärlsförändringar vid hjärninfarkt har vi valt att inducera stroke på råttor, och vi har även odlat hjärnblodkärl och blodkärlsceller från råttor vid 37° C som modeller för att mer ingående kunna studera receptorförändringar i hjärnans blodkärl.

8.1. Endotelinreceptor-reglering

Två olika sorters endotelinreceptorer förmedlar de huvudsakliga effekterna av endotelin; ET_A och ET_B. När endotelin binder till ET_A-receptorer i blodkärl hos friska djur och människor kontraherar kärlen, medan kärlen utvidgar sig när endotelin binder till ET_B-receptorer. Det har dock visat sig att olika sjukdomstillstånd kan orsaka en uppkomst av kontraktile ET_B-receptorer. I avhandlingens första studie demonstrerar vi att detta är fallet även vid experimentell hjärninfarkt. Vi observerade en uppkomst av kontraktile ET_B-receptorer i hjärnans högra mediaartär 48 timmar efter att hjärninfarkt inducerats i höger hjärnhalva, ett fenomen som inte förekom i den vänstra hjärnhalvans mediaartär eller i basilarisartären – ett hjärnkärl längre bort från skadan. Fenomenet var alltså relativt lokalt. ET_A-receptorernas funktion var inte påverkad vid tidpunkten i fråga. I en följande endotelinstudie visade vi att en uppkomst av kontraktile ET_B-receptorer, liknande den vid hjärninfarkt, sker då råttans

mediaartär odlas vid 37° C i minst 12 timmar. Denna uppkomst har sitt ursprung redan på gen-nivå i artärens celler, och en molekyl inuti cellen som heter proteinkinase C verkar vara involverad i "igångsättningen" av skeendet. Mekanismen kan därför vara intressant ur terapeutisk synpunkt vid hjärninfarkt. Två andra studier i avhandlingen visar att proteiner involverade i kärltillväxt och inflammation också kan påverka endotelinreceptorerna i råtans mediaartär. Detta är av intresse eftersom både kärltillväxt och inflammation ingår i sjukdomsbilden vid stroke. Även här har vi alltså möjliga mål för nya läkemedel.

8.2. Angiotensinreceptor-reglering

Liksom endotelin binder angiotensin huvudsakligen till två receptortyper, AT₁ och AT₂. De två receptortyperna utövar i stort sett motsatta effekter; Till exempel kontraherar AT₁-receptorerna blodkärl, orsakar celltillväxt och stimulerar törst, medan AT₂-receptorerna orsakar en utvidgning av blodkärl, inducerar celledöd och motverkar törst. AT₁-receptorblockad vid hjärninfarkt har diskuterats livligt de senaste åren och studier både på djur och människa har visat att en sådan blockad kan ha gynnsamma effekter. Vid de flesta av dessa studier har dock AT₁-receptorinhibitorer givits preventivt under lång tid före hjärninfarkten. Studier angående effekten av akut behandling med AT₁-inhibitorer vid hjärninfarkt har däremot varit motsägande. Likaså diskuteras det flitigt vad som är orsaken till att AT₁-inhibitorer fungerar skyddande. En möjlig mekanism presenterar vi i denna avhandling. Vi upptäckte att hjärninfarkt i råttor orsakar en ökad kontraktion mot angiotensin i den högra, drabbade mediaartären. Den här kontraktionen förmedlades just av AT₁-receptorer, och detta skulle enligt vår hypotes kunna orsaka ett sämre blodflöde i den redan påverkade hjärnhalvan. Här har vi alltså en möjlig förklaring till varför AT₁-receptorinhibitorer kan skydda vid hjärninfarkt. Dessutom visade det sig att genuttrycket av angiotensin convertering enzyme, ett protein som producerar angiotensin, ökar i den högra mediaartären efter hjärninfarkt, vilket kan innebära att hjärninfarkt orsakar en ökad produktion av angiotensin. Slutligen lyckades vi, i den sista inkluderade studien, bekräfta att blockad av AT₁-receptorer (i en låg dos) är gynnsamt vid hjärninfarkt i råttor. Hjärnskadan i de behandlade råttorna var betydligt mindre än i de obehandlade 48 timmar efter operationen, och dessutom visade de behandlade råttorna en tendens till färre neurologiska symptom.

9. TACK TILL (ACKNOWLEDGEMENTS)...

Min handledare:

Lars Edvinsson för din fantastiska förmåga att entusiasmera och se det positiva i alla situationer! Stort tack även för att du delat med av din tid och kunskap, och slutligen för att du lärt mig att vara som en gås (inte dum, utan att man skall låta oförrätter och kommentarer från taskiga referees "glida av som vatten").

Mina medförfattare:

Marie Henriksson för ett helt underbart roligt samarbete under 4 år, och för att du har förmågan att veta vad jag tänker innan jag vet det själv! Jag hoppas verkligen att vi kommer hålla kontakten i framtiden, som forskningskollegor, filmmakare eller alumna-försäljare...

Malin Malmsjö för att du är en jättebra förebild både vad det gäller forskning och livet i allmänhet. Tack även för trevligt resesällskap!

Erik Uddman för att du bjuder på minst ett skratt om dagen, och för all hjälp med myografer och datorer förstås!

Cang-Bao Xu för att du besitter stor kunskap som du gärna delar med dig av, och för många kul och givande diskussioner om livet i stort och smått.

Petter Vikman för att du trots ditt stora hjärta kan leverera sarkasmer som ingen annan! Även tack för all hjälp med de molekylärbiologiska metoderna.

Gunilla Gidö för att du med stort tålamod lärde mig strokemodellen och för all fortsatt hjälp och handledning.

Tadeusz Wieloch för din generositet.

Mina nuvarande kollegor:

Angelica Wackenfors, Roya Jamali, Saema Beg, Yi Liu, David Nilsson, Bengt Granström, Elisabeth Nilsson och Marie-Louise Edvinsson för att tack vare er har det nästan alltid varit kul att gå till jobbet! Ni har lärt mig jättemycket om blodkärl, statistik, hjärtan, SAH, calcium, migrän, shopping, politik, religion och livet i allmänhet, tack!

Vår sekreterare:

Christel Ekstrand för att du har förmågan att snabbt lösa alla problem med stipendier, löner, intyg, mm, mm med ett leende på läpparna (vi saknade dig sååå när du var borta!).

Mina tidigare kollegor:

Christine Lindström, Hoa Ytterberg, Jacob Hansen-Schwartz, Rikard Alm, Sebastian Möller, Karen Eskesen, Ming-Yan Hou, Ming-Fang Zhou, Henrik Lind och Emil Pantev för glada diskussioner, handledning och för att ni skapade en trevlig labb-miljö.

Andra kollegor som hjälpt mig under doktorandtiden:

Rikard Pehrson för vänskap och för ett roligt samarbete. **Tomas Deierborg** och **Maithe Perez** för en noggrann och trevlig halvtids-opponering. **Kerstin Beirup** och **Carin Sjölund** för all hjälp och handledning i operationssalen. **Mattias Bryborn** för att du skapade ett så listigt hjärnskadeanalysprogram. **Siv Dahlquist** för massor av hjälp med installationen av våra halothanförgasare mm. **Personalen på konventionella djuravdelningen** för all hjälp med att fasta och injicera våra råttor.

Familj och vänner:

Ewa och Sven Johansson för att ni alltid stöttat och hjälpt mig. Bättre föräldrar finns inte!

Julia Ahlrot för att du besitter ett enormt självförtroende som liksom sprider sig som en varm filt till omgivningen (även neurotiska syrran blir tillfälligt lugn...). **Resten av familjen Ahlrot** för att ni är så härligt stolliga. **Anja och Matti Stenman** för att utan er hade det helt enkelt inte blivit någon avhandling! Tack för att ni tagit så väl hand om Ika och alltid har ställt upp med transporter, kalas, flytt och mycket annat. **Övrig familj och alla vänner (ingen nämnd och ingen glömd)** för att ni är som luft för mig (inte obetydliga utan livsnödvändiga, och låter mig vara precis som jag är)!

Slutligen mina älskade:

Christer och Ika Stenman för att ni inte har packat era väskor och stuckit för länge sen under mina många artikel-ångestattacker, och för att ni är det bästa som någonsin hänt mig!

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