Signal Transduction and Gene Regulation in Cerebral Arteries Following Ischemia

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Signal Transduction and Gene Regulation in Cerebral Arteries Following Ischemia

Petter Vikman

Doctoral thesis

The public defense 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 Friday the 16th of June 2006 at 9 am.

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Abstract

The thesis consideres the molecular events that take place in the cerebral arteries following a stroke. The degree and temporal course of reperfusion following a stroke is pivotal for the survival of the neuronal tissue in the penumbra. Previous investigations have revealed upregulation of contractile receptors, a putative factor in the blood flow reduction following stroke that can could augment cell death. The major aim of the study is achieve a better understanding of the changes that occur in the cerebral arteries following a stroke and of the resulting activation of the signal pathways involved, along with changes in gene expression and protein regulation to further the knowledge of how arteries participate in the events that take place following a stroke.

The more specific goals have been the following:
• To investigate the gene regulation in cerebral arteries following SAH, aimed at determining what the important processes involved in the increase in contraction are.
• Investigation of MAPK activation and its relation to gene expression.
• Comparison of the ischemic models MCAO and SAH with organ culture of cerebral arteries to determine similarities and to validate the use of organ culture as a model.
• Investigation of gene expression and protein regulation in human MCA following thromboembolic stroke so as validate previous findings in connection with experimental rat models.
• Molecular characterization of smoke induced changes in cerebral arteries.

Key words: Inflammation, Extra-cellular matrix, Mitogen activated kinases, Metalloproteases Stroke, SAH, MCAO, Gene regulation, Phosphorylation

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Till
Jenny
Siri
och
Mini

Science is a wonderful thing
if one does not have to earn
one's living at it

--Albert Einstein--

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1. Original articles

I. Vikman P, Beg S, Khurana T, Hansen-Schwartz J, Edvinsson L. Gene expression and molecular changes in cerebral arteries following subarachnoid hemorrhage in rat. Journal of Neurosurgery 2006, accepted.

II. Vikman P, Edvinsson L. Gene expression profiling in the human middle cerebral artery after cerebral ischemia. European Journal of Neurology 2006, accepted.

III. Vikman P, Beg S, Henriksson M, Edvinsson L. Activation of p38, ERK1/2 and SAPK/JNK initiate transcription of inflammatory and extracellular matrix genes in cerebral arteries following cerebral ischemia in rat. 2006. *Submitted.*

IV. Vikman P, Beg S, Edvinsson L. Signal transduction, inflammation and gene activation in cerebral arteries following experimental SAH in rat. 2006. *Submitted.*

V. Vikman P, Edvinsson L. Lipid soluble smoking particles induce an inflammatory response in rat cerebral arteries via p38MAPK activation and downstream transcription factors ATF-2 and Elk-1. 2006. *Submitted.*

2. Abbreviations

5-HT	5-hydroxytryptamine
ABC	Avidin: Biotinylated enzyme Complex
AngII	Angiotensin type II
AT_1	Angiotensin receptor type 1
AT_2	Angiotensin receptor type 2
ATF-2	Activating transcription factor
BA	Basilar Artery
BOXes	Bilirubin Oxidation products
ccl20	chemokine (C-C motif) ligand 20
CNS	Central Nervous System
cxcl	chemokine (C-X-C motif) ligand
DAB	3-3-diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimetylsulfoxide
DNA	Deoxyribonuclease
DSP	DMSO soluble smoke particles
EF-1	Elongation Factor 1
Elk-1	E-26-like protein 1
ERK1/2	Extracellular signal Regulated Kinases 1 and 2
EST	Expressed Sequence Tag
ET-1	Endothelin 1
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
GAPDH	Glyceraldehyde-3-phosphate-dehydrogenase
GPCR	G-protein couple receptors
HRP	Horse Radish Peroxidase
Il	Interleukin
iNOS	inducible NO synthase
LPE	Local Pool Error test
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MCA	Middle Cerebral Artery
MCAO	Middle cerebral artery occlusion
MMP	Matrix Metalloproteinase
PCR	Polymerase Chain Reaction
PGF 2a	prostaglandin F 2 alpha
PKC	Protein Kinase C
mRNA	messenger Ribonucleic Acid
rtPA	recombinant tissue-type Plasminogen Activator
S6c	Sarafotoxin 6c
SAH	Subarachnoid Hemorrhage
SAM	Significance Analysis of Microarray
SAPK/JNK	Stress Activated Protein Kinase / c-Jun terminal NH2 kinase
TNF	Tumor Necrosis Factor
VSMC	Vascular Smooth Muscle Cells
WC	Circle of Willis
WHO	World Health Organisation

3. Introduction

Stroke is the third leading cause of death worldwide and can lead to direct death or severe disability.[1] It is caused by the disruption of the blood flow to the brain and is defined according to WHO criteria as a involving the rapid onset of neurological symptoms lasting more than 24 hours unless alleviated by surgery or interrupted by death.[2] The brain is especially susceptible to changes in blood flow due to its high rate of metabolism and the non-redundancy of its cells which have little or no regenerative properties.[3] A stroke can be either hemorrhagic or thromboembolic. Hemorrhagic strokes are often caused by the rupture of an aneurysm causing a rapid discharge of blood whereas a thromboembolic stroke is a permanent or temporary obstruction of a cerebral artery, often due to movement of a thrombus or rupture of a plaque. This arterial rupture or blockage causes inhibits of blood flow, which can be so severe that an ischemic core is produced surrounded by a penumbra. The assumption is made that the cells in the ischemic core cannot be salvaged whereas those in the penumbra can survive given favorable conditions. Research has been concerned mainly with neuroprotective agents, yet major clinical trials of these have had poor outcomes.[4]

This thesis considers the molecular events that take place in the cerebral arteries following a stroke. The degree and temporal course of reperfusion following a stroke is pivotal for the survival of the neuronal tissue in the penumbra. Previous investigations have revealed upregulation of contractile receptors, a putative factor in the blood flow reduction following stroke that can could augment cell death.[5-8] The major aim of this thesis is to achieve a better understanding of the changes that occur in the cerebral arteries following a stroke and of the resulting activation of the signal pathways involved, along with changes in gene expression and protein regulation to further the knowledge of how arteries participate in the events that take place following a stroke.

3.1 Types and pathophysiology of strokes

3.1.1 Subarachnoid hemorrhage, SAH

The most common cause of SAH is the spontaneous rupture of an aneurysm. This gives rise to a discharge of blood into the subarachnoid space, a rapid increase in intracranial pressure, constriction of the perforating arteries and a reduction in cerebral blood flow.[9] The condition is biphasic, its having an early/immediate phase in which there is a reduction in cerebral blood flow, followed by a late phase characterized by varying degrees of cerebral ischemia and sometimes noticeable vasospasm, usually appearing between 2 and 14 days after the stroke, its strength being primarily related to the amount of extravasated blood, especially when rebleeding. Although the vasospasm can occur at the place of the rupture, it is also known to appear elsewhere. The arterial contraction can be strong enough to cause cerebral ischemia or even infarctation.[10] Hence, even if a patient survives the initial bleeding, he/she may still suffer from this second pitfall, that of the cerebral vasospasm.

Extensive work has been done to determine the reasons for vasospasm developing, different hypotheses having been presented to explain it: release of neurotransmittors, creation of reactive oxygen species due to extravasated blood,[11] and inflammation and angiogenesis occurring in the affected areas.[12,13] The oxidation products include 8-iso-PGF2α (derived from arachidonic acid), oxy-hemoglobin and BOXes. These are vasoactive substances that can cause powerful and long lasting arterial constriction, yet none of these substances can completely explain the development of vasospasm.[11] Previous studies in our laboratory have shown there to be an increase in arterial contractility dependent in part on the upregulation of the ET_B and 5-HT_{1B} receptors,[7,8] which could help explain the development of vasospasm.

An effective treatment is yet to be presented despite the current knowledge regarding the development of vasospasm. The major treatments of SAH available today are with Ca^{2+} channel blockers and use of angioplasty[14,15] but this treatment have so far not been proved in randomized clinical trails for SAH.[16,17] There is ample evidence regarding the presence of vasospasm following SAH, but no conclusion can be drawn as to why it develops and there is no adequate therapeutic means of counteracting it.

3.1.2 Thromboembolic strokes

The disruption of a plaque or thrombus that is later lodged in a cerebral artery is a common cause of thromboembolic strokes. It causes a local reduction in cerebral blood flow and cerebral ischemia creating an ischemic core surrounded by a penumbra. This is not a static state since if there is little or no perfusion the ischemic core will grow whereas if a strong and rapid reperfusion takes place the core becomes smaller. The major treatment today is by means of thrombolytic drugs, rtPA, although its effectiveness varies with the time at which treatment is given. Unless treatment with rtPA occurs within 3 hours, there is a risk of a hemorrhagic transformation of the stroke.[14] Although extensive research has been devoted to the development of neuroprotective drugs, the results obtained by them in clinical trials have thus far been poor.[4]

Research in this laboratory has shown that there is an upregulation of contractile receptors similar to that seen after SAH in rat.[5-7] Some research has shown in man that a blocking of one of these contractile receptors, AT_1, leads to a reduction in mortality and morbidity following a stroke,[18] and there are indications of it having a similar effect in experimental animal models.[6] Other factors found have a clear effect on physiology after a thromboembolic stroke are inflammation[19] and immune cell infiltration.[20]

3.2 Mitogen activated kinases (MAPKs)

3.2.1 Introduction

Following ischemia, as indicated already, there is an upregulation of contractile receptors in the cerebral arteries.[5-8] There is also evidence that suggest the activation of PKC and ERK1/2 to be involved in this receptor upregulation.[21-25] There are indications too of MAPK activation in neuronal cells occurring after cerebral ischemia[26] and of this affecting the expression of inflammatory cytokines.[27] Treatment with antisense MAPKs has been shown to affect the degree of vasospasm that occur[28] and altered ratios of MAPKs in hypertensive rats affects the

vasoconstriction.[29] Results regarding this, taken in their entirety provide compelling evidence for the importance of MAPKs following cerebral ischemia.

3.2.2 Properties and regulation

The MAPKs are important for a number of processes in different cell types and under a variety of conditions. The three major MAPKs are p38, ERK1/2 and SAPK/JNK, each of them located at the end of a dynamic chain of kinases. A kinase is a protein able to add a phosphogroup, phosphorylate a protein containing an OH group in a serine, threonine, or tyrosine residue. Activation is achieved by the addition of phosphogroups on specific residues to bring about conformational changes, which open open up the active site of the protein thus enabling interaction or phosphorylation of downstream targets to take place. The signaling cascade that is

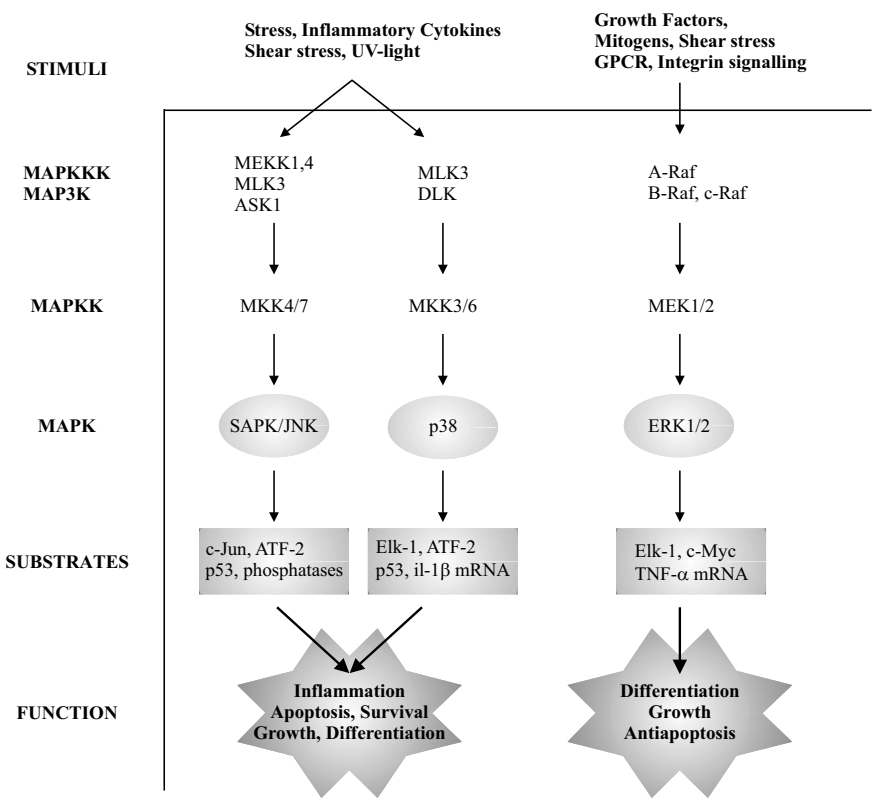

Figure 3.1 MAPK activation cascade.

produced for each of the MAPKs is complex showing both positive and negative interactions with upstream proteins that determines activation of each of the MAPKs.

Each MAPK is part of a signal pathway containing a MAPKKK that can activate MAPKKs, which in turn activate MAPKs (Figure 3.1). A kinase is able to phosphorylate several downstream targets. Accordingly; each cellular stimulus that results in MAPKKK activation can cascade through an increase in the number of active downstream kinases, which increases the signal strength at each step.

The negative feedback that occurs is a major factor to account for in discussing the activation of MAPKs. In contrast to the activating signals that leads to phosphorylation of the MAPKs are dephosphorylation signals.[30] They are just as important for signaling as the kinases are, increasing the phosphatase activity impairs the cellular response to stimulus whereas under normal conditions the phosphorylation would be balanced by dephosphorylation, thereby ensuring that the activating signal is of a proper strength and length.

Accordingly, the activation of MAPKs is complex, one needing to take into account not only of the positive signals that activate individual pathways, but also of possible synergistic effects and negative feedback loops that affect endpoint activation. The same signal can in different tissues give different responses depending on the strength and duration of the signal.[30] In general, the stimulus needs to exceed a certain threshold in order to enable an activation cascade to occur, ensuring distinct activation at the proper times. Such an activation cascade leads to a cellular response, the character of which depend on the signaling pathways activated there, the type of cells involved, in which cell type and the cellular environment.

3.2.3 Activating signals and their function

There are several different stimuli that are able to activate the p38 and SAPK/JNK pathways, whereas ERK1/2 activation differ somewhat. p38 and SAPK/JNK are considered inflammatory pathways and tend to be activated by inflammatory cytokines and cellular stress, ERK1/2 being considered more mitogenic and being activated by growth factors.[31-34] There is a large degree of redundancy between the

different activating signals,[35] however despite gene disruption experiments having revealed that the different MAPKs have separate functions.[36]

These MAPKs elicit some of their effect through the phosphorylation of transcription factors, thus initiating DNA binding and transcriptional regulation. This transcription factor activation is somewhat redundant between the various MAPKs, with the same transcription factor being activated by two or more MAPKs. There are also more direct effects of the MAPK activity in the form of cytokine mRNA transportation from the nucleus,[35] increased translation and stability of the mRNA[33,37,38] and the general upregulation of transcription through the acetylation of histone H3.[39] MAPK activation is also able to affect cellular apoptosis, in part through p53 activation.[31]

3.3 Inflammation and Matrix Metalloproteases (MMPs)

The fact that there is an inflammatory response following a stroke, either of a hemorrhagic or a thromboembolic character, is well known[12,40] and there are reports indicating that the inflammatory response is just as important as reperfusion.[19] Included in this response are oedema, immune cell infiltration and increases in cytokine, chemokine and MMP expression all of them factors coupled with stroke in man.[41] An increase in oedema can reduce the blood flow, augment the ischemia following a stroke, and directly affect neuronal cells through mechanical stress thus increasing the neurological damage.[31] The chemokines and the MMPs are known to participate in immune cell infiltration into the CNS[42,43] and it has been shown that inhibition of the infiltration of immune cells decreases arterial contraction following stroke.[40]

There are also secondary negative effects combined with these direct effects. There has been shown to be a strong immune suppression following stroke, this seen as an increased bacterial titer in the blood of rats following experimental MCAO and through impaired lymphocyte cytokine production.[44] This immune suppression has not yet been fully elucidated, it is thought to be due to activation of the central nervous system.[44]

As mentioned earlier, MMPs are a part of the inflammatory response where they participating in the recruitment of immune cells by facilitating their migration through the blood-brain barrier.[43] They are proteases capable of degrading extracellular matrix proteins, thereby opening a route for immune cell infiltration. The degree of damage to the blood-brain barrier is a marker for the severity of vasospasm following SAH,[45] and research has shown that alteration of the extracellular matrix proteins can augment vasospasm.[46] It is not suprising, therefore, that several MMPs have been linked with severity of stroke in both man[47] and in rat.[48] There is conflicting evidence, however, there being reports of beneficial effects of MMPs in CNS recovery and regeneration.[49,50]

4. Aims of the thesis

The general aim of the thesis has been to investigate molecular events that occur prior to and following after a stroke in cerebral arteries. The more specific goals have been the following:

- To investigate the gene regulation in cerebral arteries following SAH, aimed at determining what the important processes involved in the increase in contraction are.

- Investigation of MAPK activation and its relation to gene expression.

- Comparison of the ischemic models MCAO and SAH with organ culture of cerebral arteries to determine similarities and to validate the use of organ culture as a model.

- Investigation of gene expression and protein regulation in human MCA following thromboembolic stroke to validate previous findings in connection with experimental rat models.

- Molecular characterization of smoke induced changes in cerebral arteries.

5. General methods

The methods used throughout this thesis are such molecular techniques as microarray (paper I and II), real-time PCR (all the papers) and immunohistochemistry (all the papers). They have been applied to cerebral arteries, both human (paper II) and rat (paper I and III-V). The rat cerebral arteries have been subjected either to organ culture (papers III and V) or to an ischemic stroke model. The two cerebral ischemia models employed are experimental SAH (papers I, III and IV) and MCAO (paper III). Functional responses were investigated by use of myographs to investigate the contractility of the arteries; this in paper V, as were the use of DSP (lipid soluble smoke particles).

5.1 Molcular techniques

5.1.1 RNA preparation and cDNA synthesis

The RNA preparations for the microarray and for the real-time PCR involved use of Trizol according to the manufacturers' instructions. The RNA was then resuspended in 10 µl of nuclease free water, the 260/280 values being measured using a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Sweden). The typical 260/280 values were between 1.7 and 1.9, indicating a high level of purity. The cDNA synthesis involved use of superscript III according to the manufacturers instructions (Applied Biosystems, Sweden) in 40 µl reactions using 1 µg of RNA in each reaction. In the case of microarray, the RNA wasused in labeling reactions instead of cDNA production.

5.1.2 Microarray

Microarray is a technique for quickly screening the expression of thousands of genes at a time. The screening is done by extracting RNA from the cells of interest and labeling it in a reverse transcriptase reaction, using biotinylated bases. The biotinylated bases enable later detection to occur. The labelled RNA, so called probes, are hybridised to a solid medium containing complementary sequences for the genes

of interest. These complementary sequences are either spotted onto a microarray chip, then termed a cDNA spotted array, as in paper II or are created directly on the chip as in the Affymetrix technology employed in paper I. Unbound or unspecifically bound probes are removed during stringent washes to ensure later signal specificity. Antibodies directed against biotin are then employed for visualizing the number of probes that are bound. The fluorescence from the antibodies is detected at the appropriate wavelength, the signal strength being measured. The amount of fluorescent or signal strength correlates with the amount of the bound probe, thus indicating the amount of expression of the gene in question. Because of certain technical issues as probe labeling, risk of unspecific binding and risk of differences in washing efficiency, this technique yields only semiquantitative data.

5.1.3 Real-time PCR

Real-time PCR allows quantitative measurements of RNA to be made through use of a PCR reaction. This is done by first preparing RNA and then converting this to cDNA as described above. A PCR reaction is then performed using gene specific primers. A fluorescent dye, SYBR green is used for detection since its fluorescence increases several thousand folds when it is bound to double stranded DNA. Thus, as the reaction progresses there is an increase in fluorescence that directly correlates with the amount of product amplification that takes place. A Ct value, the cycle number when the fluorescence has passed a set threshold value, is then used to calculate the relative concentration in the sample. A negative control is added to each run to check for contamination and primer-dimer formation. To control for differences in the starting material and in the cDNA reaction efficiency, the expression of one or more housekeeping genes is utilized, β-actin, EF-1 and GAPDH have been used here as housekeeping genes.

5.1.4 Immunohistochemistry

Immunohistochemistry is a method that allows a specific protein in a sample to be detected. This has been carried out here with use of fluorescence and DAB staining. Sample preparation is the same for both types of immunohistochemistry. The sample is placed in TissueTech (Gibco, Sweden) and is frozen. The samples are sectioned

into 10 μm thick slices using a calibrated Microm HM500M cryostat (Microm, Germany) and the sections are then fixated and permeabilized in -20ºC acetone. The samples are rehydrated using PBS and are incubated together with the primary antibody at an appropriate concentration over night. All dilutions are in 5 or 10% serum to ensure antibody specificity. Excess antibody is removed through washes, after which incubation together with with a fluorescent secondary antibody is performed. For the DAB staining procedure, a biotinylated secondary antibody is used instead of fluorescence. An HRP-streptavidin conjugate is bound to the biotinylated antibody through incubation. DAB is then added, creating a brown staining where HRP is present. The fluorescence is detected at the appropriate wavelength using a confocal microscope (Zeiss, Germany) and the DAB stainings by use of a bright field microscope. Only secondary antibodies were used as control. The images were analyzed using ImageJ (http://rsb.info.nih.gov/ij/).

We treat this as a semiquantitative method, in much the same way as the microarray, since all the comparisons between samples are made on samples stained at the same time, using the same antibody dilutions and identical microscope settings. All incubation times are likewise kept the same, exposure of the fluorescent antibodies to the laser being kept to a minimum. A comparison of the samples was made in terms of degree of fluorescence.

5.1.5 DSP extraction

Three cigarettes (0.8 mg nicotine per cigarette) were "smoked" by a water aspirator, the smoke being directed through a cotton wool filter. The smoke particles retained in the filter were dissolved in 1 ml DMSO as described by Zhang et al,[51] 1 μl of such a preparation being added to the incubation medium prior to incubation.

5.2 Ischemic models and functional investigations

5.2.1 Experimental SAH

Male Sprague-Dawley rats (350-400 g) were anaesthetized and kept anaesthetized by use of Halothane. Respiration was monitored by regularly withdrawing arterial blood samples for blood gas analysis in a Radiometer blood gas analyser ABL 520.

Catheters were inserted to monitor blood pressure and intracranial pressure. Finally, a 27G blunt canula with side holes was introduced stereotactically 7.5 mm anterior to the bregma in the midline at an angle of 30° to the vertical, the tip of the needle being placed in the prepontine cistern. After 30 minutes of equilibration, 250 µl of blood was injected intracranially at a pressure equal to the mean arterial blood pressure. Our model differed from the model originally devised by Svendgaard et al[52] in that the rats were hydrated subcutaneously using 40 ml isotonic sodium chloride at the end of the operation and on day one to reduce mortality. The rat was kept under anaesthesia for another 60 minutes to allow recovery from the cerebral insult that occurs, after which the catheters were removed and the incisions were closed. The rat was then revitalized and extubated. For a detailed description see Prunell et al.[52]

5.2.2 MCAO

Male Wistar rats were kept anesthetized by inhalation of 1.5% halothane through a mask. To confirm proper occlusion and subsequently a proper reperfusion of the right MCA, a laser-Doppler probe (Perimed, Sweden) was fixed to the skull (1 mm posterior to the bregma and 6 mm from the midline on the right side), to measure the blood flow in the area supplied by the right MCA. A polyethylene catheter was inserted into a tail artery for measurements of mean arterial blood pressure, pH, pO_2, pCO_2 and plasma glucose. Thereafter, an 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 with sutures. A filament was inserted into the internal carotid artery via an incision in the common carotid artery, and was advanced until the rounded tip reached the entrance of the right MCA. The resulting occlusion was made visible by laser-Doppler flowmetry as an abrupt reduction in cerebral blood flow by 75-90%. The rats were then allowed to wake up. Two hours after occlusion the rats were reanaesthetized briefly to allow for withdrawal of the filament and reperfusion to be achieved. The inclusion criterion was a proper occlusion (>75% reduction of regional blood flow) as measured by laser-Doppler. For a detailed description see Memezawa et al[53] and Stenman et al.[5]

5.2.3 Organ culture

The organ culture has been described previously be Adner and collegues.[54] Male Sprague-Dawley rats were anesthetized and decapitated. The brains were removed and immediately chilled in a cold bicarbonate buffered solution. Major cerebral arteries, the right and left MCA and BA, were removed and were placed in DMEM supplemented with penicillin (100U/ml) and streptomycin (100µl/ml), amphotericin B (25µg/ml) prior to incubation at 37ºC / 4% CO_2. DMSO and DSP were added prior to the addition of arteries to the medium.

5.2.4 Myograph investigations

Myograph experiments were used to investigate the contractile properties of the arteries.[55,56] The arteries were cut into cylindrical segments and were mounted onto two 40 µm diameter stainless steel wires in the Mulvany-Halpern myographs. One of the wires was connected to a force transducer attached to an analog-digital converter unit. The other wire was attached to a movable-displacement device allowing for adjustments of arterial tension. The responses were recorded on a computer by use of the software Chart™. The segments were immersed in a temperature controlled (37 ºC) bicarbonate buffer which was continuously gassed with 5 % CO_2 in O_2 resulting in a pH of 7.4. The contractile capacity was determined by exposure to a K^+ rich buffer, this value being used then as a reference value. Concentration-response curves for ET-1, S6c and AngII were obtained using cumulative application of them at increased concentrations. Maximum concentration was denoted E_{max}, and pEC_{50} representing half the maximum response.

5.3 Statistics and data presentation

The statistics used to determine differentially expressed genes in the microarray experiments were the LPE test at a 0.01% False Discovery Rate[57,58] (LPE, S+ Array Analyser software) in paper I and SAM (http://www-stat.stanford.edu/~tibs/SAM/) in paper II. All the real-time PCR, immunohistochemistry and myograph data is presented as mean ± S.E.M.

The results were analysed using the Grubbs test, samples deemed as outliers being removed. A two sided Students t-test was used for comparing two groups in paper I and II, the Mann-Whitney test in papers III-V, and the Kruskall-Wallis test together with the Dunns post test for comparisons between more than two groups. * denotes a p-value ≤ 0.05, which was considered significant.

6. Results and comments

The articles contained in the thesis will first be presented in terms of how the work progressed in the laboratory. There will then be a more thorough discussion of the results reported in the articles and of their relation to the literature. These five articles deal with stroke-related changes in cerebral arteries ranging from the period prior to the stroke (paper V) to intermediate changes (papers I, III and IV) on to endpoint changes after a fatal stroke (paper II) (Figure 6.1).

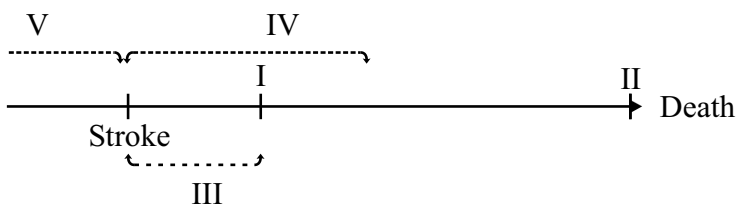

Figure 6.1 Time line showing the papers relative placement in relation to a stroke

6.1 Presentation of papers

6.1.1 Gene expression and molecular changes in cerebral arteries following subarachnoid hemorrhage in rat. (Paper I)

Previous investigations have shown there to be an upregulation of contractile ET_A, ET_B and 5-HT receptors in cerebral arteries 48 hours ours following SAH.[7,8] Through the work of Svendgaard et al[59] there is evidence of a decrease in cerebral blood flow in rats following SAH, using this particularly well studied experimental model for SAH.[52,60] There have been a number of hypotheses concerning the reasons for the increase in arterial contractility observed after SAH, such as the release of neurotransmitters, the formation of reactive oxygen species, and the occurrence of inflammation and angiogenesis.[10,11,60,61] These processes may contribute to or enhance the arterial contractility observed.[12,13] There is little information, however, regarding molecular changes in the cerebral arteries. Accordingly, the effort in paper I was directed at extending the knowledge regarding changes in gene expression at the time 24 hours after SAH has occurred. This time point chosen as one preceding the maximum blood flow reduction that occurs after about 48 hours in our experimental

model.[52,59,60] To achieve this, gene expression in cerebral arteries 24 hours post-SAH, was investigated using microarray technology and real-time PCR and protein amounts by use of immunohistochemistry.

We made use of Affymetrix RAE203 A and B chips during the microarray which together contains sequences corresponding to over 35000 of the genes or ESTs found in rat. The expression data was mined using the local pooled error test,[57,58] the genes found to be significantly regulated being sorted according to their function. This showed that the major gene groups found to be regulated were inflammatory, extracellular-matrix-regulating, apoptosis and metabolic genes **(**Table 6.1**)**.

Table 6.1 The major gene groups found regulated by microarray in cerebral arteries 24 hours after SAH.

Gene name	Gene Title	Accession number	Expression
Receptors (3)			
Gucy1a3	guanylate cyclase 1, soluble, alpha 3	NM_017090	0.571187
Inflammatory and Immuno related (21)			
Cxcl2	chemokine (C-X-C motif) ligand 2	NM_053647	18.16244
Il6	interleukin 6	NM_012589	10.69899
Scya4	small inducible cytokine A4	U06434	2.224858
Extra cellular matrix and adhesion (9)			
Mmp13	matrix metalloproteinase 13	M60616	55.20392
Mmp9	matrix metalloproteinase 9	NM_031055	8.441764
Vtn	vitronectin	NM_019156	0.602555
Apoptosis and intracellular pathways (12)			
Nos2; Nos2a	inducible NO synthase	NM_012611	6.01174
Superoxides (5)			
Metabolism / cell function (15)			
Calcium related (5)			
Intracellular proteins (8)			
Unspecified (60)			

Regulation of the metabolic genes was considered to be due to the ischemic episode following SAH and to be more of a marker for SAH rather than driving it. Accordingly, these genes were not investigated further. To quantify the changes observed using microarray, which is a semi-quantitative method, quantitative real-time PCR was employed. The genes chosen for further investigation were selected on the basis of changes in expression of them in combination with the information available regarding their function (Il6, cxcl2, MMP9 MMP13, iNOS, vitronectin,

Scya4 and Gucy1a3). Several receptors previously reported as being important after a stroke were also included in the investigation, ET_A, ET_B, 5-HT_{2A}, 5-HT_{1B}, 5-HT_{1D}, AT_1 and AT_2. Seven of the genes investigated were found to be significantly regulated as observed by real-time PCR (Figure 6.2). Immunohistochemistry was used to verify the changes in gene regulation observed being translated into protein, our

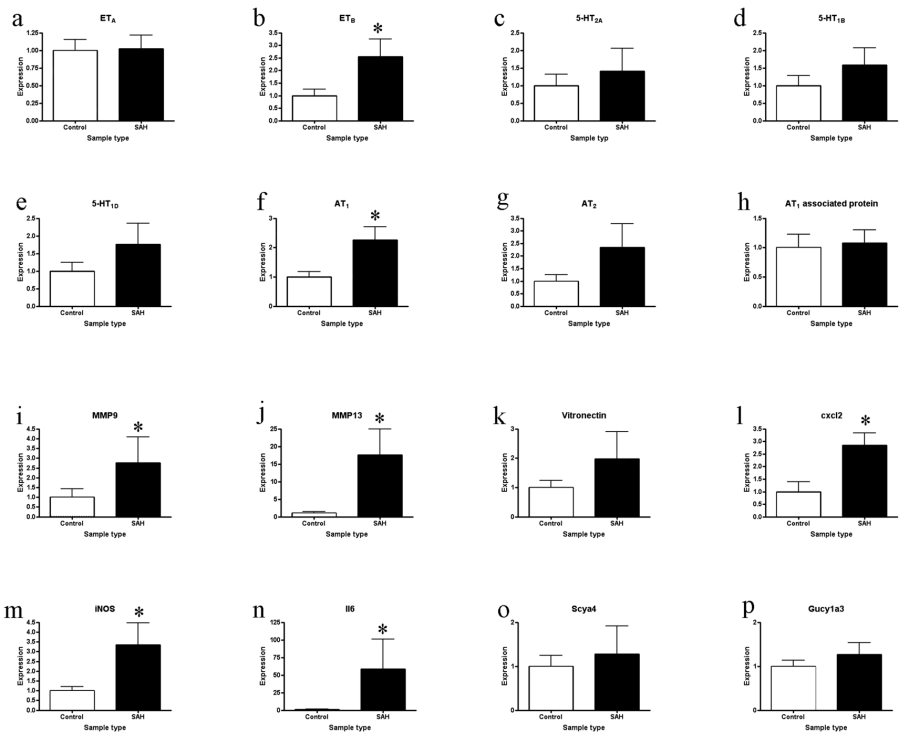

Figure 6.2 Gene expression in cerebral arteries 24 hours following SAH as investigated with real time PCR.

finding a significant upregulation here of ET_B, 5-HT_{1B} and 5-HT_{1D} correlating with the real-time PCR data (Figure 6.3). Of the microarray genes found significantly upregulated Il6, MMP9 and iNOS are previously reported in conjunction with cerebral ischemia,[20,41,62] whereas cxcl2 and MMP13 are quite novel findings. An upregulation of ET_B,[5,7] 5-HT_{1B},[8] and AT_1[6] has been reported previously to be found 48 hours after cerebral ischemia, whereas we showed here that the upregulation occurs already after 24 hours and that for ET_B, 5-HT_{1B} and 5-HT_{1D} there are increased amounts of protein in the smooth muscle layer of the arteries.

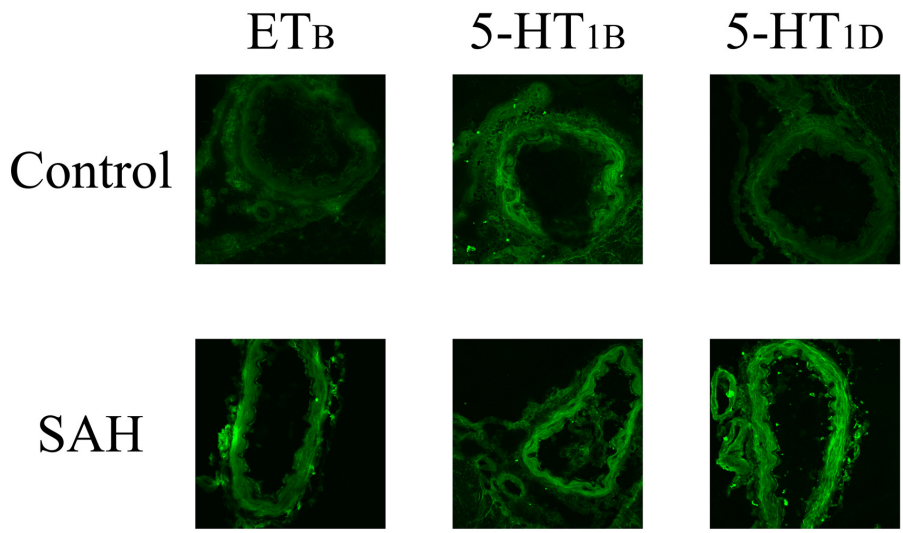

Figure 6.3
Immunohistochemistry investigation of ET_B, 5-HT_{1B} and 5-HT_{1D} receptor amounts in cerebral arteries 24 hours following SAH, This investigation showed that there were increased amounts of receptors in the arteries following SAH.

6.1.2 Gene expression profiling in the human middle cerebral artery after cerebral ischemia. (Paper II)

As mentioned earlier there is ample evidence of increased expression of vasocontrictive receptors in cerebral arteries following ischemia and after organ culture.[5-8,63-66] In paper II we investigated whether these receptors were upregulated in human cerebral arteries following ischemia as well. The expression of ET_A, ET_B, AT_1,

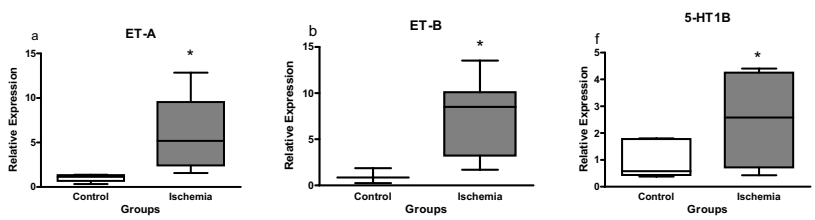

Figure 6.4 Real time PCR investigation of endothelin and 5-HT_{1B} receptors amounts in human MCA following a thromboembolic stroke.

AT_2 and 5-$HT_{2A/1B/1D}$ receptors were investigated in human MCA following a thromboembolic stroke and were compared with that in the MCA taken from subjects

who were deceased due to extracranial causes. Protein levels were investigated using immunohistochemistry. We here found that there was a significant upregulation of ET_A, ET_B and 5-HT_{1B} receptors as based on real-time PCR (Figure 6.4) and also there to be a significant upregulation of the endothelin and 5-HT_{1B} and AT_1 receptors in terms of the protein levels (Figure 6.5). Theses findings are consistent with previous ones.[5-8,65] Since results based on the samples were found to be valid rather than then reflecting unspecific degradation, we decided to further investigate the gene

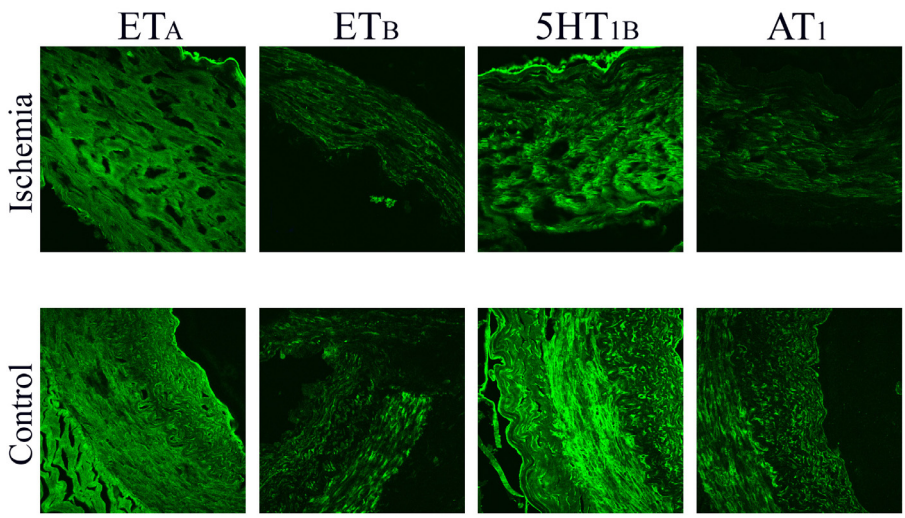

Figure 6.5
Immunohistochemistry investigation of endothelin and 5-HT_{1B} and AT_1 receptors amounts in human MCA following a thromboembolic stroke showing increased amounts in the stroke arteries.

expression in these arteries with use of microarray. This involved our investigating gene expression in the MCA leading to the ischemic region and we found 82 genes upregulated and 17 downregulated significantly by use of SAM (http://www-stat.stanford.edu/~tibs/SAM/). The genes found to be significantly regulated included those involved in cellular signaling, intra and extracellular-matrix-remodeling. Real-time PCR was used to confirm the changes in expression obtained by microarray. We were thus able to conclude that the cerebral arteries in both rat and man upregulate contractile receptors following ischemia. The investigation based on microarray revealed changes in gene expression that supported the occurrence of dynamic changes in human cerebral arteries following a thromboembolic stroke.

6.1.3 Activation of p38, ERK1/2 and SAPK/JNK initiate transcription of inflammatory and extracellular matrix genes in cerebral arteries following cerebral ischemia in rat. (Paper III)

The two investigations just described concerned molecular changes in cerebral arteries after ischemia. There are similarities regarding upregulation of receptors following ischemia in these two experimental models, MCAO and SAH,[5-8,65] a similarity shared with organ culture.[54,64-67] I decided, therefore, to investigate whether these two ischemic models and organ culture shared the regulation of other ischemia related genes and whether there were similarities in the activation of signal transduction pathways. The expression of inflammatory and extracellular-matrix-related genes found in paper I, along with il-1β an TNF-α was investigated on the basis of previous indications of these genes being linked with both thromboembolic and hemorrhagic stroke.[45,47,68-70] First the phosphorylation of three different MAPKs (p38, ERK1/2 and SAPK/JNK) and their downstream transcription factors (ATF-2, Elk-1 and c-Jun) were investigated in post-ischemic cerebral arteries (SAH and MCAO) and were compared with results for organ culture. These MAPKs shared various activation signals and all of the are affected by shear stress, a common factor in these models.[31] Activation was investigated by means of

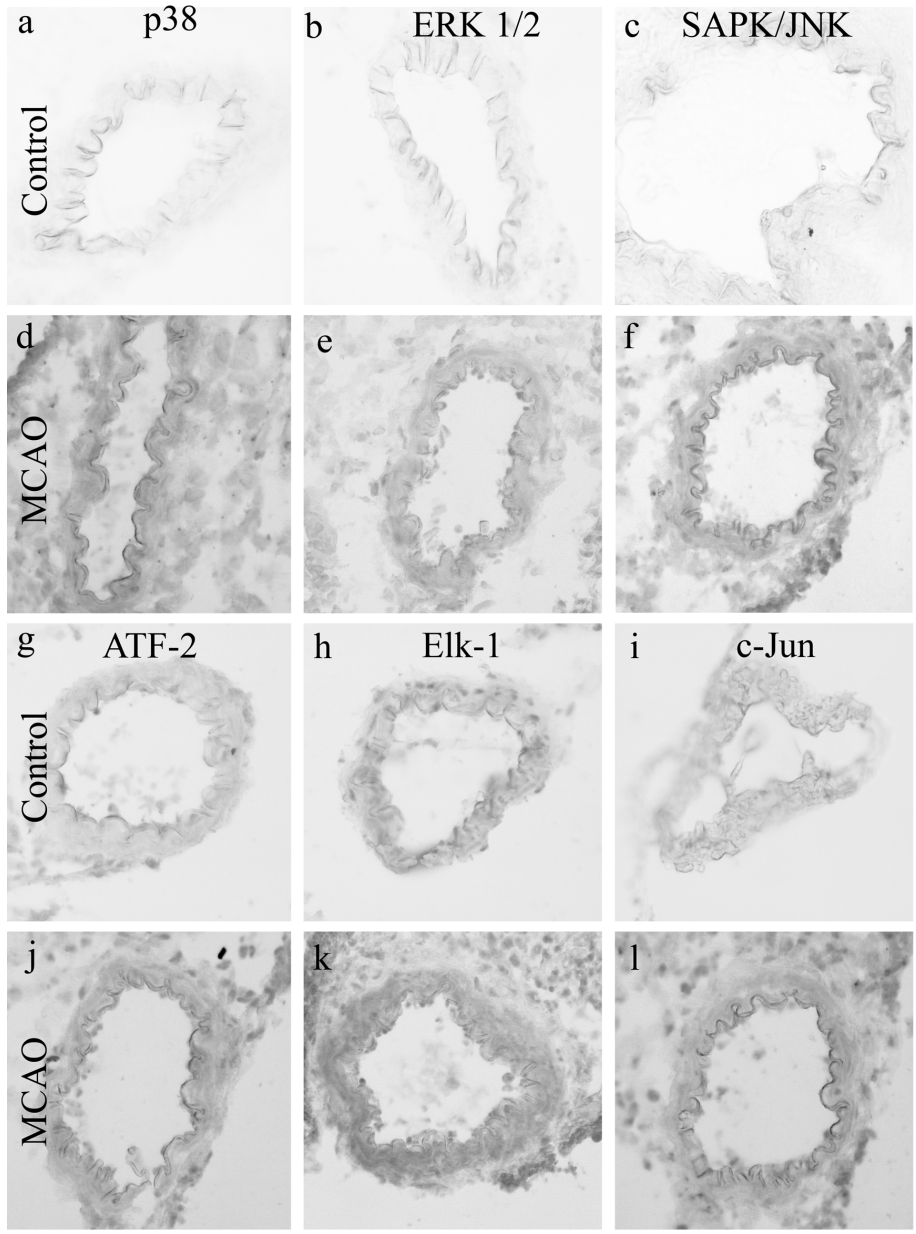

Figure 6.6. MAPK activation in fresh MCA and following MCAO. There is increased amounts of p38, SAPK/JNK and ERK1/2 MAPK and their downstream transcription factors ATF-2, Elk-1 and c-Jun.

immunohistochemistry, using antibodies specific for the phosphorylated form of the MAPKs and the transcription factors. There was a strong activation of the MAPKs following both cerebral ischemia and organ culture, whereas little or no activation was found in fresh arteries (Figure 6.6). The phosphorylation on both the contralateral and ipsilateral side was investigated for MCAO, phosphorylation being found on the contralateral side, although not as strong as on the ipsilateral side. A similar activation of the downstream transcription factors (ATF-2, Elk-1 and c-Jun) was found which correlates to MAPKs activation.

The expression of the genes found in the microarray investigation in paper I (Il6, cxcl2, iNOS, MMP9 and MMP13) and two other inflammatory genes found to be important after ischemia (TNF-α and il-1β) were also investigated using real-time PCR. The inflammatory genes and the MMPs that were investigated showed increased expression after both cerebral ischemia and organ culture, except for iNOS in the MCAO model (Figure 6.7). To verify the ability of the transcription factors to activate the transcription of these genes, the promotors of the genes were investigated. This was done using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and MATCH (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi). Analysis of the promotors showed there to be binding sites for the transcription factors in the promotors of the genes investigated, indicating that the

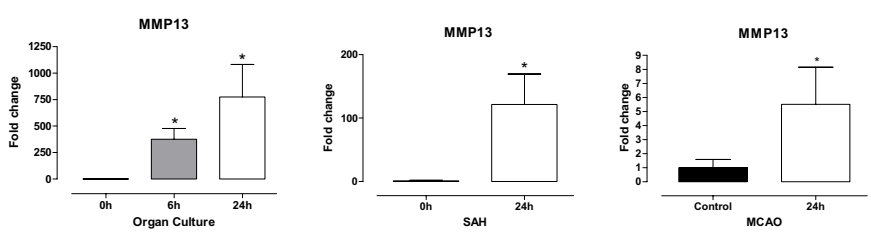

Figure 6.7 The gene expression of MMP13, one of the genes investigated after SAH, MCAO and organ culture.

MAPKs activated could be responsible for the transcriptional upregualtion observed. We thus concluded that there are similarities in the MAPK activation following cerebral ischemia and following organ culture. This activation could explain the observed gene upregulation and could also help explain the similarities in regulation referred to. This indicates that organ culture can be used as a tool to investigate molecular changes in cerebral arteries following ischemia.

6.1.4 Signal transduction, inflammation and gene activation in cerebral arteries following experimental SAH in rat. (Paper IV)

The investigation in paper I revealed that the cerebral arteries show an increased expression of inflammatory and extracellular-matrix-related genes following SAH. To further elucidate when and how these changes take place, we decided to conduct a detailed temporal study of molecular changes in the arteries. We decided here to

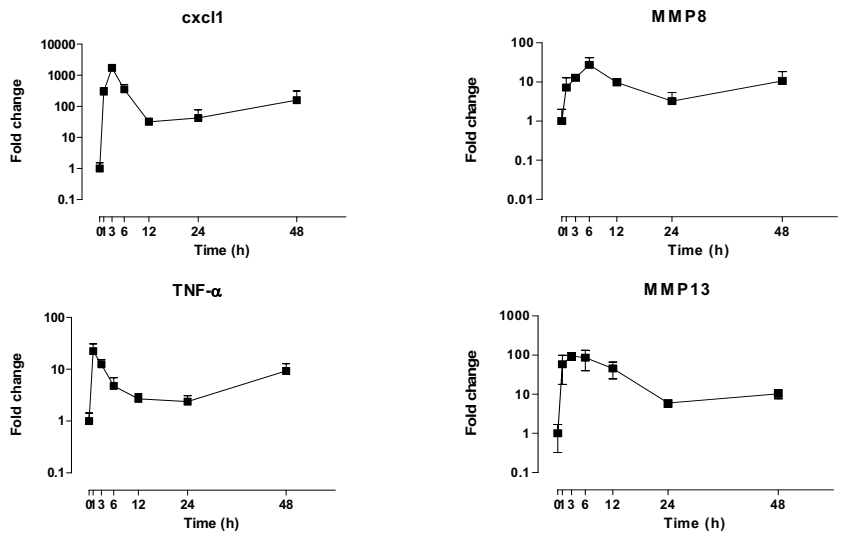

Figure 6.8 Gene expressions of inflammatory genes and extracellular matrix related genes following SAH.

follow MAPK activation and gene regulation from 1 to 48 hours following SAH. The protein levels of certain genes were also investigated using immunohistochemistry. The list of genes chosen for real-time PCR was extended as compared with that in paper I. We investigated the expression here of inflammatory and extracellular-matrix-related genes: Il6, TNF-α, Il-1β, cxcl1, cxcl2, ccl20, MMP8, MMP9, MMP13 and iNOS. The inflammatory genes are cytokines and chemokines, two gene groups previously reported to appear after ischemia.[42,71] The role of MMP9 after SAH has been documented[68] just as the importance of the extracellular matrix has been.[72] Thus, there exist some information in the literature, although little in conjunction to the arteries.

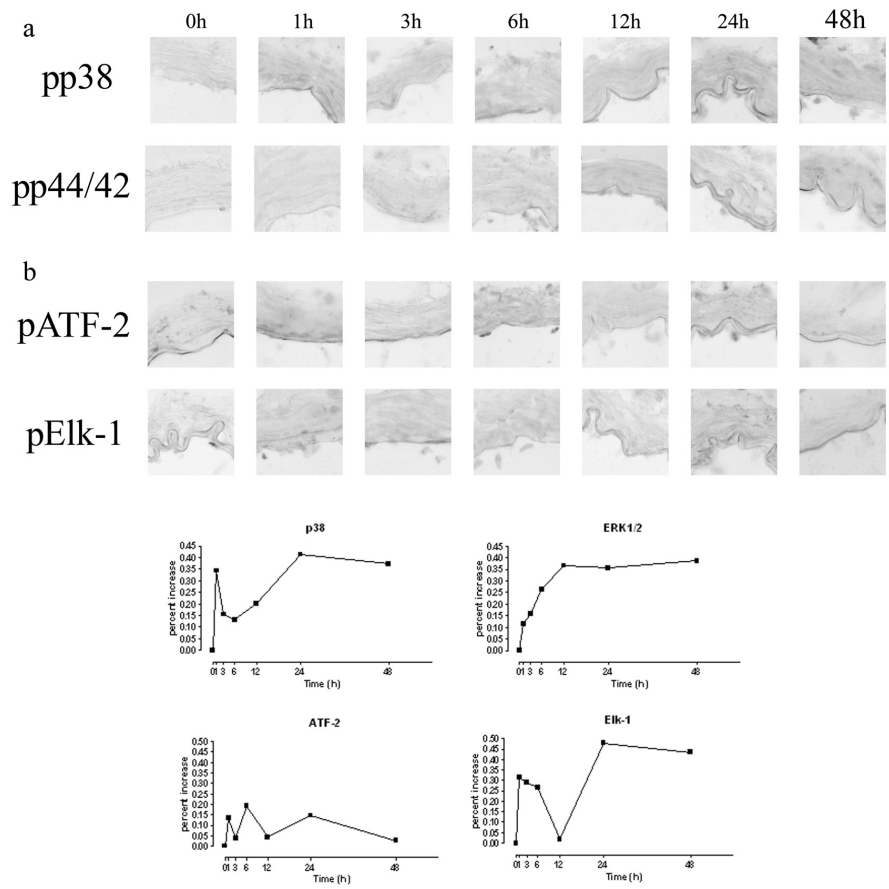

Figure 6.9 Activation of MAPKs in cerebral arteries following SAH. The activation of p38

The inflammatory genes investigated (Il6, TNF-α, Il-1β, cxcl1, cxcl2 and ccl20) showed an early peak in expression, followed by a slight decrease and a subsequent increase at 48 hours (Figure 6.8). The extracellular-matrix-related genes (MMP8, MMP9, MMP13 and iNOS) showed a similar expression pattern as the inflammatory genes except for MMP9 (Figure 6.8), which had a slightly later early peak. The MAPKs that were investigated showed an increase in activation following SAH as did their downstream transcription factors (Figure 6.9). In investigating activation by use of image analysis, we noted it to occur early, followed by a slight decline and a subsequent activation at 24 and 48 hours again for p38, ATF-2 and Elk-1. ERK1/2 showed an increase in activation during the period of 1 to 12 hours were it stayed

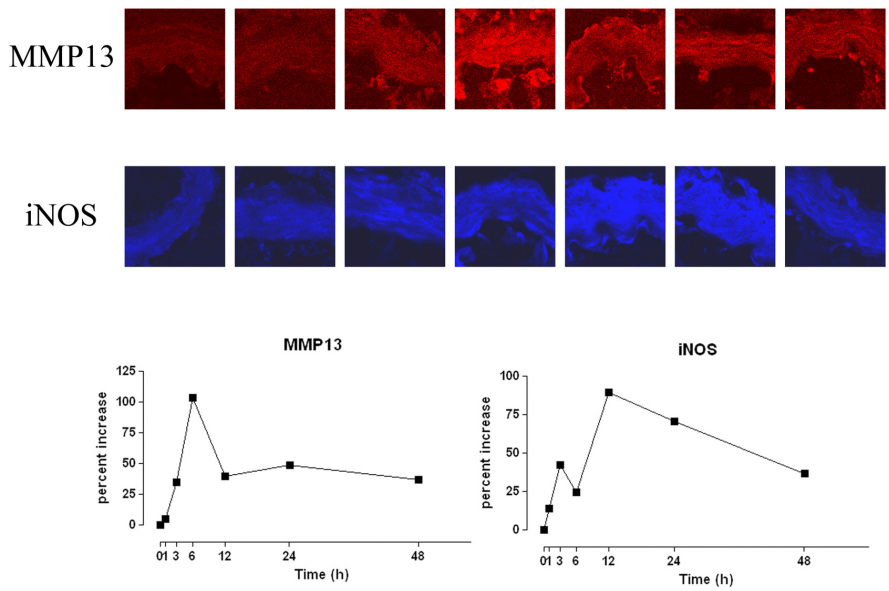

Figure 6.10. Immunohistochemistry and image analysis investigation of MMP13 and iNOS protein amounts in BA following SAH.

activated through 24 and 48 hours (Figure 6.9**)**. The protein analysis of MMP13 and iNOS showed there to be elevated levels of protein in the cerebral arteries following SAH, which corresponded to the real-time PCR data (Figure 6.10). These results indicated that the cerebral arteries to actively participate in the inflammatory processes that have been seen following SAH in the CNS neurons.[60] The transcriptional regulation and MAPK activation were similar, indicating that the MAPK activation could be responsible for the transcriptional regulation that occured. Promotor analysis of the genes, which had not been investigated in paper III, showed these genes to also contain binding sites for the transcription factors, providing further support for this conclusion. Higher protein levels in the regulated genes (Figure 6.10) were observed as early as 3 hours after SAH, showing the gene regulation to be translated into functional protein. Thus, the gene regulation observed was translated into functional protein that could affect cerebral circulation as early as 3 hours after SAH.

6.1.5 Lipid soluble smoking particles induce an inflammatory response in rat cerebral arteries via p38MAPK activation and downstream transcription factors ATF-2 and Elk-1. (Paper V)

Paper I-IV concerned changes that take place in cerebral arteries following cerebral ischemia. In paper V we decided to investigate how smoking particles could affect the cerebral arteries and whether this could explain the increased incidence of stroke in smokers.[73,74] This investigation was done on cerebral arteries incubated with DSP or DMSO. Previous studies have shown that nicotine *per se* or water soluble smoking particles have no effect on receptor expression, therefore DSP was employed. This contains the lipid soluble smoking particles having hydrophobic properties which give them the ability to traverse cellmembranes and the possibility to directly affect the cells. The investigation concerned the activation of MAPKs, (p38, ERK1/2 and SAPK/JNK) and their downstream transcription factors. The gene expression of several inflammatory and extracellular-matrix-regulating genes (Il6, cxcl2, iNOS, MMP9 and MMP13) as well as vasopressive receptors (ET_A, ET_B, 5-HT_{2A}, 5-HT_{1B}, 5-HT_{1D}, AT_1 and AT_2) was also investigated. In addition, we investigated the contractile response of the receptors to determine whether any regulations that took place affected this contractility. Protein levels of MMP13 and vasopressive receptors were assessed with immunohistochemistry.

There was found an increased activity of p38 in the cerebral arteries treated with DSP as compared to those treated with DMSO. There was also an increase in activation of the downstream transcription factors ATF-2 and Elk-1 (Figure 6.11). We also found a significant upregulation of the inflammatory genes following organ culture and a tendency towards even higher expression in the DSP treated arteries, albeit not significant. The expressions of MMPs was significantly elevated in the arteries treated with DSP as compared with both organ culture and the fresh arteries (Figure 6.12).

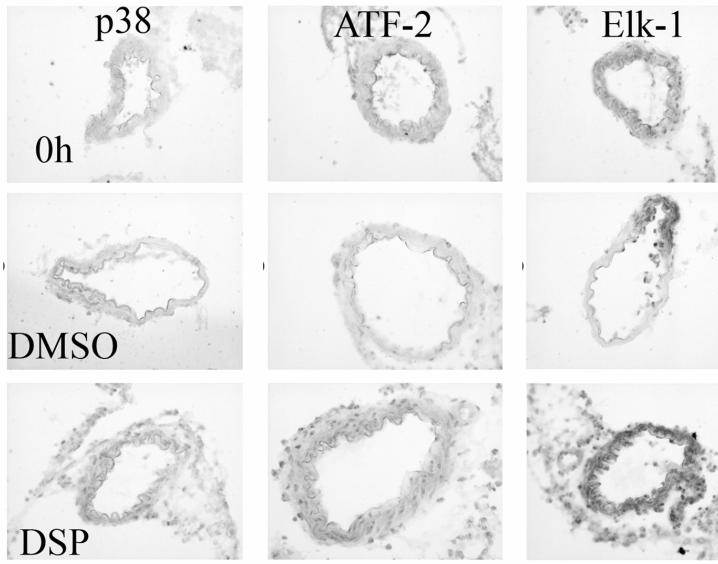

Figure 6.11 Immunohistochemistry analysis of p38, ATF-2 and Elk-1 in BA incubated with either DMSO or DSP. This revealed increased activity in the arteries treated with DSP.

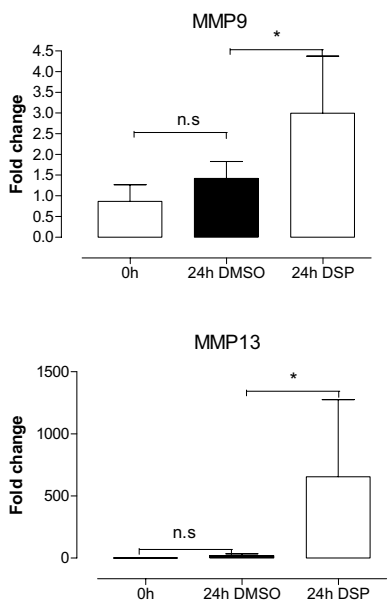

Figure 6.12 The gene expression of MMP9 and MMP13 following organ culture together with DSP.

Of the different vasopressive receptors that were investigated an increased expression of AT_1 receptors in comparison with the DMSO treated was observed whereas the others were not significantly regulated. Immunohistochemistry revealed there to be increased amounts of both MMP13 and AT_1 receptors in the cerebral arteries after DSP treatment in comparison to DMSO treatment while there was no increase in the number of AT_2 receptors (Figure 6.13). The myograph investigation of the

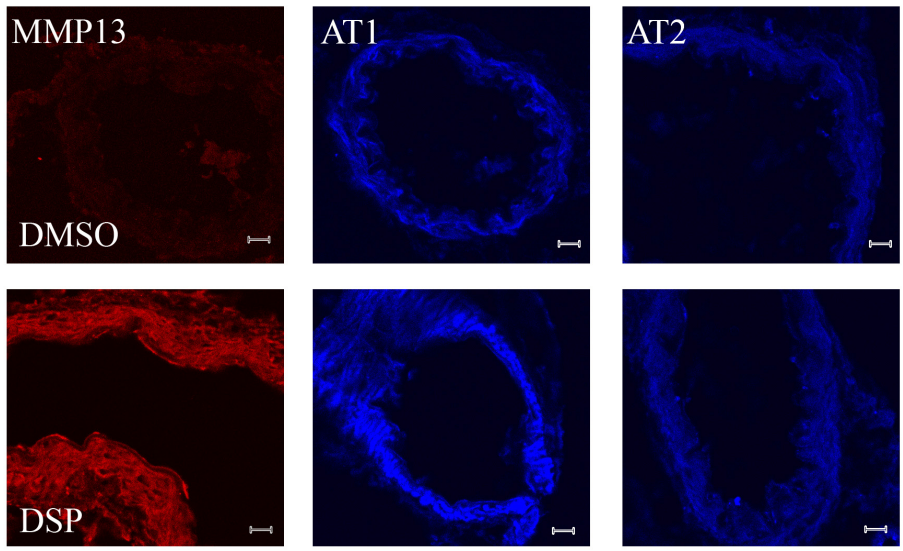

Figure 6.13
Immunohistochemistry revealed increased amounts of MMP13 and AT_1 in the BA treated with DSP in comparison to the DMSO treated. Note that there was no difference of the AT_2 receptor amounts.

arteries revealed an increase in response to Ang II in the DSP treated as compared with the DMSO treated arteries, whereas there were no difference in the S6c response (Figure 6.14). This increase was not affected by the AT_2 receptor specific antagonist PD123319 (100mM), whereas it was abolished by candesartan (10^{-7}M), a specific AT_1 receptor antagonist.[75] These results indicate that the cerebral arteries respond to cigarette smoke through an inflammatory response being introduced through p38 activation and through secretion of MMPs. An increase in MMP secretion in plasma has been reported previously[76] but the present investigation shows that the cerebral arteries to produce it locally, putatively increasing the importance due to the local production. The arterial contractility was also increased following DSP treatment. Together these various factors could explain the increased risk of a stroke for smokers.

6.2 General Discussion

The papers presented deal with different aspects of the changes that occur in cerebral arteries following ischemia. They also concern different periods of time as can be seen in Figure 6.1 which indicates their relative place in relation to a stroke. The discussion that follows concerns primarily these results and how they relate to the literature. Above all, matters of inflammation (chemokines and cytokines), the extracellular matrix, and MMPs gene expression and how these processes are regulated and putative effects of this regulation are discussed.

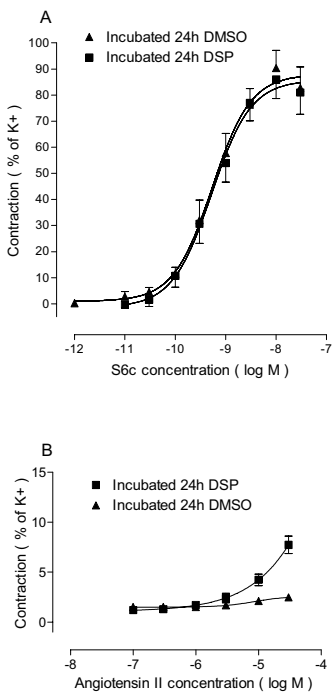

Figure 6.14 Functional studies revealed no difference in the response to S6c whereas there were an increased response to AngII in arteries treated with DSP in comparison to the DMSO treated arteries.

6.2.1 Arterial activation

I have shown that the cerebral arteries express inflammatory cytokines, chemokines and MMPs following a stroke. The initial investigation revealing this gene regulation was paper I in which an upregulation of these genes was reported. The investigation there concerned the gene expression 24 hours after SAH, a relative late point in time for an inflammatory response. It was not possible therefore with the basis of only the results of paper I to determine whether the arterial inflammation was a primary effect due to the ischemic episode itself or a secondary effect due to a general inflammatory response in the surrounding tissue. Paper IV concerned arterial changes during the period 1 to 48 hours post SAH showing that there to be upregulation of the inflammatory genes and the MMPs in the cerebral arteries as early as one hour post-SAH. These results indicate that the response evident in the cerebral arteries is due to the initial events that took place following the stroke, namely the changes in shear stress, the presence of extravasated blood and

ischemia since the response was so rapid. There would be an inflammatory response in the tissue surrounding the cerebral arteries as well, but since the gene regulation in the arteries took place within an hour, it would appear too short a period of time for this to first develop and to then affect the arteries.

There is ample evidence concerning the development and importance of inflammation and of MMPs after thromboembolic strokes as well.[19,41,47,77] There is also evidence of both SAH and MCAO upregulating contractile receptors in the cerebral arteries.[5-8] Paper III concerned similarities in gene regulation after global (SAH) and focal (MCAO) ischemia and a comparison of these with the gene regulation in organ culture, a method used extensively for studying the upregulation of such receptors.[22,23,64,65] This comparison showed that the cerebral arteries upregulated the expression of inflammatory genes and MMPs in both models of ischemia as well as after organ culture. Thus, the cerebral arteries appear to actively participate in the inflammatory response in a manner which is similar following both global and focal ischemia. The investigation also validated the use of organ culture as a model for studying some of the molecular changes that are seen in cerebral arteries following a stroke.

To study how this upregulation occurs I investigated the activation of three MAPKs, p38, ERK1/2 and SAPK/JNK in paper III and IV. The investigation in paper IV being aimed at elucidating temporal aspect of these MAPKs during the period after a stroke and paper III being aimed at elucidating whether the similarities in gene regulation was based on comparable activation in signal transduction between these models and organ culture. Paper IV showed that there was very early activation of the MAPKs, suggesting that MAPK activation is responsible for the increase in gene expression. This activation of the MAPKs was shown in paper III to occur in the arteries following MCAO and organ culture as well indicating the similarities between the two ischemic models and organ culture to include not simply gene regulation but also signal transduction. The reason for the MAPK activation is still unclear, but factors common to these models are changes in shear stress and cellular stress, factors known to clearly affect MAPK activation.[31,78] This likeness in MAPK activation between the two ischemic models and organ culture further justifies the use of organ culture to investigate molecular changes following a stroke.

These results concerned with gene regulation and MAPK activation in cerebral arteries following ischemia indicate that the cerebral arteries actively participate in the response seen following a stroke and not simply react to the surrounding tissue damage. We decided to examine how the arteries respond to lipid soluble smoke particles, in particular if there was a molecular response that could explain the increased risk of smokers suffering a stroke. Paper V indeed indicated that the cerebral arteries actively participate in this process as well with an increase in inflammatory genes and MMPs.

These changes were all observed in rat cerebral arteries following stroke. There are earlier reports of an inflammatory response and MMP expression in man is being of importance following a stroke.[41,62] In paper II we investigated the gene regulation in human MCA following thromboembolic stroke, to determine whether results from these experimental stroke models in rat are comparable to changes seen in man. I could note that there indeed was a high degree of similarity between rat and man concerning the gene regulation following a stroke. There was upregulation of vasopressive receptors and a gene regulation in the human MCA, indicating these to actively participate in the inflammatory response and in the remodeling of the extracellular matrix. Observe that the period of time for the investigation dealt with was much later in Paper II than in the experimental models (Figure 6.1). Since the samples were obtained from subjects deceased due to stroke, the samples probably represents endpoint changes in the arteries, whereas the findings obtained in connection with the experimental models applied to the period during which the ischemic core is developing. Yet, even though the exact genes investigated differed the same general processes appear to apply.

In my opinion therefore, the cerebral arteries actively participate in the response following a stroke, in part through MAPK activation which increases the gene expression of inflammatory and extracellular-matrix-regulating genes (Figure 6.15).

6.2.2 Effects of the arterial inflammatory response

I have shown that the cerebral arteries are capable of directly influencing the inflammatory response through cytokine and chemokine expression, both in rat and man. The expression of the cytokines following a stroke can affect the arteries directly, thus affecting the regulation of cerebral blood flow,[71,79,80] possible through the MAPK activating effects that occur[32] or through the secondary effects based on

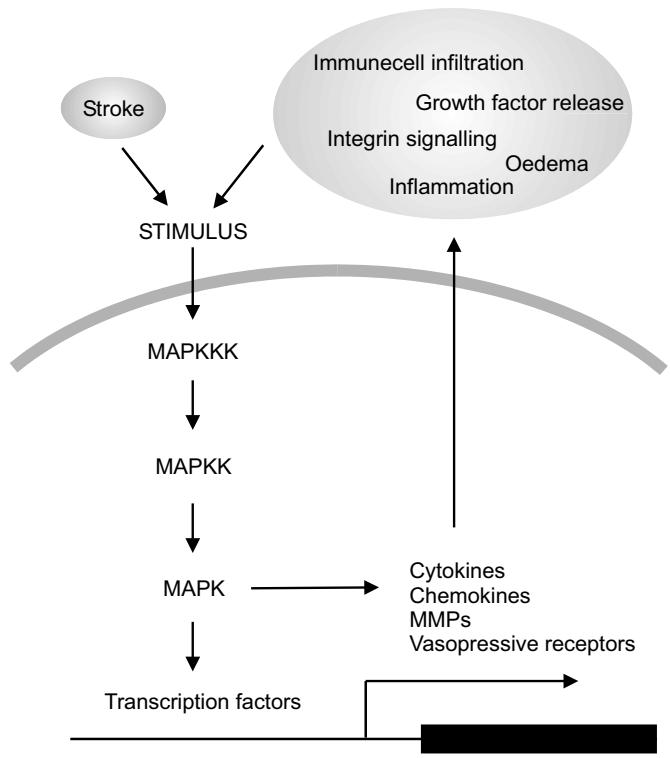

Figure 6.15 Increased transcription of cytokines, chemokines and MMPs through MAPK activation. This activation cascade can create a self-propagating cycle unless

the immunomodulating properties of the cytokines. Determining the exact effect of the cytokines that are produced is not within the scope of this thesis, but some effects of TNF-α is worth mentioning. TNF-α, a cytokine the production of which is known to be affected by p38 activation, both transcriptionally and translationally[81] has been shown to upregulate ICAM-1 on endothelial cells, thus increasing immune cell influx which can negatively affect the outcome after a stroke.[40] It can also increase arterial contraction, thereby affecting the cerebral blood flow.[82,83] Increased expression of cytokines is also able to activate MAPKs, a self-propagating cycle will take place unless sufficient negative feedback enters the system.[32,84] (Figure 6.15).

The transcriptional activation of chemokines is an important part of the inflammatory response. Chemokines work through their activation of specific receptors, inducing cell mobility towards a gradient. They play an important role both during development[85] and in inflammatory diseases.[42] An increase in chemokines (cxcl1, cxcl2 and ccl20) attract immune cells as well as activate platelets thus inducing clot formation.[86] As a result, the increased expression of chemokines is able to increase the infiltration of immune cells, which can be detrimental to the development of a stroke[20] and induce clot formation, which can exacerbate the stroke.

Arterial inflammation has been shown to increase the risk of stroke,[87,88] linking molecular changes observed here after DSP treatment with the increased risk smokers have of suffering a stroke. The effects of a DSP induced inflammatory response are slightly different, DSP-induced arterial activation not being as marked as after a stroke. Yet smoking can affect the arteries over a period of decades, easily making initial small changes much larger over time.

6.2.3 Extracellular matrix and MMPs

The expression of MMPs can to some extent be viewed as part of the inflammatory response since they participate in both inflammation and extracellular-matrix-remodeling.[89] Despite this the effects of MMP expression are in part separate from the immune response. Paper IV and V show that following a stroke there is a marked increase in the amount of MMP13 protein and the same occurs in response to DSP exposure in cerebral arteries. The increased in expression of MMP13 was observed in connection with stroke as early as 3 hours post stroke, which means that the increased expression is during the early phase of the development following SAH. These findings in conjunction with the findings in other studies showing the importance of MMP9 and MMP13 as predictive markers for stroke outcome [45,47] indicates that increased MMP expression has a severe negative effect on the outcome of stroke.

Part of the effect elicited by the MMPs could be through the breakdown of the basal membrane, an effect that can be highly detrimental to arterial function, especially for the microvasculature, since the arteries use the extracellular matrix for support.[68] This

degradation produces secondary effects through the release of growth factors that were previously bound[90] and through the creation of small peptides, degradation products with angiogenic and inflammatory properties.[91] In conjunction with this, MMP expression could increase inflammation by facilitating the infiltration of immune cells.[43,92] Excessive expression of MMPs for a prolonged period of time are also known to inhibit cutaneous wound healing, suggesting that uncontrolled MMP expression could inhibit healing processes generally.[93] An increased expression of MMPs could thus have higly detrimental effects through augmentation of the inflammatory response,[19,20] excessive extracellular matrix breakdown,[93] and the uncontrolled release of growth factors.[71,90] The effect of MMPs on the cerebral arteries prior to stroke would in many ways be similar to that of the inflammatory response. MMP expression would augment the inflammatory response in the arteries and thereby increasing the risk of suffering a stroke. [87,88]

MMP expression, like cytokine expression, can reinforce its own production. The released growth factors referred to could reactivate MAPK through their receptor signaling.[94] Activation could also proceed by the way of integrin degradation, where changes in integrin binding can affect MAPK activation.[31] This could produce a self-propagating cycle of MMP expression, activating MAPKs, leading to still further MMP expression.

6.2.4 Importance of expression and synergistic effects

The present results indicate the cerebral arteries to actively participate in the inflammatory response following a stroke and to help drive it. Similarly, they initiate processes after the exposure to smoke particles that are linked with an increased incidence of stroke. Why is the fact then that the cerebral arteries actively participate in this response important? Is not the production of proinflammatory markers likewise observed in surrounding tissue sufficient to drive a pathophysiological response? Several of the factors that increase following stroke are able to modify the cerebral blood flow by affecting the arterial function. A local production of such agents can thus directly affect the arteries in a negative way. The arteries are also the major gateway between the CNS and the rest of the body, so that the production of

chemokines and cytokines there directly affect the circulating blood cells, increasing the rapidity of the response to the stroke.

There are also the synergistic effects of the inflammatory and extracellular-matrix-related genes. They are not only dependent upon MAPK activation for their production but can also reactivate MAPK activation. Thus, the initial activation, if sufficiently strong, can easily create a self-propagating cycle based on any of the possible means of reactivation. The fact that MAPK activation plays an important role for apoptosis, its extent depending upon the length and strength of activation, makes this possibility for reactivation intriguing to investigate further.

6.3 Major conclusions

The cerebral arteries actively participate in the response following a stroke in both man and rat. They can also participate actively in processes that could increase the incidence of stroke in smokers, through the activation of MAPK which in turn activate the transcription of inflammatory and extracellular-matrix-regulating genes as well as of specific receptors (Figure 6.16). The processes investigated are able to reactivate MAPK, which can lead to very powerful and prolonged effects of the initial activation. Because of the synergistic nature of the processes investigated, a single-factor approach seems unlikely to work completely. There is a need on the patient's part for an inflammatory response following a stroke so that the necrotic and apoptotic cells as well as extravasated blood are removed. Modulating of the MAPK activation and selectively affecting the expression of inflammatory and extracellular-matrix-related genes could thus allow of a more controlled response to occur.

7. Swedish summary

7.1 Bakgrund

Hjärnan är ett av de viktigaste organen i kroppen och även ett av de känsligaste. För att fungera optimalt behöver den riklig tillgång på socker eftersom hjärnan inte använder sig av vare sig fett eller proteiner som näring. Ett rikligt blod flöde är av yttersta vikt för att kontinuerligt förse hjärnan med den energi som behövs och för att frakta bort nedbrytnings produkter som koldioxid. Detta blodflöde sköts av kärl som leder till hjärnan, så kallade hjärnkärl. Utöver att sköta dessa transporter så fungerar kärlen som väktare. I övriga delar av kroppen så kan olika näringsämnen och immunceller vandra ganska fritt, infiltrera, genom kärlen och ut i omkringliggande vävnad. I hjärnan är all sådan aktivitet reglerad och stoppas av blod-hjärn-barriären, en del av kärlen. När olika ämnen och immunceller ska transporteras till hjärnan sker detta genom en aktiv, kontrollerad transport, vilken styrs av kärlen. Denna kontrollerade process vaktar därmed på vad som i slutändan kan passera ut till hjärnvävnaden. Hjärnkärlen fungerar alltså inte som passiva rör för blodet utan de skyddar även hjärnan från oönskad påverkan.

Det finns tillfällen när dessa funktioner påverkas negativt och slutar fungera, till exempelvis efter en stroke, som förr kallades ett slaganfall. Det finns två typer stroke, de som beror på en blödning och de som beror på en propp. Båda dessa typer orsakar en minskning av blodflödet i hjärnan, en minskning som snabbt orsakar stora skador, detta gör att det viktigaste efter ett stroke är att återfå ett så normalt blodflöde som möjligt. De behandlingar som finns idag för att återfå flödet och begränsa skador är tyvärr begränsade, de har inte alltid den positiva effekt som man önskar eller måste påbörjas väldigt snabbt för att få någon effekt.

Efter den direkta, akuta fasen av ett stroke sker stora förändringar i hjärnan och en rad processer startas som kan bidra till att öka storleken på skadan. Bland de viktigaste processerna är det inflammatoriska svaret, som består av invandring av immunceller och svullnad. Hur dessa processer utvecklas har visats vara nästan lika viktigt för hur hjärnskadan utvecklas som den initiala blodflödes minskningen.

7.2 Syfte med Avhandlingen

Denna avhandling är fokuserad på de förändringar som sker i hjärnkärlen efter en stroke. Fokus har varit på hur kärlen påverkas och även om och hur de påverkar de processer som sker. Jag har även undersökt kärlförändringar efter påverkan av cigarettrök för att leta efter en förklaring till den ökade risk rökare har att drabbas av stroke.

7.3 Metoder och Resultat

Bland de metoder som har använts är två stycken modeller för stroke i råtta och en odlings modell med enbart kärl för att undersöka rök relaterade förändringar. Kärlen har sedan undersökts med molekylära metoder för att påvisa förändringar.

Delarbete I var en screening för att förutsättningslöst undersöka vilka processer som verkar ske i ett kärl efter stroke. Här visades att kärlen deltog i det inflammatoriska svaret och att de påverkade blod-hjärn-barriären. De deltog i det inflammatoriska svaret genom att tillverka ämnen som aktiverar och som ökar immuncell infiltrationen. Blodhjärn-barriär påverkan var genom ämnen som kan bidra till att delvis bryta ner denna. Det som studie I inte gav svar på var dock om kärlen direkt reagerade på det initiala stroket eller om de reagerade på att det skett en skada efteråt i omkringliggande vävnad. Detta undersöktes i delarbete IV som visade på att kärlen direkt reagerade på att det skett ett stroke. Jag såg här att de direkt började tillverka ämnen som kan påverka immunförsvaret och skada blod-hjärn-barriären.

Både delarbete I och IV undersökte förändringar i en modell som simulerar en blödande stroke. Oftast brukar en blödande stroke behandlas annorlunda än en stroke orsakats av en propp, då de av hävd setts som olika. I delarbete III undersöktes om kärlen reagerade likadant på dessa två typer av stroke även om den initiala skadan såg annorlunda ut. Vi kunde här se att kärlen reagerade förvånansvärt lika på dessa skador.

Undersökningen av hur rökning kan påverka kärlen gjordes i delarbete V. Vi såg här att cigarettrök kunde påbörja inflammatoriska processer och även skada blod-hjärn-barriären. Dessa processer är kända för att påverka kärl negativt, även när de inte är kopplade till cigarettrök kan de öka risken för ett stroke.

Det mesta av undersökningarna i de olika delarbetena har skett i olika experimentella djur modeller. I delarbete II undersöktes patientkärl för att verifiera dessa modellerna. Vi kunde här se att de processer som vi sett aktiverade i experimentella modeller, även var aktiva i kärlen från patienter kärl vilket stöder de experimentella modellerna.

7.4 Slutsats

Baserat på dessa fem delarbeten drog jag därför slutsatsen att (i) de hjärnkärlen aktivt deltar i och bidrar till utvecklingen efter en stroke, (ii) att bland de processer som är aktiva finns både inflammatoriska processer och även processer som skadar blod-hjärn-barriären, (ii) rökning startar processer som påverkar kärlen negativt, liknande de som sker efter en stroke och (iv) att dessa förändringar sker i både patienter och i våra experimentella modeller.

Förhoppningen är att dessa resultat i framtiden ska kunna underlätta att utveckla nya behandlings metoder för att kontrollera förloppet i hjärnans kärl efter en stroke.

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