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MAPK and pro-inflammatory mediators in the walls of brain blood vessels following cerebral ischemia

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MAPK and pro-inflammatory mediators in the walls of brain blood vessels following cerebral ischemia

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Doctoral Thesis

Lund University
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Academic dissertation

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Abstract

Stroke is a serious neurological disease which may lead to death and severe disability. Inflammation that evolves within a few hours after stroke plays an important role in the pathogenesis of stroke. Therefore, the aim of the present thesis was to examine the role of pro-inflammatory mediators in cerebrovascular pathophysiology following stroke. The main focus was directed towards the expression and production of cytokines and inducible nitric oxide synthase (iNOS), the activation of matrix metalloproteinases (MMPs) and the mitogen activated protein kinase (MAPK) pathway in the cerebrovascular walls after ischemic and hemorrhagic strokes. The results show that cerebral arteries and brain parenchyma microvessels participate actively in the inflammatory response with production of pro-inflammatory mediators following focal and global ischemia. This involves activation of the ERK1/2 MAPK pathway at early time-points in the smooth muscle cells (SMCs) of the cerebral vessels, leading to production and upregulation of pro-inflammatory cytokines, iNOS and MMPs, and is associated with increase in infarct volume and reduced neurological function. Inhibition of the MAPK-ERK1/2 signaling pathway upstream with administration of a MEK1/2 inhibitor and/or a Raf inhibitor at 6 hours after cerebral ischemia prevented the upregulation of vascular pro-inflammatory mediators, decreased infarct volume and improved CBF and neurological function.

The main findings in this thesis, suggest that modulation of Raf-MEK-ERK1/2 signaling may represent a potential target for the development of novel anti-inflammatory therapy approaches to alleviate the tissue damage associated with cerebral ischemia.

Key words: Middle cerebral artery occlusion , Subarachnoid hemorrhage , Inflammation, TNF-α, IL-6, IL-1ß, Matrix metalloproteinases, iNOS, Mitogen activated protein kinase

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To the memory of my mother

To: my Father

and my husband Sina

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LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers:

I. **Maddahi A**, Chen Q, Edvinsson L. Enhanced cerebrovascular expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 via the MEK/ERK pathway during cerebral ischemia in the rat. *BMC Neurosci. 2009 Jun 4;10:56*

II. **Maddahi A**, Edvinsson L. Cerebral ischemia induces microvascular pro-inflammatory cytokine expression via the MEK/ERK pathway. *J Neuroinflammation.2010 Feb 26; 7:14. Erratum in: J Neuroinflammation. 2011; 8:18.*

III. **Maddahi A**, Ansar S, Chen Q, Edvinsson L. Blockade of the MEK/ERK pathway with a raf inhibitor prevents activation of pro-inflammatory mediators in cerebral arteries and reduction in cerebral blood flow after subarachnoid hemorrhage in a rat model. *J Cereb Blood Flow Metab. 2011 Jan;31(1):144-54*

IV. **Maddahi A**, Povlsen G, Edvinsson L. Regulation of enhanced cerebrovascular expression of pro-inflammatory mediators in experimental subarachoid hemorrhage via the MEK/ERK pathway. *Preliminary accepted in J Neuroinflammation.*

V. **Maddahi A**, Kruse LS, Chen QW, Edvinsson L. The role of tumor necrosis factor-α and TNF-α receptors in cerebral arteries following cerebral ischemia in rat. *J Neuroinflammation. 2011 Aug 28;8:107*

ABBREVIATIONS

AP-1	Activator protein-1
AT_1	Angiotensin II receptor type 1
BA	Basilar artery
BBB	Blood brain barrier
BSA	Bovine serum albumin
CNS	Central nervous system
CVS	Cerebral vasospasm
cDND	Complementary deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle`s medium
DCI	Delayed cerebral ischemia
EF-1	Elongation factor-1
ERK1/2	Extracellular signal-regulated kinases 1 and 2
ET_B	Endothelin receptor type B
Elk-1	E-26-like protein-1
GPCR	G-protein coupled receptor
GFAP	Glial fibrillary acidic protein
HRP	Horseradish peroxidase
ICP	Intracranial pressure
IL-6	Interleukin-6
IL-1ß	Interleukin-1ß
iNOS	Inducible nitric oxide synthase
JNK	c-jun NH_2-terminal kinase
MAPK	Mitogen activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MMP-9	Matrix metalloproteinase-9
MABP	Mean arterial blood pressure
mRNA	Messenger ribonucleic acid
NOS	Nitric oxide synthase
NF-κB	Nuclear factor-κB
pO2	Partial pressure of oxygen
pCO_2	Partial pressure of carbon dioxide
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SAH	Subarachnoid hemorrhage
S.E.M.	Standard error of the mean
SMC	Smooth muscle cell
TNF-α	Tumor necrosis factor
TIMP-1	Tissue inhibitor of metalloproteinase protein-1
TTC	2.3.5-triphenylterazolium
VSMC	Vascular smooth muscle cell

INTRODUCTION

Stroke is a serious neurological disease which may lead to death and severe disability [1, 2]. There are two major types of stroke: ischemic and hemorrhagic stroke. Both are associated with disruption of blood flow to a part of the brain with rapid depletion of cellular energy and oxygen, resulting in ionic disturbances and eventually neuronal cell death [3]. The pathologic process that develops after stroke is divided into acute (within hours), sub-acute (hours to days), and chronic (days to months) phases [4, 5]. Obviously, the most effective therapy requires the earliest possible intervention e.g. with removal of a thrombus. However, no specific treatment, apart from thrombolysis, that acts effectively to protect the neurons during the acute phase has yet been developed.

Experimental and clinical data show an acute and prolonged inflammatory response in the brain after a stroke. Several investigators have reported that inflammation evolves within a few hours after stroke, and plays an important role in the development of the cerebral lesions [6]. This inflammatory reaction involves activation of resident cells (mainly microglia), infiltration and accumulation of various inflammatory cells (including neutrophils, leukocytes, monocytes, macrophages), and production of pro-inflammatory mediators in the injured brain areas [6, 7]. It has been established that the inflammatory reaction triggered by stroke affects not only the neuronal tissue itself but has impact also on the cerebral arteries [7].

Stroke is a vascular disease and despite extensive research in the area, the physiology and pathophysiology of the neurovascular unit, the complex network of endothelial cells, smooth muscle cells, inflammatory cells and mediators are not fully understood, which is necessary in order to develop effective therapies. The aim of the present thesis was to examine the role of pro-inflammatory mediators in cerebrovascular pathophysiology following stroke. The main focus was directed towards the expression and production of cytokines and inducible nitric oxide synthase (iNOS), the activation of matrix metalloproteinases (MMPs) and mitogen activated protein kinase (MAPK) pathway because microarray work [8] and published data [9] primarily pointed at these. These parameters and the relationships between them were studied in the cerebrovascular walls after ischemic and hemorrhagic strokes.

This study lends further support to the view that inflammatory mediators are important contributing factors in brain injury after stroke. It provides evidence that blocking the intracellular signaling pathways involved in the transcription of these mediators may have therapeutic potential, as it may prevent or at least attenuate the inflammatory processes elicited by stroke.

Ischemic stroke

Ischemic stroke is the most common type of stroke (85% of cases). It is caused by a transient or permanent occlusion of a cerebral artery most often by a thrombus or an embolus [10, 11]. When an ischemic stroke occurs, blood flow to an area of the brain is reduced and the brain cells are starved of oxygen and nutrients, which quickly leads to neuronal cell death and the development of an infarct. The infarct region is divided into two parts: 1) A central part or an ischemic core, where the neurons die and have no chance to survive without rapid reperfusion. 2) A peripheral area or an ischemic penumbra, which surrounds the core [12]. Cells in the penumbra are impaired and cannot function due to compromised metabolism, but do not die immediately and have therefore become a prime target for neuroprotective treatments [13-15]. A number of neurochemical and pathophysiological events are triggered within the ischemic penumbra. As a result of energy depletion, there is disruption of ion homoeostasis, excessive release of excitatory neurotransmitters such as glutamate, calcium channel dysfunction, generation of oxidative stress and free radicals, activation of stress signaling, cell membrane disruption, inflammation, ultimately leading to necrotic and apoptotic cell death [1, 4, 15, 16].

The effect of ischemia on brain cells results not only in loss of structural integrity of brain tissue but affects also blood vessels, partly through the activation of inflammatory events and excess production of vasoconstrictor substances and increased receptor expression [17]. The early inflammatory response, which often is associated with the blood vessels, starts immediately or a few hours after the onset of the ischemia and contributes to the irreversible damage [18-21]. Currently, there are two major ways used for treating ischemic stroke: (i) Dissolution of the clot in the occluded artery by a thrombolytic drug, rt-PA (recombinant tissue-plasminogen activator) [22] and, (ii) administration of neuroprotective agents [23].

Treatment with rt-PA is limited by time and should be administered within 4.5 hours after the onset of stroke to reduce the risk of hemorrhagic transformation [24, 25]. Moreover, rt-PA is associated with the risk of disruption to the blood-brain barrier (BBB) which is due to activation of matrix metalloproteinases [26]. Despite intense research, the results obtained with neuroprotective drugs in clinical trials have not revealed positive results [27, 28].

Hemorrhagic stroke

Hemorrhagic stroke (15% of all strokes) is often associated with hypertension, and is due to the rupture of an arterial aneurysm or a vascular malformation [1, 29]. Hemorrhagic stroke is divided into two categories: intracerebral and subarachnoid hemorrhage. Intracerebral hemorrhage (ICH) is due to the rupture of a small artery (arterioles) which bleeds within the brain tissue. It is often associated with chronic high blood pressure and the symptoms often begin with severe headache. Subarachnoid hemorrhage (SAH) occurs when an artery or an arterial aneurysm on the surface of the brain ruptures and bleeds into the space between the pia mater and the arachnoid (subarachnoid space) [1].

The most common cause of the SAH is the spontaneous rupture of an arterial aneurysm. This is associated with acute rise of the intracranial pressure (ICP), reduction of cerebral blood flow (CBF), rapid discharge of blood into the basal cisterns, and delayed cerebral ischemia (DCI), each of which may be fatal. The SAH is most common in women and younger people (below 55 years old). Around 50-70% of patients with SAH die or suffer severe disability, and is the cause of up to 10% of all strokes [30-33].

The disease is biphasic, with an early/short-lived phase that occurs immediately after SAH with a reduction in CBF, followed by a chronic or prolonged phase which is characterized by a varying degree of pathological contraction of cerebral arteries, known as vasospasm [34, 35]. The vasospasm (narrowing of arteries) typically occurs within 5-15 days after SAH and is present in approximately one-third of patients and is accompanied by DCI [36, 37]. It can occur not only at the site of the hemorrhage, but also in brain arteries at a distance from the bleeding. The narrowing of the cerebral vessel lumen leads to reduction in local blood flow and in cerebral metabolism, causing severe cerebral ischemia, with increase in mortality of 1.5-3 folds during the first two weeks after SAH [37-39]. Despite intense research, the

pathogenesis of DCI after SAH is not well understood and no specific pharmacological treatment is available.

Current treatment recommendations involve management in an intensive care unit. The blood pressure is maintained with consideration to the patient's neurologic status. In addition, calcium channel blockers, endothelin-1 receptor antagonists, hemodynamic management and endovascular treatment are also used, but these treatments are expensive, time-consuming and only partly effective [40].

Many theories have been advanced to explain the mechanisms responsible for vasospasm and DCI that occur after SAH such as, endothelial damage [41-43], enhanced smooth muscle cell (SMC) contractility, morphologic changes in vessel walls [44], enhanced levels of free radicals [45-47], increased production and release of potent vasomotor substances such as endothelin-1 (ET-1) and angiotensin II (Ang II) [48, 49], local inflammation and immunological reactions in the vascular wall [50-52]. Yet, the exact mechanisms underlying the vasospasm and the DCI remain unknown [53].

There is evidence that the amount of blood in the subarachnoid space is related to development of vasospasm [54]. Oxyhemoglobin from extravasated blood may be an important trigger of vasospasm and DCI after SAH [55-57] by inducing inflammation [50, 58]. It may in addition correlate with structural damage to the vessel wall [59], release of spasmogenic substances, and inhibition of endothelium dependent relaxation [60, 61]. It is suggested that the extravasated blood could induce generation of free radicals that subsequently may exert a direct local toxic effect on the cerebral arteries [62, 63].

G-protein coupled receptors following stroke

Recently, a novel aspect of the pathophysiology of stroke has been suggested, namely that the upregulation of vasoconstrictor receptors in the cerebral arteries after stroke may be an important mechanism in the development of the final damage [64]. Vasoconstrictor receptors such as those of angiotensin II receptor type 1 (AT_1) and endothelin-1 receptor type B (ET_B) belong to the seven transmembrane G-protein coupled receptor (GPCR) family [65-67]. They are upregulated in the SMCs of cerebral vessels within and associated with the ischemic

region after focal ischemic stroke [68] and after SAH [69]. This results in enhanced contractility of the vessels, which further impairs local blood flow and aggravates tissue damage. Importantly, the receptor ligands (angiotensin II and endothelin-1) are formed in the cerebrovascular endothelium. In addition, contractile responses mediated by AT_1 and ET_B receptors were found to be increased in SMCs of human cerebral arteries after organ culture [70]. Experimental stroke induces upregulation of cerebrovascular contractile receptors in the SMCs which are caused by increased receptor gene transcription induced via activation of specific intracellular signaling pathways (such as MEK-ERK1/2 and PKC pathways) [64]. Importantly, inhibition of these signaling pathways prevents the receptor upregulation, reduces infarct volume after ischemic stroke and improves neurological score and CBF after SAH [71, 72]. This may indicate that the increased cerebrovascular contractility caused by the upregulated receptors contributes to worsening of the brain damage.

Inflammation in general and following stroke

Inflammation is the body's defense against injurious factors and foreign antigens, *e.g.*, trauma, infection and toxins, and is considered to be both a beneficial and a detrimental element of a pathological process. It is a complicated and multifaceted response, characterized by acute and chronic phases [73, 74].

Among many mechanisms involved in the pathogenesis of stroke, inflammation is increasingly recognized as a key factor. However, all the mediators of the inflammatory response have not been clearly identified [6, 75-77]. There is evidence to suggest that inflammation and immune responses are involved in all three stages of the ischemic cascade, from the acute intravascular process triggered by the interruption of the blood supply to the parenchymal processes that lead to brain damage and subsequently to tissue repair. The early inflammatory response contributes to the ischemic injury, whereas late responses may represent endogenous mechanisms of recovery and repair [78] **(Figure 1)**. When there is a switch from detrimental to beneficial effects might depend on the strength and the duration of the stroke and knowledge about the mechanisms involved is crucial for determining the time-window for effective pharmacotherapy [79].

As mentioned above, reduction in CBF after stroke can result in energy depletion and subsequent neuronal cell death. This triggers an immune response that results in activation of a variety of inflammatory cells and molecules [51, 80, 81]. In the acute phase (minutes to hours), extravasated blood following SAH (or following reperfusion after arterial occlusion in transient ischemia) induces generation of reactive oxygen species (ROS). They may stimulate ischemic cells to produce inflammatory molecules such as cytokines and chemokines which in turn may activate microglial cells and increase leukocyte infiltration. These produce more cytokines, causing an increase in adhesion molecules, which are normally required for the adherence and accumulation of leukocytes and neutrophils to vascular endothelial cells and infiltration of brain parenchyma. In the sub-acute phase (hours to days), increased activation of inflammatory cells results in further production of pro-inflammatory mediators including more cytokines, extracellular MMPs, as well as iNOS which generates nitric oxide (NO) and more ROS [79, 82]. The intravascular accumulation of leukocytes and of platelets results in occlusion of microvessels, leading to hypoxia and further increases in levels of ROS [83, 84]. Activation of mast cells and macrophages can in turn lead to release of histamine (a strong vasoactive substance) and production of more cytokines and proteases [85]. In addition, degradation of extracellular matrix components by MMPs (mostly MMP-9) leads to BBB disruption which contributes to secondary brain damage by releasing serum and blood elements into the brain tissue resulting in vasogenic brain edema and post-ischemic inflammation [83].

Disruption and permeability of the BBB can be either transient or permanent depending on severity of the insult. Permanent disruption is associated with endothelial swelling, astrocyte detachment and blood vessel rupture in the ischemic area, while transient BBB disruption is caused by endothelial hyperpermeability to macromolecules in the penumbra area. This follows a biphasic pattern with an initial opening 2-3 hours after the onset of the insult and a second opening 24-48 hours after reperfusion leading to edema and increased intracranial pressure. All these events involve pro-inflammatory cytokines, adhesion molecules and production of MMPs [86, 87].

Cerebral blood vessels are the first to be exposed to the ischemic insults and their reaction to injury sets the stage for the inflammatory response. Post-ischemic inflammation thus involves activation of microglial and endothelial cells accompanied by migration of peripheral circulating inflammatory cells into the brain such as leukocytes, neutrophils, platelet, mast

cells and macrophages. These events amplify signaling along inflammatory cascades increasing the accumulation of toxic molecules that enhance the secondary damage leading to more cell stress, edema, hemorrhage and finally cell death **(Figure 1)** [76, 79, 84].

On the other hand, many pro-inflammatory mediators play a positive role in late stage of stroke. For example, MMPs have been reported to promote brain regeneration and neurovascular remodeling in the later repair phase [79, 88, 89]. Moreover, macrophages and microglial cells also contribute to tissue recovery by scavenging necrotic debris, by producing anti-inflammatory cytokines and by facilitating plasticity [90] **(Figure 1)**. Yet, despite these beneficial effects there is evidence that administration of anti-inflammatory drugs may reduce infarct volume and improved outcomes in animal models of stroke [91]. On the other hand, to date, clinical trials with anti-inflammatory agents have not been able to demonstrate improved clinical outcome [92, 93].

With better knowledge about which cells and molecules that participate and which mechanisms regulate the inflammatory reactions triggered by cerebral ischemia, it may be possible to identify novel targets for suppression of inflammation following cerebral ischemia and thereby develop more effective stroke therapies.

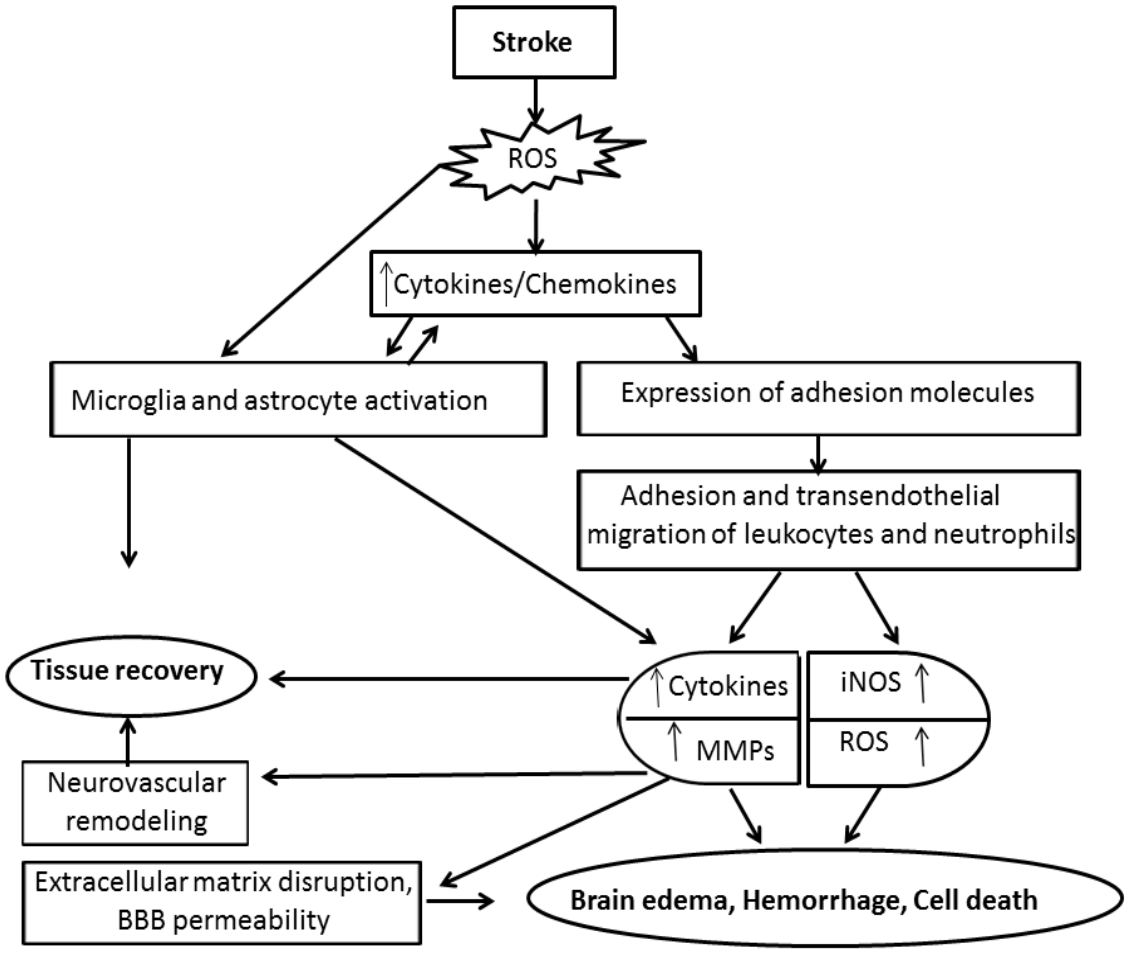

Figure 1. Main inflammatory pathways that respond to injury after a stroke. The generation of ROS and free radicals that occur after stroke triggers inflammatory responses. This involves activation of cytokines and chemokines which leads to activation of inflammatory cells such as microglia and leukocytes causing more production of inflammatory mediators (cytokines, iNOS, MMPs and more ROS) which then lead to brain edema, hemorrhage and cell death. Thus, these early inflammatory responses contribute to ischemic injury, whereas late responses may represent endogenous mechanisms of recovery and repair through activation of anti-inflammatory cytokines, scavenging necrotic debris by microglia and neurovascular remodeling by MMPs.

Major inflammatory mediators in cerebral ischemia

In this present thesis, I have studied the expression of some of the major cytokines (IL-1ß, IL-6, TNF-α, TNF-R1 and R2), of MMP-9 (BBB associated) and of iNOS (potential toxic molecule) in cerebral vessel walls. Increased levels and activation of these factors may lead to exacerbation of vasoconstriction, resulting in decreased CBF and enhanced neuronal damage following a stroke.

Cytokines

Cytokines are recognized as small proteins, generally associated with inflammation, immune activation, cell differentiation and hematopoiesis [94]. Most cytokines are pleiotropic and have multiple biologic activities that generally act over a short distance, during short periods of time and at low concentrations. They are produced and expressed by different cell types such as astrocytes, macrophages, monocytes, microglia, platelets, endothelial and smooth muscle cells, neurons, fibroblasts and neutrophils [52, 95, 96]. Normally, they have a beneficial role, but when their expression increases in an imbalanced fashion they become detrimental [97]. Evidence for the involvement of cytokines in the pathology following stroke comes from the detection of their high levels in CSF and plasma of patients [98, 99]. It is thought that increased production and activation of such cytokines in vessel walls after cerebral ischemia/reperfusion may facilitate and expand the ischemic core by inducing secondary brain damage (brain swelling, impaired microcirculation, hemorrhage and inflammation) that typically develops after a delay of hours or days after the original ischemia, trauma or SAH [100]. It is well known that cytokines are involved in the upregulation and activation of adhesion molecules, MMPs, leukocytes, microglial, increased leukocyte-endothelium interaction and increase in vasoconstrictor substances like ET-1 following cerebral ischemia [52, 76, 101]. Tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1ß (IL-1ß) are the main cytokines which initiate inflammatory reactions and induce expression of other cytokines and inflammatory mediators after stroke. Ischemic brain has been shown to produce increased levels of TNF-α, IL-6 and IL-1ß, which are considered as a part of the damaging response [102]. Inhibiting the expression of these pro-

inflammatory cytokines has been reported to reduce brain infarct size in animal models of stroke [103].

TNF-α

TNF-α is a pleiotropic cytokine and exists as either a transmembrane or soluble protein. It is involved in the disruption of the BBB, as well as in inflammatory, thrombogenic and vascular changes associated with brain injury [104]. This cytokine promotes inflammation by stimulation of acute-phase protein secretion, enhances the permeability of endothelial cells to leukocytes, and the expression of adhesion molecules and other cytokines into the ischemic area [105, 106]. In addition, it has been suggested to stimulate angiogenesis after cerebral ischemia through induced expression of angiogenesis-related genes [107, 108]. It is known as a strong immunomediator, which is rapidly upregulated early in neuronal cells in and around the ischemic penumbra, and is associated with neuronal necrosis or apoptosis [105]. TNF-α effects are mediated via two receptors, TNF-R1 and TNF-R2, on the cell surface [109]. TNF-R1 is expressed on all cell types, can be activated by both membrane-bound and soluble forms of TNF-α and is a major signaling receptor for TNF-α. The TNF-R2 is expressed primarily on endothelial cells, responds to the membrane-bound form of TNF-α, and mediates limited biological responses [109]. There is evidence that TNF-α and its receptors may activate nuclear factor-κB (NF-κB), a transcription factor whose activation leads to expression of several genes involved in inflammation and cell proliferation [110-112]. In addition, NF-κB is involved in signaling cell death as well as cell survival, and the balance between these signals determines the toxic degree of TNF-α [112, 113].

TNF-α appears then to be not only neurotoxic but also neuroprotective. Increased TNF-α levels have been observed in brain tissue, plasma and CSF in several CNS diseases such as Alzheimer's, multiple sclerosis and Parkinson's [114-116]. Accordingly, a recent study demonstrated that blocking TNF-α significantly reduced infarct size after both permanent and transient MCAO, suggesting the involvement of TNF-α in neuronal cell damage [104]. In contrast, there is evidence to suggest that brain injury after ischemia becomes worse in mice lacking TNF-R1, suggesting that TNF-α mediates neuroprotection through this receptor [117]. The function of TNF-α appears to differ between brain regions. TNF-α released for instance in the striatum is considered as neurodegenerative, while release in the hippocampus has been

suggested to promote neuroprotection [112]. Several investigators have suggested that the detrimental effects are activated in the early phase of the inflammatory process whereas the beneficial effects take place in the later phases [79].

IL-6

IL-6 is an endogenous and hematopoietic cytokine that plays multiple roles in the central nervous system during infection and after traumatic injuries. It is involved in induction of B-cell differentiation and helps to attract T-lymphocytes into the brain, contributing to exacerbation of the inflammatory response [79]. To exert its biological effects, IL-6 binds to its receptor, IL-6Rα, which can be either soluble or membrane-bound [118]. IL-6 is often induced together with TNF-α and IL-1ß in different conditions, and circulating IL-6 plays an important role in the induction of acute phase reactions [119]. Several studies have revealed that the expression of IL-6 is detected at an early time point, 4-6 hours after onset of ischemia, and at a later point at 24-48 hours, and that it remains detectable for up to 14 days [120, 121]. The level of IL-6 in CSF is significantly increased from days 3-6 in patients with vasospasm compared to patients with no symptoms of vasospasm, suggesting that IL-6 might be involved in inducing CVS after SAH [122]. In addition, in animal models of stroke, there is an enhanced expression of IL-6 in neuronal cells [123]. However, the exact role of IL-6 in cerebral ischemic is still unclear. For example, the high levels of IL-6 in plasma of patients with acute brain ischemia, are strongly associated with stroke severity and long-term clinical outcome [124]. On the other hand, IL-6 deficient mice show similar infarct size compared to the wild type, suggesting that it does not participate in ischemia pathogenesis [125].

IL-1ß

IL-1ß is a member of the IL-1 family and is rapidly produced in the brain after cerebral ischemia [105, 126]. IL-1ß is involved in development of brain damage following cerebral ischemia and blockade of IL-1ß converting enzyme activity, reduced infarct size and improved behavioural deficit [127]. IL-1ß acts by binding to its two transmembrane receptors, type I IL-1 receptor (IL-1R1) and type II IL-1 receptor (IL-1R2) initiating signaling cascades

that result in expression of inflammatory genes [128-130]. In addition, IL-1ß plays an important role in the acute stress-induced worsening of behavioural and neurological outcomes and increased infarct size after MCAO in rat [131]. A previous study indicated that IL-1ß expressed in vascular SMCs after SAH, mediates SMC apoptosis and results in enhanced aneurysm formation [132]. Several investigations have shown that IL-1ß, IL-6 and TNF-α follow approximately the same time course of expression after both global and focal cerebral ischemia. Thus, there is an early increase in the levels of TNF-α (1 h), IL-6 and IL-1ß (3-6 h) and a later increase at 2 days post-MCAO, which have been observed in cerebral cortex, striatum and hippocampus [121, 133, 134].

Matrix Metalloproteinases

The MMPs represent a family of zinc-dependent proteolytic enzymes with the ability to break down extracellular matrix (ECM) proteins and to cleave other non-ECM molecules ranging from growth factors, cytokines and binding proteins to cell surface receptors [135]. MMPs are involved in extracellular matrix remodeling, wound healing and angiogenesis. They are normally found in the cytosol in an inactive form, but when cleaved by proteases, such as plasmin or other MMPs, they convert to their active form [136]. The proteolytic activity of MMPs is tightly controlled by tissue inhibitors of MMPs (TIMPs). TIMPs are specific endogenous molecules, which by binding to pro-MMPs inhibit the activation of MMPs. Imbalance between production of MMPs and TIMPs plays an important role following stroke [137, 138]. Pfefferkorn and Rosenberg showed in an experimental stroke model that inhibition of MMP reduces infarct volume, brain edema and hemorrhage [139], supporting the notion that MMPs mediate the degradation of the neurovascular matrix and thereby promoting injury of the BBB.

Increased expression of MMP levels may contribute to inflammation, particularly those of MMP-2 and MMP-9 which have been shown to be upregulated in cerebral ischemia, however at different time points [140, 141]. Permanent MCAO in rats resulted in production of MMP-2 peaking at 5 days post-ischemia while MMP-9 peaked at 24-48 hours when the BBB was maximally opened. It was concluded that MMP-9 had a more significant role as compared to MMP-2 [141-144]. At the same time, MMPs seem to play yet another role in the later phase

of cerebral ischemia, being involved in vascular plasticity and recovery through increase of vascular endothelial growth factor (VEGF) signals [145].

MMP-9

MMP-9 or gelatinase B is a pro-inflammatory protease that is produced during inflammatory responses by astrocytes, microglia, endothelial cells, neutrophils and macrophages. MMP-9 activates both AP-1 and NF-κB transcription factors which in turn activate pro-inflammatory cascades [146]. MMP-9 is able to degrade major components of the endothelial basal lamina (type IV collagen, laminin and fibronectin), playing an important role in the disruption of the BBB thereby contributing to the development of edema following ischemia/reperfusion [142, 143, 147]. In support, it has been reported that administration of a MMP-9 inhibitor prevented the degradation and abolished the BBB disruption after focal ischemia in rat [142]. MMP-9 is also expressed in human brain tissue after ischemic and hemorrhagic stroke [148]. Furthermore, degradation of basal lamina through activation of MMP-9 after cerebral ischemia leads to loss of astrocytes and endothelial cell contacts, resulting in hemorrhagic transformation [149]. Besides this, an early increase in MMP-9 expression has been observed in microvascular walls, which has been suggested to be the primary cause of microvasular hemorrhage after cerebral ischemia [138]. Accordingly, MMP-9 deficient animals showed a smaller infarct area in an experimental stroke model, as compared to wild type controls [150]. Additionally, it has been reported that the levels of MMP-9 in plasma appear to correlate with infarct volume and severity of stroke [151].

iNOS

Another potent biological molecule that is expressed during inflammatory reactions in the CNS is nitric oxide (NO) which is induced by nitric oxide synthase (NOS) [152]. NO is an important signaling molecule, involved in numerous physiological processes such as neuronal communication, host defense and regulation of vascular tone. Three different isoforms of NOS exist: neuronal NOS (nNOS), endothelial NOS (eNOS) and the inducible form (iNOS). nNOS and eNOS are constitutively expressed and calcium-dependent, whereas iNOS is

expressed after immunologic challenge and neuronal injury and is calcium-dependent under most circumstances [153].

Activation of iNOS produces toxic levels of nitric oxide and is considered one of the key inflammatory mediators produced by different cells [76, 154]. There is evidence that iNOS can enhance glutamate release, resulting in ATP depletion in the ischemic infarct area after transient focal cerebral ischemia in rats [155]. In addition, it has been reported that iNOS is not present in the CNS under physiologic conditions, but its expression can be stimulated by cytokines [51]. The activity of iNOS is strongly linked to that of COX enzymes because of the interaction between the two systems. There is evidence that the production of iNOS increases with the activity of COX-2, which results in generation of more free radicals [52]. Moreover, iNOS may in addition cause DNA damage in cerebral ischemia through the formation of peroxynitrite [156].

A previous study has revealed that iNOS expression is significantly increased in vascular endothelial and smooth muscle cells at 7 days post-SAH, and that its intensity is greatest in animals with angiographic vasospasm [157]. Furthermore, the mRNA level of iNOS is increased mostly in vascular tissue at 1-7 days in different experimental SAH models [158]. Expression of iNOS has also been reported in both permanent and transient MCAO at 12-48 hours post-ischemia in inflammatory cells of the brain parenchyma and in cerebral blood vessels [159, 160]. The involvement of iNOS in cerebral ischemia is confirmed by the observation that mice lacking the iNOS gene have significantly reduced infarct volumes compared with wild-type controls [161]. Accordingly, administrations of iNOS blockers after cerebral ischemia attenuate the damage, decrease the infarct volume and improve neurological outcome [162, 163].

Intracellular Signaling

Cerebral ischemia activates several cell signaling pathways that are crucial for cell survival or damage, initiating complex cascades of events at genomic, molecular and cellular levels in all types of cells in the CNS. Inflammation, which occurs after both ischemic and hemorrhagic stroke, is very important in this context and may participate as a further enhancer in the ischemic cells [164]. Studies of signal transduction pathways that regulate the inflammatory

genes have mostly focused on mitogen activated protein kinases (MAPKs), one of the cascades activated in response to cerebral ischemia [165].

In this thesis, the attention has been directed towards evaluation of the role of Raf-MEK-ERK1/2 signaling transducers in regulating the expression of pro-inflammatory mediators in cerebral vessels following both ischemia and hemorrhagic stroke. The work provides some clues to explaining beneficial effects observed with inhibitors of these cascades in experimental models.

Mitogen activated protein kinases

MAPKs are involved in the transduction of cellular responses, mediating signaling from the extracellular environment to the nucleus and other intracellular targets [166, 167]. In response to extracellular stimuli, MAPKs regulate a broad range of intracellular activities from metabolism, motility, inflammation, differentiation and proliferation to cell death and survival [168]. The transduction of signals is made through activation of protein kinases and protein phosphatases [168, 169]. The MAPK family consists of three major groups, including extracellular signal-regulated kinase (ERK), p38 and stress-activated protein kinase c-Jun N-terminal kinase (SAPK/JNK). Each MAPK signaling pathway contains a three-tiered kinase cascade comprising a MAPKKK, that can activate MAPKK, which in turn activates and phosphorylates MAPKs [170]. Activation of MAPK pathways regulates the activity of a number of transcription factors that are present in the cytoplasm or the cell nucleus, such as Elk-1, ATF-2, C-Myc, NF-κB, and AP-1 components, as well as c-Fos and c-Jun [170, 171]. Activation of these transcription factors leads in turn to the expression of target genes, resulting in biological responses. It has been demonstrated that various MAPKs have overlapping functions, with the same transcription factor sometimes being activated by two or more MAPKs [172]. ERK1/2 is thus involved in differentiation, proliferation, meiosis, learning and memory in nerve cells and is activated by oxidative stress and mitogenic stimuli such as growth factors, cytokines and GPCRs [173-175]. In addition to the ERK pathway, the p38 and JNK pathways have been demonstrated to be involved in inflammation, cell survival and apoptosis, and can be activated by inflammatory cytokines and changes in shear stress. These kinases are activated by phosphorylation on both threonine and tyrosine residues,

which may phosphorylate intracellular enzymes and transcription factors [172, 176]. The balance between ERK and p38-JNK has been suggested to regulate cell fate, mediating survival or death [177].

MAPK and inflammation in cerebral ischemia

As mentioned before, cerebral ischemia is a pathophysiological condition caused by decreases in blood supply to the brain that results in deprivation of oxygen and glucose, leading eventually to cell death, inflammation and tissue repair [178]. In response to inflammatory stimuli that activate macrophages and initiate leukocyte infiltration, intracellular signaling pathways are activated and carry the signals needed to further activate the production of inflammatory mediators [179]. Cytokines such as TNF-α, IL-1ß and IL-6 can act as intracellular messengers because they have low molecular weights [180]. They act through binding to their respective receptors and/or Toll like receptors. Activation of the receptors triggers major intracellular signaling pathways, leading to activation of transcription factors such as NF-κB and AP-1, which in turn produce more cytokines involved in secondary damage [179]. Regulation of the expression of these genes by MAPK signaling, especially via ERK1/2, plays important roles in cerebra ischemia [167].

Several investigators have suggested a role for the ERK pathway in the regulation of cytokine expression following cerebral ischemia. For example, studies have shown that TNF-α can increase the permeability of the BBB via activation of the ERK1/2 pathway and increase the expression of TNF-R1 and TNF-R2. Treatment with a MEK1/2 inhibitor inactivates this signaling pathway and decreases the expression of the TNF receptors [181, 182]. However, it has been suggested that the main biological response to p38 activation involves the production and activation of inflammatory genes such as cytokines, COX_2 and collagenase-1, while, inhibition of p38 may reduce pro-inflammatory cytokines in several inflammatory cells [179, 183].

Activation of all three major MAPK pathways has been reported in experimental cerebral ischemia [9, 175, 184, 185] and activation of ERK1/2 is reported in humans after ischemic stroke [186]. Activation of JNK and p38 appears to be detrimental after a stroke and their

inhibition decreases infarct size and prevents neuronal apoptosis [177, 187-189], while ERK1/2 activation can be both beneficial and detrimental [190].

There is evidence pointing at the activation of ERK1/2 in cerebral arteries after MCAO [72], after SAH [49] and in cultures [185]. In addition, several studies have reported on the involvement of the MEK/ERK/MAPK pathway in the regulation of CVS after experimental SAH [191, 192]. It has been suggested that activation of the ERK1/2 pathway increases neurological damage by increasing ROS and oxidative stress-related cell death, promoting inflammation after stroke [190]. ERK1/2 activity may also stimulate inflammation by upregulation of IL-1ß, which results in necrosis [180].

Wang and co-workers revealed that the activation of ERK1/2 in the brain following ischemia/reperfusion is associated with cell death and brain injury while inhibition of ERK1/2 by a specific MEK1/2 inhibitor provided neuroprotection in cerebral ischemia through suppression of IL-1ß expression [193]. Administration of inhibitors of the MEK/ERK1/2 pathway has been found to attenuate ischemic injury and improve neurological outcome [68, 194, 195]. On the other hand, ERK1/2 activity may also block apoptosis by increasing the level of the anti-apoptotic protein BCL-2 or by inhibiting the pro-apoptotic protein Bad [168]. Moreover, it has been reported that ERK1/2 mediates protection after cerebral hypoxic-ischemic injury through activation of neurotrophins such as brain derived neurotrophic factor (BDNF), resulting in survival of neurons in the neonatal brain [196]. According to earlier studies, the protein kinase ERK1/2 is activated in the early phase after stroke [71].

Here, in this work, we suggest that this activation is probably an early “switch-on” mechanism involved in the increased release or formation of vasoconstrictor receptors, cytokines and of other mediators. Cytokine stimulation, in turn, activates p38 and JNK pathways which results in induction of more inflammatory genes, causing more damage. Therefore, early inhibition of this pathway may provide novel interesting targets for anti-inflammatory therapy following stroke **(Figure 2)**.

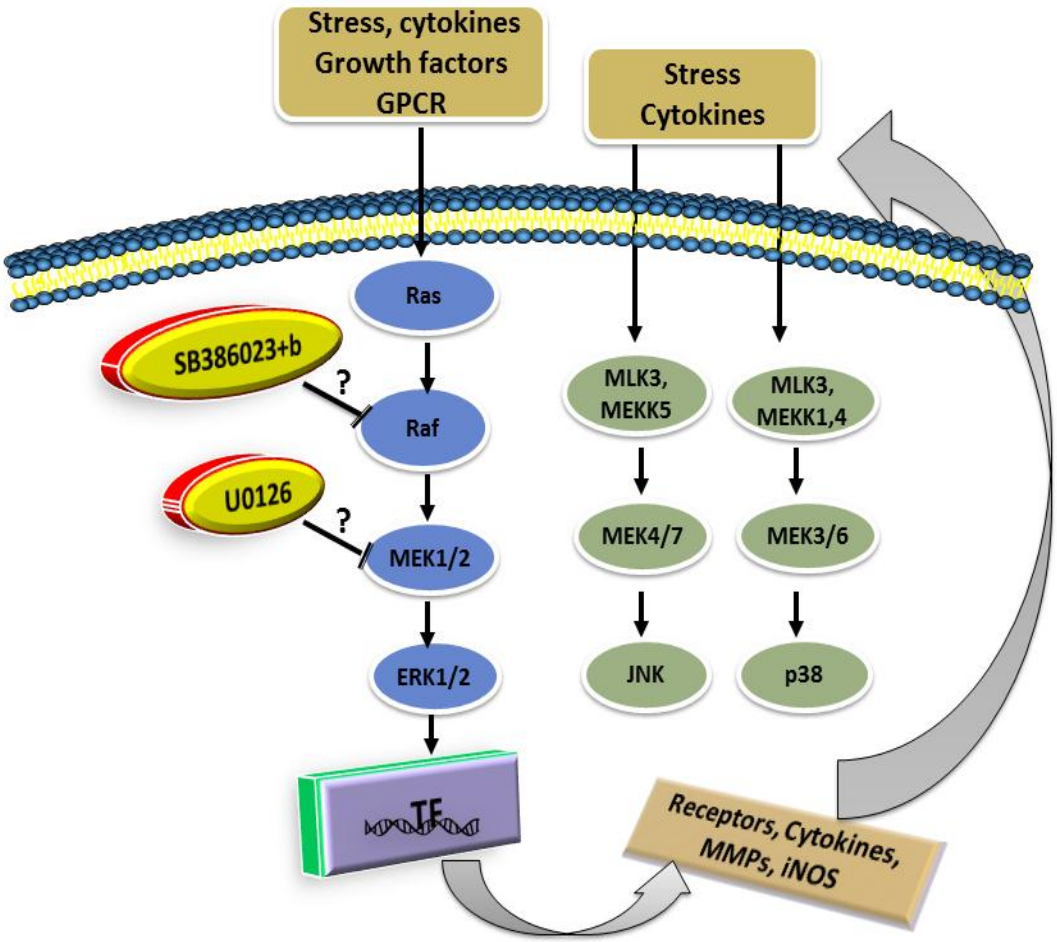

Figure 2. Regulation and production of pro-inflammatory mediators through early activation of the ERK1/2 MAPK pathway, which is stimulated by stress, cytokines and CGRP.

Hypothesis

We hypothesize that the increase in expression of cerebrovascular pro-inflammatory mediators that is seen after cerebral ischemia occurs via increased inflammatory gene transcription induced via activation of MAPK-MEK-ERK1/2 signaling pathway. This results in reduced CBF, larger brain damage and worsened neurological function. By blocking this signaling pathway in time, it will prevent the enhanced transcription of inflammatory genes and is associated with improved outcome after stroke.

AIMS

The general aim of this thesis is to investigate the role of pro-inflammatory mediators and their regulation in the wall of cerebral vessels following cerebral ischemia. More specifically:

- To investigate the expression of MMP-9 and TIMP-1 in cerebrovascular SMC following focal cerebral ischemia and to determine if their expression is regulated via the MEK/ERK pathway.
- To investigate the expression of pro-inflammatory cytokines in the walls of cerebral vessels after MCAO and to compare the inhibition of the inflammatory reaction with **1)** a specific MEK1/2 inhibitor (U0126) to block transcription, and **2)** a combined blockade of the AT_1 and ET_A receptors.
- To determine if the time-course and upregulation of pro-inflammatory mediators in the walls of cerebral arteries and microvessels after SAH is associated with the MEK-ERK1/2 pathway.
- To investigate if treatment with specific Raf or/and MEK1/2 inhibitors given as late as 6 hours after induction of SAH would prevent the upregulation of pro-inflammatory mediators, prevent SAH-induced decrease in CBF and improve functional neurological outcome.
- To examine if the expression of TNF-α and TNF receptors in the wall of cerebral arteries in two *in vivo* models (MCAO, SAH) and in an *in vitro* model of isolated cerebral arteries segments (organ culture), is regulated via the MEK/ERK pathway.

GENERAL METHODS

Animal surgery procedure

MCAO model (papers I, II and V)

Transient middle cerebral artery occlusion (MCAO) was induced in male Wistar rats by an intraluminal filament technique described by Memezawa *et al* [197]. The rats were housed under controlled temperature and humidity with free access to water and food. Anesthesia was induced using 4.5 % halothane or isoflurane in N_2O:O_2 (70:30); thereafter the rats were kept anesthetized by inhalation of 1.5 % halothane or isoflurane on a mask. To confirm a proper occlusion of the right MCA, a laser-Doppler probe was fixed on the skull measuring regional cortical blood flow. A polyethylene catheter was inserted into a tail artery for measurements of mean arterial blood pressure (MAP), pH, pO_2, pCO_2, and plasma glucose. A rectal temperature probe connected to a homeothermal blanket was inserted for maintenance of a body temperature of 37 ° C during the operational procedure. 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 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. The resulting occlusion was made visible by laser-Doppler as an abrupt reduction of cerebral blood flow of about 80-90 %. Finally, the filament was fixed by a suture and the rats were allowed to wake up. Two hours after occlusion, the rats were re-anesthetized to allow for withdrawal of the filament, and subsequently achieve reperfusion as verified by laser-Doppler recording [198, 199].

SAH model (papers III, IV and V)

Subarachnoid hemorrhage (SAH) was induced in male Sprague-Dawley rats by a model originally described by Svendgaard *et al* [200] and in detail by Prunell *et al* [201]. In this model, fresh and non-heparinized blood is administered into the subarachnoid space at an intracranial pressure (ICP) equal to the mean arterial pressure. The rats were anesthetized, intubated and artificially ventilated with inhalation of 0.5-1.5% halothane (paper III) or 1-2% isoflurane (paper IV) in N_2O/O_2 (70:30) during the surgical procedure. Respiration was monitored by regularly withdrawing blood samples to a blood gas analyzer. A temperature probe was inserted into the rectum of each rat to record the body temperature, which was maintained at 37ºC by a heating pad. An arterial catheter was placed in the tail artery to measure blood pressure and a catheter to measure the ICP was placed in the subarachnoid space. A laser-Doppler probe was placed to measure cortical cerebral blood flow (CBF). A 27G blunt cannula with a side hole facing right was placed 6.5 mm anterior to the bregma in the midline at an angle of 30º to the vertical plane placing the tip of the needle just in front of the chiasma opticum. After 30 minutes of equilibration, 250 µl of blood was withdrawn from the tail catheter and injected manually into the prechiasmatic cistern at a pressure equal to the mean arterial blood pressure. Subsequently, rats were maintained under anesthesia for another 60 minutes in order to allow the animal to recover. The ICP catheter was cut and sealed with a removable plug 2 cm from the tip. The tail catheter, the needle and the laser-Doppler probe were removed and incisions closed. The rats were then revitalized and extubated.

Drug administration

U0126 (papers I, II and IV)

U0126 is a specific MEK1/2 inhibitor that inhibits the ERK1/2 pathway by binding to and inhibiting the enzyme activity of MEK1/2, inhibiting thereby the activation and phosphorylation of ERK1/2 [180]. U0126 was used in both MCAO and SAH experimental models. In the MCAO model, 30 mg/kg body weight of U0126 (obtained from Sigma, St Louis, MI, U.S.A.) were administered intraperitoneally either immediately after starting reperfusion (0 hours) or at 6 or 12 hours after the start of reperfusion; in both cases, the first

injection was followed by a second one at 24 hours. The animals were then sacrificed 48 hours after occlusion (papers I and II). In the SAH experimental model, 0.22 µg/kg body weight of U0126 were administered intracisternally in two strategies: (i) treatment started at 6 hours after SAH induction, was repeated at 12, 24 and 36 hours and animals were then sacrificed at 48 hours; or (ii) treatment started at 6 hours after SAH induction, was repeated at 12 and 24 hours and animals were sacrificed at 72 hours post-SAH (paper IV). The U0126 doses were chosen on the basis of previous studies [68, 202].

SB386023+b (paper III)

SB386023+b is a specific B-Raf inhibitor, which inhibits MAPKKK upstream of MEK/ERK1/2 pathway [203]. The substance (20 µl of a 10^{-6}M solution; kind gift from Dr. AA Parsons, GSK; UK), were administered intracisternally. Treatment started at different time points after SAH induction (0, 6 or12 hours) with repeated injections over a period of 36 hours. Animals were subsequently sacrificed at 48 hours post-SAH. The dose of SB386023+b was chosen based on a previous study on isolated arteries [185].

Neurological evaluation (papers I, II and IV)

Neurological evaluations were performed for all survival MCAO and SAH animals. The MCAO animals were examined neurologically before recirculation and immediately before they were sacrificed, at 48 hours after MCAO, using an established scoring system described in **Table 1** [198, 199]. A rotating pole test was used to evaluate gross sensorimotor function (integration and coordination of movements as well as balance) of SAH animals. This examined the ability of the animals to traverse a rotating pole, which was either steady or rotating at different speeds (3 or 10 rpm) [204]. The performance of the rat was scored according to the described in **Table 2**.

Table 1.

Score	Interpretation
0	No visible deficits.
1	Contralateral forelimb flexion, when held 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.

Table 2.

Score	Interpretation
1	Animal is unable to balance on the pole and falls off immediately.
2	Animal balances on the pole but has severe difficulties crossing the pole and moves less than 30 cm.
3	Animal embraces the pole with the paws and does not reach the end of the pole but manages to move more than 30 cm.
4	Animal traverses the pole but embraces the pole with the paws and/or jumps with the hind legs.
5	Animal traverses the pole with normal posture but with more than 3-4 foot slips.
6	Animal traverses the pole perfectly with less than 3-4 foot slips.

Brain damage evaluation (papers I and II)

Coronal slices (2 mm thick) were obtained from brains of MCAO operated rats and stained by 1 % 2, 3, 5-triphenyltetrazolium chloride (TTC) dissolved in buffer solution at 37˚C for 20 minutes. Following TTC staining, normal brain tissue appeared bright red, while regions of damage were pale-white. The size of the ischemic damage was calculated as a percentage of the total brain volume calculated from the slices, using the software program Brain Damage Calculator 1.1 (MB Teknikkonsult) [72].

Cerebral blood flow measurement (paper III)

The SAH rat was intubated and artificially ventilated with inhalation of 0.5% to 1.5% halothane in N2O/O2 (70:30) during the surgical procedure. The anesthesia and the respiration were monitored by regularly withdrawing arterial blood samples for blood gas analysis. A catheter to measure MABP was placed in the right femoral artery and a catheter for blood sampling was placed in the left femoral artery. This catheter was connected to a constant velocity withdrawal pump for mechanical integration of tracer concentration. Another catheter was inserted in one femoral vein for injection of heparin and for infusion of the radioactive tracer. After 30 minutes of equilibration, a bolus injection of 50 µCi of ^{14}C-iodoantipyrine 4[N-methyl-^{14}C] was administered intravenously and subsequently, 122 µl of arterial blood was withdrawn over 20 seconds. Thereafter, the rat was decapitated, the brain removed and chilled to -50°C. The ß-radioactivity scintillation counting was performed on the blood samples with a program that included quench correction. The ^{14}C activity in the tissue was determined on cryo-sections of the brain. The sections were exposed to X-ray films together with ^{14}C methyl methacrylate standards and exposed for 20-30 days. The ^{14}C content was determined in several brain regions and CBF was calculated from the brain tissue ^{14}C activity determined by autoradiography using the equation of Sakurada [205] and Gjedde [206].

Organ culture (paper V)

The organ culture has been described previously by Adner and co-workers [207]. Male Wistar rats were anesthetized with CO_2 and decapitated. The brains were quickly removed and chilled in ice-cold bicarbonate buffer solution. The cerebral arteries were removed and dissected free from the brain and surrounding tissue. The artery segments were placed individually in wells with 2 ml serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with streptomycin (100 µg/ml) and penicillin (100 U/ml). Incubation was performed at 37°C in humidified 5 % CO_2 in air for 24 or 48 hours in the presence or absence of the intracellular signal inhibitors (a NF-κB inhibitor, IMD-0354; 30 nM; the specific MEK1/2 inhibitor, U0126; 10µM, the specific B-Raf inhibitor, SB386023-b; 10µM). These substances were added to the medium in the beginning and at 24 hours of the culture. IMD-

0354 was shown to specifically inhibiting the phosphorylation of IκB by IκB kinases, thus preventing NF-κB release and activation [208].

Molecular techniques

Real-time PCR (paper III)

Real time polymerase chain reaction (RT-PCR) is a sensitive method used for the detection of mRNA expression of a specific gene in a tissue homogenate. Cerebral arteries were immediately dissected out free from the brain and cleaned from connective tissue and blood. Total cellular RNA was extracted from the cerebral arteries using the Trizol RNA isolation kit according to the supplier's instructions (Invitrogen, Taastrup, Denmark). Total RNA was determined using a Gene Quant Pro spectrophotometer measuring absorbance at 260/280 nm. Reverse transcription of total RNA to cDNA was performed using the GeneAmp RNA kit (Perkin-Elmer Applied Biosystems, USA) in a Perkin-Elmer DNA thermal cycler, using random hexamers as primers.

Real-time quantitative PCR was performed in a GeneAmp 5700 sequence detection system using the GeneAmp SYBER® Green kit (Perkin-Elmer Applied Biosystems, USA) with the cDNA synthesized above as template. Specific primers were designed by using the Primer Express software program and no-template controls for each primer pair were included in all experiments. The real–time PCR consists of a system that is able to evaluate the amount of DNA in each PCR cycle via the detection of a fluorescent dye binding double-strand DNA. The amount of mRNA for each gene was calculated relative to the amount of the housekeeping genes elongation factor-1(EF-1) and ß-actin, which were used as endogenous standards as they are continuously expressed in cells. For detailed description of the real-time PCR method, please see the paper III.

Immunohistochemistry (papers I-V)

Indirect immunofluorescence staining was used for the detection and localization of specific proteins in cerebral arteries, microvessels and surrounding brain tissue. Briefly, the cerebral arteries and surrounding brain tissue were dissected out, placed into Tissue TEK, frozen on dry ice, and sectioned into 10-µm-thick slices in a cryostat. Cryostat sections were fixed for 10 minutes in ice cold acetone and thereafter rehydrated in phosphate buffer solution (PBS) containing 0.25% Triton X-100 for 15 minutes. The tissue sections were then permeabilized and blocked for 1 hour in blocking solution and thereafter were incubated over night at 4 ˚C with the primary antibody of interest. The sections were subsequently washed with PBS and incubated with the appropriate secondary antibody conjugated with a fluorophore for 1 hour at room temperature. After washing with PBS, the slides were mounted with anti-fading mounting medium and sections were photographed with a confocal microscope at the appropriate wavelengths. The same procedure was used for the negative controls except that either the primary antibody or the secondary antibody was omitted to verify that there was no autofluorescence or unspecific labeling. Fluorescence intensity was used as a semi-quantitative measure of the level of expression of the proteins in the samples and was determined using the ImageJ software (http://rsbweb.nih.gov/ij/).

Western blot (papers I, III and V)

Western blotting is a method commonly used in order to identify and quantify a certain protein in a sample of tissue homogenate or extract. The cerebral arteries were harvested, frozen in liquid nitrogen and homogenized in cell extract denaturing buffer. Whole cell lysates were sonicated on ice and the supernatants were collected as protein samples. Total protein concentration was determined using a Bio-Rad DC kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were loaded onto a gel and separated by sodium dodecyl sulfate-PAGE. Molecular weight markers were loaded on each gel for protein band identification. After separation, proteins were transferred to a nitrocellulose membrane by electroblotting. The membrane was then blocked for 1 hour at room temperature and incubated with the primary antibody of interest overnight at 4ºC, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. The labeled

proteins were developed using the LumiSensor Chemiluminescent HRP Substrate kit (GenScript, Piscataaway, NJ, USA). The membrane was visualized using a Fujifilm LAS-1000 Luminescent Image Analyzer and band intensity was quantified using Image Gauge Version 4.0. The levels of ß-actin were used as a control for the normalization of the target proteins and data were expressed as a percentage of control.

Statistics

Statistical analyses were performed using the nonparametric Kruskal-Wallis with Dunn´s post hoc test for comparison between more than two groups and Mann-Whitney test for comparison between two groups, using Graph Pad Prism v.5 (Graph Pad software, Inc., La Jolla, CA). Data were expressed as the mean ± standard error of the mean (S.E.M) and *P*-values less than 0.05 were considered significant.

Ethics

For MCAO the experimental procedures were approved by the University Animal Ethics Committee in Sweden (M43-07). For SAH all procedures were carried out strictly in accordance with national laws and guidelines and were approved by the Danish Animal Experimentation Inspectorate (license no. 2066/561-1139) and the Ethical Committee for Laboratory Animal Experiments at the University of Lund.

RESULTS AND DISCUSSION

Cerebrovascular expression of pro-inflammatory mediators following MCAO (papers I and II)

MMP-9 and TIMP-1 expression

The BBB plays an important role in protecting the neuronal environment. Endothelial cells of brain arteries and capillaries have tight junctions, which can restrict molecules from moving between the blood and the brain. MMP-9 has been reported to be involved in the disruption of the BBB by degrading the tight junction proteins claudin-5 and occludin between the endothelial cells and extracellular matrix molecules constituting the basal lamina surrounding the endothelial cells. When the integrity of the BBB is lost, inflammatory cells and fluid can pass to the brain, causing hemorrhage, vasogenic edema and neuronal cell death [142, 209, 210].

There exist evidence pointing at angiotensin II and endothelin-1 increase following cerebral edema. They may induce elevated MMP-9 expression in rat vascular SMCs and astrocytes through activation of AT_1 and ET_B receptors after focal cerebral ischemia and in culture [211, 212]. Interestingly, administration of inhibitors towards these receptors reduced MMP-9 expression and ischemic injury [211, 212]. Therefore, the aim of **paper I** was to examine the early changes in the expression of MMP-9 and TIMP-1 in the walls of brain vessels at 48 hours post-MCAO.

Results from immunohistochemistry showed markedly enhanced expression of these proteins in the SMCs of the middle cerebral arteries (MCAs) and in associated microvessels within the ischemic region **(Figure 3)** and not in vessels on the contralateral side. This was confirmed with western blot analysis that showed the protein levels of MMP-9 and TIMP-1 were significantly increased in MCAs after MCAO as compared to control groups **(Figure 4)**. The results are in agreement with a previous study that reported increased in MMP-9 mRNA levels in the MCA at 24 hours after focal ischemia [9]. Another studies confirmed the presence and increase in MMP-9 mRNA and protein levels in the ischemic region at 24 hours

after MCAO with reperfusion. This was associated with reduction in tight junction proteins in cerebrovascular endothelial cells and administration of a MMP blocker and in MMP-9 knock-out animals prevented the degradation of tight junction proteins, reduced the BBB opening and vasogenic edema [142, 143]. Moreover, It has been reported that in rats with 2 hours transient MCAO, a maximally increase in MMP-9 was associated with maximal brain sucrose uptake at 48 hours after reperfusion [146].

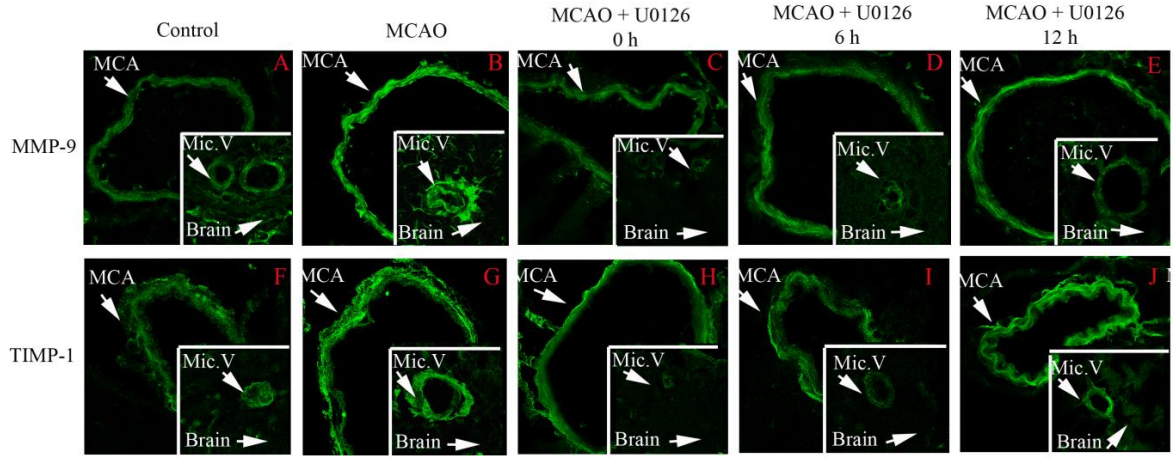

Figure 3. Confocal microscopy images of the MCA, cerebral microvessels (Mic.V), and surrounding brain tissue (Brain). Immunofluorescence labeling corresponding to MMP-9 **(A-E)** or TIMP-1 **(F-J)**. Images represent the vehicle control group (contralateral side) **(A, F)**, MCAO plus vehicle group (epsilateral side) **(B, G)**, MCAO plus U0126 with start at 0 hour **(C, H)**, with start at 6 hours **(D, I)** or at 12 hours **(E, J)** groups. There was a significant increase in MMP-9 protein levels in the smooth muscle cell layer of ischemic vessels as compared to vessels from the control group. TIMP-1 expression was upregulated in SMCs and in the proximity of the adventitia layer of ischemic vessels as compared to control vessels. Treatment with U0126 starting at zero and 6 hours, but not 12 hours after occlusion, prevented the increase in MMP-9 and TIMP-1 protein expression.

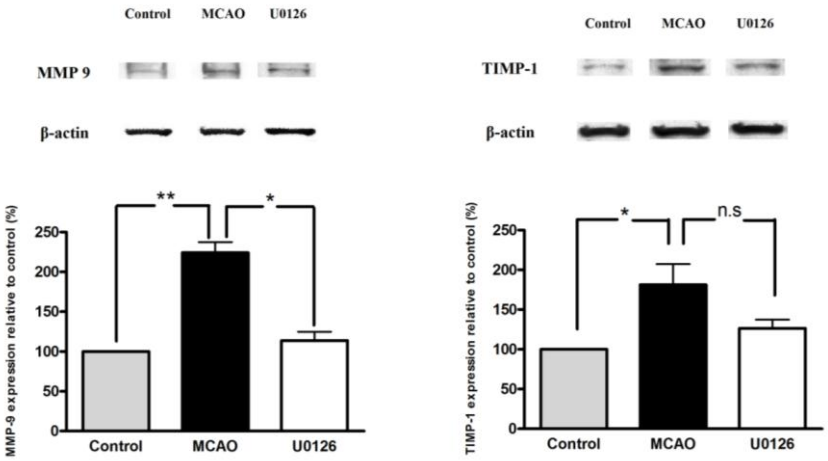

Figure 4. Western blot showing MMP-9 and TIMP-1 protein expression levels in the MCA 48 hours after MCAO using ß-actin as a loading control. Treatment with U0126 at 0 hour post occlusion decreased the MCAO-induced enhanced expression of MMP-9 and TIMP-1 proteins. Data are expressed as mean ± S.E.M., n = 4. *$P < 0.05$, **$P < 0.01$.

In addition, we showed that TIMP-1 was increased 48 hours after MCAO, probably in effort to balance the elevated expression of MMP-9 after induction of MCAO. Thus, we suggest that an imbalance between MMPs and TIMPs expression following cerebral ischemia may result in opening of the BBB and increase vessels permeability in reperfusion injury, contributing to cerebral edema and more brain damage.

To determine the cellular source of MMP-9 and TIMP-1, we performed co-localization studies using a SMC-actin specific antibody. MMP-9 immunoreactivity was localized to the cytoplasm in SMCs of the cerebral vessels. TIMP-1 was also localized in the SMCs of the medial layer but it was mainly located closer to the adventitia layer of the cerebral vessel walls **(Figure 5)**. To confirm this, we performed co-localization studies using CD31 (as a marker of endothelium cells) neither MMP-9 nor TIMP-1 revealed any major co-localization with CD31; hence, the upregulation occurs in the medial layer. In addition, some vessels were studied after mechanical removal of endothelium. After this procedure the localization of the above proteins in the SMCs was still the same and the SMC localization confirmed. Interestingly, following double staining with GFAP (a selective marker of astrocytes), we noted that the expressions of MMP-9 and TIMP-1 were not associated with glial or astrocyte end-feet in the vessel walls. This confirmed and supports that the transcriptional upregulation takes place in the vascular SMCs themselves. There was a rich network of GFAP-positive

astrocytes in the cerebral cortex tissue and around the microvessels. This is in agreement with a previous study that demonstrated the presence of astrocytic end-feet surrounding the microvasculature [213].

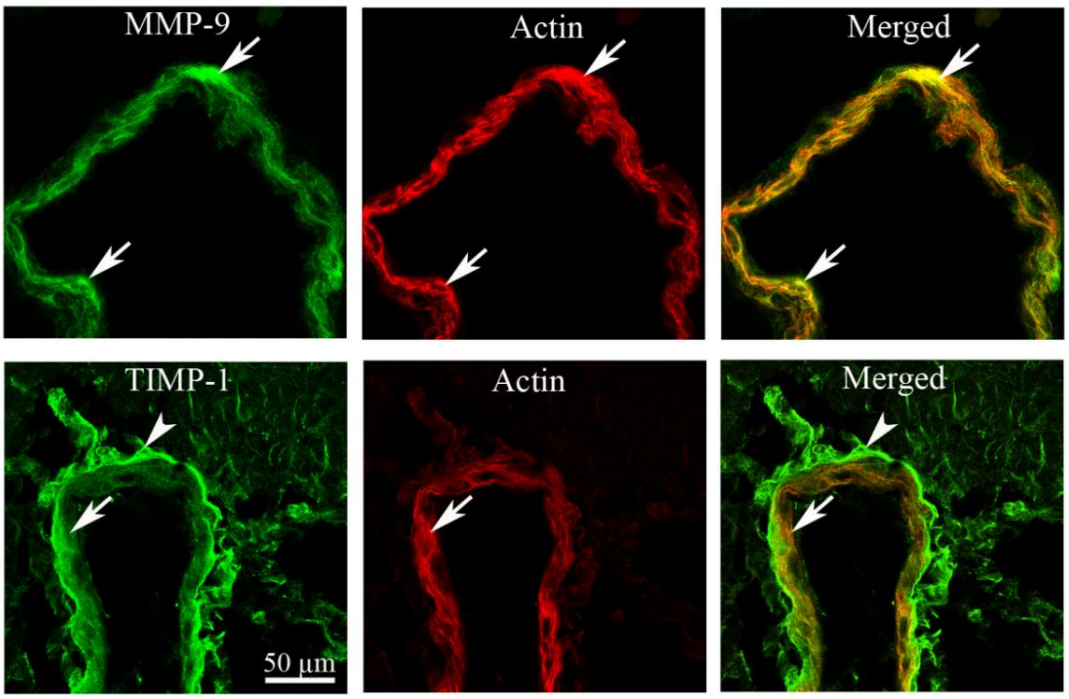

Figure 5. Double immunofluorescence staining for MMP-9 or TIMP-1 and SMCs actin in the MCA after MCAO. Photographs show the localization of MMP-9 and TIMP-1 (green) and of actin (red) in smooth muscle cells, and their co-localization (yellow fluorescence in the merged picture).

Cytokines and iNOS expression associated with infarct volume and neurological scores

It is well established that the neuroinflammatory process is complex and involves numerous pathways and molecules in the brain. However, relatively little information is available on the role of the cerebrovascular SMCs in this process following cerebral ischemia. Therefore, the next step was to investigate early changes in cytokine expression in the wall of cerebral vessels after focal ischemia.

Previously, Vikman and co-workers revealed, using microarray and qPCR analysis, that upregulation of cytokine genes occurs in the walls of the cerebral arteries at 24 hours after cerebral ischemia and after organ culture [8, 9]. To investigate if the upregulated genes are

translated to proteins, immunohistochemistry was used to detect TNF-α, IL-6 and IL-1ß protein expression in brain vessel walls at 48 hours after MCAO. We observed significantly enhanced expression of TNF-α, IL-6 and IL-1ß proteins in the walls of MCA and brain microvessels **(paper II)**. Notably, this enhanced expression was primarily located in the cytoplasm of the SMCs (co-localization with actin), while a weak expression was in addition seen for IL-6 and IL-1ß in the endothelial cells. Taken together with the results obtained by Vikman at 24 hours, our results support the notion that a transcriptional event is involved.

It is thought that cytokines such as TNF-α, IL-6 and IL-1ß are involved in the development of secondary brain damage and are associated with increased infarct size through upregulation and activation of adhesion molecules, leukocyte infiltration and MMP-9 activation [76, 96, 214].

At 48 hours after MCAO, we also revealed that acute cerebral ischemia followed by reperfusion in the rat is accompanied by an infarct volume of 25 ± 2% of total cerebrum and a poor neurological score **(Figure 6)**. The results are in agreement with those of previous studies. In parallel there is elevated expression of TNF-α and IL-1ß in the cortex after both transient and permanent MCAO in rat [104, 215]. It has been reported that intracerebroventricular injection of antibodies against TNF-α and IL-1ß starting at 30 minutes before permanent MCAO or immediately after reperfusion following transient MCAO reduced infarct volume [104, 215]. Vila and co-workers have reported high levels of IL-6 and TNF-α in plasma and CSF of patients within the first 48 hours after ischemic stroke onset, which correlated with early neurological deterioration, raised body temperature, and a larger infarct volume [216]. In addition, stroke is associated with enhanced expression of some GPCRs that mediate vasoconstriction. We have recently revealed that cytokines can, at least *in vitro,* enhance this expression [217]. Thus, there may appear a link between the expression of cytokines and the brain damage.

In conjunction with an inflammatory reaction there is formation of iNOS [218]. This is another inflammatory mediator which is expressed in the brain following cerebral ischemia and its expression can be stimulated by cytokines [51]. In addition, iNOS is involved in secondary brain injury through production of COX and free radicals, which are putative mediators of BBB disruption and brain edema, leading to increased infarct volume [219, 220]. Therefore, we decided to investigate if there is upregulation of iNOS in the vessel walls at 48

hours post-ischemia. In concert with the findings for cytokines and MMP-9, we noted a marked expression of iNOS in the SMCs of both MCA and microvessels on the ischemic side compared to the contralateral side. This finding is supported by a previous study by Iadecola of iNOS mRNA and protein expression in the ischemic brain at 48 hours after MCAO [221].

One important observation we made was that the upregulation of cytokines, iNOS and MMPs occurred not only in large cerebral arteries but also in associated microvessels in the affected brain region. This indicates that both large cerebral arteries and associated microvessels in the ischemic region are actively participating in inflammatory responses and production of inflammatory mediators following focal ischemic stroke. This provides the first direct evidence of an associated vascular mechanism, involving both large cerebral arteries and brain microvessels.

MEK-ERK1/2 pathway regulating upregulation of MMPs, cytokines and iNOS following MCAO

Previously, we have shown that 48 hours after MCAO caused activation of the MEK-ERK1/2 pathway in the SMCs of cerebral vessels associated with the ischemic region [68].

In **papers I** and **II**, we demonstrate that several pro-inflammatory mediators (MMP-9, TIMP-1, TNF-α, IL-6, IL-1ß and iNOS) are upregulated at the protein level in the vascular walls at 48 hours after focal ischemia. In order to elucidate if the upregulation is associated with activation of the signal transduction pERK1/2 protein, we blocked this pathway upstream by a specific MEK1/2 inhibitor (U0126), which has high affinity for binding to MEK1/2. Inhibiting MEK1/2 activity leads to inhibition of phosphorylation and activation of ERK1/2 downstream but not upstream of MEK1/2 [222]. In addition, U0126 is well tolerated by the animals and does not alter any physiological parameters even when we used a higher dose (30 mg/kg) (to reach the abluminal side of the BBB), than that used by others in culture (10 µM) and or *in vivo* (200 µg/kg) to inhibit the MEK-ERK1/2 pathway in cells [185, 223]. The results showed that U0126 administration abolished the enhanced expression of vascular MMP-9, TIMP-1 **(Figure 3)**, cytokines and iNOS both in the large cerebral artery and in associated microvessels in the ischemic region. Importantly, this worked if U0126 was given immediately following the reperfusion (0 hour) or starting 6 hours after the start of

reperfusion. In addition, treatment with U0126 significantly reduced infarct volume and improved the neurological score when given at 0 hour or 6 hours after reperfusion **(Figure 6)**. This is in agreement with a previous study that showed that U0126 treatment decreased the ischemic area and improved neurological scores when given in conjunction with the occlusion after MCAO [72].

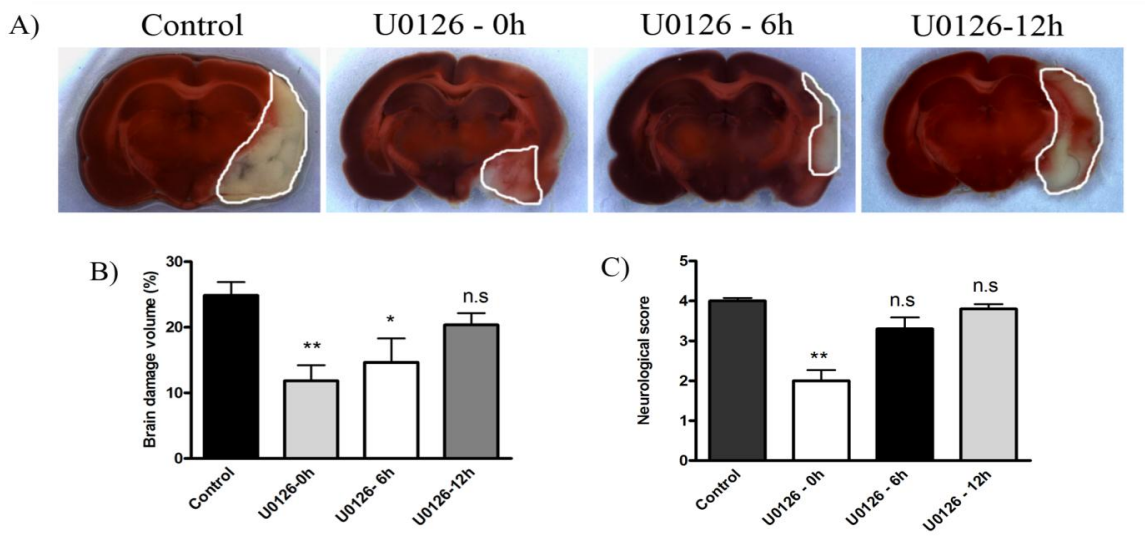

Figure 6. A) Typical examples of coronal brain sections that show smaller ischemic areas in animals treated with U0126 when administrated immediately after reperfusion (0 hour), or at 6 hours after reperfusion versus animals treated with vehicle (Control). **B)** Measurements of the brain damage (% of total volume) showed a significant decrease in infarct size on the ischemic side in animals treated with U0126 starting at 0 hour (11.8 ± 2%**) and 6 hours (14.6 ± 3%*) after MCAO as compared to the control group (25 ± 2%) and treated after 12 hours (20.3 ± 1%). **C)** Neurological scores for vehicle treated rats (Control) and rats treated with U0126 at 0, 6 and 12 hours after MCAO. Data are expressed as mean ± S.E.M. and n = 6 -7. *P < 0.05, ** P< 0.01.

Furthermore, we showed that pERK1/2 immunoreactivity was increased in the SMCs of both cerebral artery and microvessels but not in the adjacent brain tissue in the MCAO group as compared to the control group. This suggests that following ischemia the pERK1/2 activity occurs in the vascular walls and not in the surrounding brain tissue. The activity was inhibited by U0126 administration when given at 0 or 6 hours after the reperfusion **(Figure 7)**. This agrees with a previous study that showed U0126 administration diminished pERK1/2

immunoreactivity in the hippocampus and thereby protected brain against damage after forebrain ischemia in gerbil [223].

If the MEK1/2 inhibitor was given 12 hours after the reperfusion, there was no significant change in all above proteins as well as no effect on decreased the infarct volume and improved neurological functions. We suggest that there is a “switch-on” mechanism that is accessible to early antagonism but if therapy is given after this (as at 12 hours after reperfusion) it will be too late to modify the outcome.

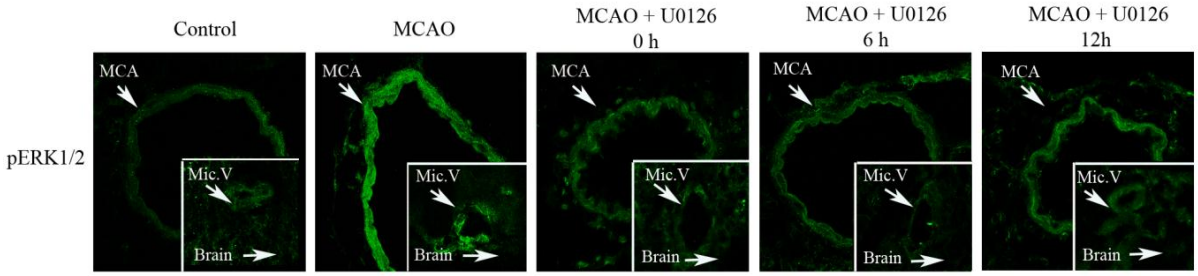

Figure 7. Immunofluorescence staining for pERK1/2 in the ischemic MCA, cerebral microvessels (Mic.V), and surrounding brain tissue (Brain) in Control (contralateral side), MCAO (ipsilateral side) and MCAO plus U0126 at different time points. There was an increase in pERK1/2 in the SMC layer of ischemic vessels (MCA and Mic.V) as compared to control. Treatment with U0126 starting at 0, or 6 hours after reperfusion prevented this increased 48 hours following MCAO.

We have also shown that the other MAPK, p38 and JNK were only slightly affected in vessel walls by MCAO. In addition, U0126 does not affect phosphorylation of p38 or JNK in cerebrovascular SMCs at 48 hours after focal ischemia. This is in agreement with previous study that demonstrated that U0126 did not affect these parameters in cultured neurons [223]. These findings also support the specificity of U0126 for the MAPK-ERK1/2 pathway.

Taken together, the results have revealed that a reduction in infarct volume occurred in parallel with reduction in expression of vascular pro-inflammatory molecules and this is associated with decreased pERK1/2 activity in the walls of cerebral arteries and microvessels. Thus, we suggest that the upregulation of cytokines, MMP-9, TIMP-1 and iNOS involves transcriptional events since the increases observed were all paralleled by an upregulation of pERK1/2 and they were normalized by inhibiting this pathway with U0126.

Combined inhibition of endothelin and angiotensin receptors following MCAO

Endothelin-1 (ET-1) and angiotensin II (Ang II) are produced in endothelial cells in the body, in all vascular regions including the cerebral circulation and are present in the systemic circulation [17, 224]. Both are involved in inflammatory processes in addition to having potent vasomotor effects [225, 226]. Increased and release of ET-1 from endothelial cells is reported after hypoxia/ischemic injury of the brain [227]. Their effects are mediate by specific GPCRs in the vessel walls [65, 67]. Consequently, an inhibition of these mediators, by directly targeting their receptors might provide a good way to reduce infarct volume after MCAO. Particularly since ample published data have shown positive effects [17]. The use of specific endothelin [228] or angiotensin receptor antagonists [229] have depending on experimental conditions provided reduction in infarct size. In our work we found that combined receptor blockade showed stronger effects [230]. The results in **paper II** confirmed that the dual blocked of ET_A and AT_1 receptors caused significant reduction in infarct volume and improved the neurological scores with no effect on mean arterial blood pressure. In addition, this dual blocked abolished the IL-6 and TNF-α expression and slightly reduced the IL-1ß expression, possibly via an anti-inflammatory effect. However, the receptors blockade did not change the upregulation of pERK1/2 and iNOS. Thus, ET-1 and Ang II responses via these receptors might be involved in the process of regulating the cytokine response after MCAO followed by reperfusion.

The results provide evidence that the reduction in ischemic brain damage by inhibition of the MEK-ERK1/2 pathway with U0126 is a more promising approach since this involves not only inhibition of receptor upregulation, but may involves several mechanisms and complex interactions, such as modulation of the inflammatory response.

Cerebrovascular expression of pro-inflammatory mediators after SAH (papers III and IV)

Several studies have suggested that oxyhemoglobin from extravasated blood may result in induction of inflammation and vascular alterations after SAH [50, 58]. This inflammation may have an important role in the development of DCI and outcome of SAH [51, 231, 232]. Focal cerebral ischemia (papers I and II) results in a local inflammatory response and upregulation of vascular pro-inflammatory mediators in the occluded artery and microvessels in the ischemic region. This raises the question whether a similar upregulation of vascular pro-inflammatory mediators may be found after SAH, which is a disease that involves a global cerebral ischemia because of the initial increase in ICP and reduction in CBF. [233].

MMP-9 and TIMP-1 expression

Several studies have suggested that MMP-9 contributes to the development of DCI and to poor outcome following SAH through degradation of vascular and microvascular basal lamina proteins, associated with BBB dysfunction and cerebral edema [233, 234]. A previous study reported that MMP-9 knockout mice exhibited better neurological scores, less brain edema formation, and lower chronic ICP at 3 days after SAH using an endovascular puncture model [233]. Prunell [201] compared 3 methods to induce SAH. The puncture method was associated with 40% mortality and large inter individual variations. We based our choice on the reproducibility of the injection method and the much lower mortality rate.

The results from our immunohistochemistry experiments showed that the present model of SAH (injection of 250 µl of autologous blood into basal cisterns) results in enhanced expression of MMP-9 and TIMP-1 proteins in the SMCs both in major cerebral arteries (BA and MCA) and in brain microvessels at 48 and 72 hours. The results were similar when compared to results from the MCAO and reperfusion model but for SAH more wide spread in different cerebral regions. Further, the results were confirmed with two quantitative methods, real time PCR for gene expression (mRNA) and western blot for the quantitative detection of proteins **(Figure 8)**. Our findings are in agreement with a previous study showing that MMP-

9 is upregulated in brain blood vessels affected by intracranial aneurysms [101] and in cerebral aneurysm walls following SAH in animals [235]. Thus, we suggest that MMP-9 might be an important clinical target for the therapy after SAH in humans. In addition, increases in TIMP-1 protein and mRNA have been observed at 48 hours post-SAH which reveals an imbalance in MMP-9/TIMP-1 regulation. This agrees with our results from the MCAO model, suggesting similarities in cellular activation.

Cytokines and iNOS upregulation associated with CBF reduction

Previous study showed that elevation of IL-1β, IL-6 and TNF-α in the subarachnoid space of patients with SAH is associated with development of vasospasm and DCI [98]. However, the pattern of cytokine expression differs depending on stroke type and localization.

In the present work, we have reported that the above cytokines are expressed in the wall of cerebral vessels at 48 and 72 hours post-SAH in our experimental SAH model. Similar to MCAO, results from immunohistochemistry and western blot analysis showed significant upregulation of TNF-α, IL-6 and IL-1ß proteins in the walls of large cerebral arteries **(Figures 8 and 9)** and in microvessels but not in the surrounding brain tissue in SAH groups as compared with sham (control) groups. Furthermore, the TNF-α expression is located to the SMCs of the media layer (co-localization with SMC actin), while IL-6 and IL-1ß are located in both SMCs and endothelial cells **(Figure 9)**.

As mentioned earlier, iNOS is not present in brain tissue under normal conditions but its expression can be activated by cytokines [51]. We found that the iNOS protein is weakly present in cerebral vessels, but its expression is enhanced in cerebrovascular SMCs after SAH. Thus, upregulation of iNOS in vascular walls after cerebral ischemia might be correlated to production of cytokines.

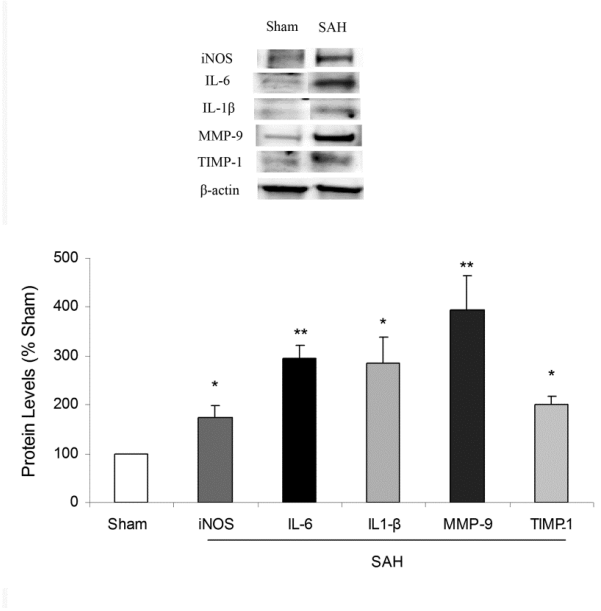

Figure 8. Western blot showing significant increase of iNOS, IL-6, IL-1ß, MMP-9 and TIMP-1 protein expression levels in the cerebral artery 48 hours after SAH as compared to sham. ß-actin was used as a loading control. Data from four experiments (each performed on vessels from 3 rats) are expressed as mean ± SEM, n = 4. **P* < 0.05, ***P* < 0.01.

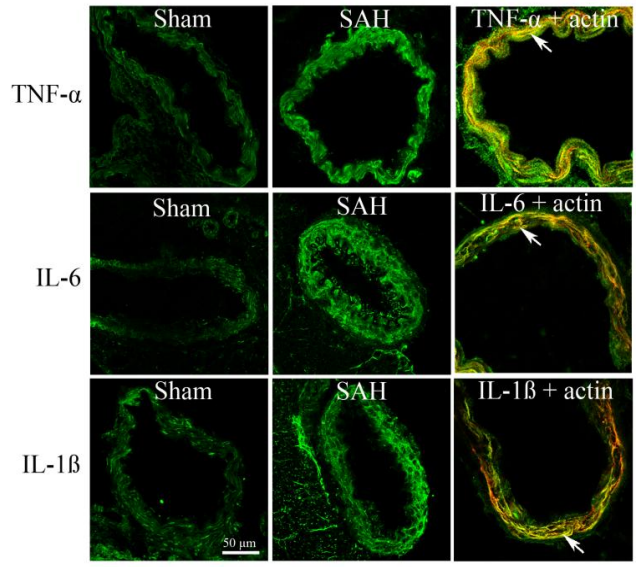

Figure 9. Immunofluorescence staining for TNF-α, IL-6 and IL-1ß in the wall of cerebral arteries in the sham and SAH groups and the double staining of each protein with actin in SMCs. There was a significant increase in TNF-α, IL-6 and IL-1ß protein levels in the wall of cerebral arteries in SAH as compared to sham. This expression was mostly located in the SMCs in the medial layer (white arrows and yellow colour in co-localization images).

In addition, we observed that there was a significant decrease in CBF at 48 hours in the SAH group as compared to the sham group (from 140 ± 6 to 63 ± 2 ml/100g/min). Since the maximum blood flow reduction occurred at 48 hours in experimental SAH [51, 236, 237], we chose this time point to investigate the expression of pro-inflammatory mediators in vascular walls. In fact, CBF reduction at 48 hours is associated with maximum upregulation of cerebrovascular constrictor receptors (ET_B, AT_1 and 5.-HT_{1B}) [238] as well as of cytokines, MMP-9 and iNOS. This is consistent with studies that have demonstrated enhanced

expression of cytokines via activation of the MAPK signaling pathway after cerebral ischemia [98, 239].

Time-course of MMP-9 and cytokine expression following SAH

We noted that at 48 and 72 hours after SAH there was increased expression of TNF-α, IL-6, IL-1ß and MMP-9 in cerebral vessel walls as compared to the sham group. To further elucidate when and how these changes take place, we decided to conduct a study of the molecular changes that occur over time in the cerebral arteries. More specifically, we aimed to determine if the time-course and upregulation of pro-inflammatory mediators in cerebral arteries and microvessels after SAH is associated with the MEK-ERK1/2 pathway. The protein levels of TNF-α, IL-6, IL-1ß and MMP-9 at 0-96 hours after SAH were investigated using immunohistochemistry and the fluorescence intensity measured by using the program ImageJ software. The results showed that TNF-α, IL-6, IL-1ß and MMP-9 proteins were elevated in cerebral artery walls over time, with maximum at 48-72 hours after SAH **(Figure 10)**. Examination of IL-6, IL-1ß and MMP-9 immunoreactivities revealed that during the first 12 hours after SAH there was a slight transient increase at 6 hours in IL-6, IL-1ß and MMP-9 protein expression in cerebral artery walls. At 24 hours and onwards also TNF-α **(Figure 10)** showed robustly elevated expression. Interestingly, the cytokines and MMP-9 elevation occurred in parallel with increments of local SMC pERK1/2 [71]. This indicates that the response event in the cerebral arteries is due to the initial events that took place following the SAH, namely changed in shear stress and the presence of extravasated blood cells. We did not determine a time-course for iNOS because it has been performed before [240]; it was then observed that the iNOS expression was increased in the wall of cerebral artery following SAH and peaked at about 12 hours and then slightly decreased during 24-48 hours [240].

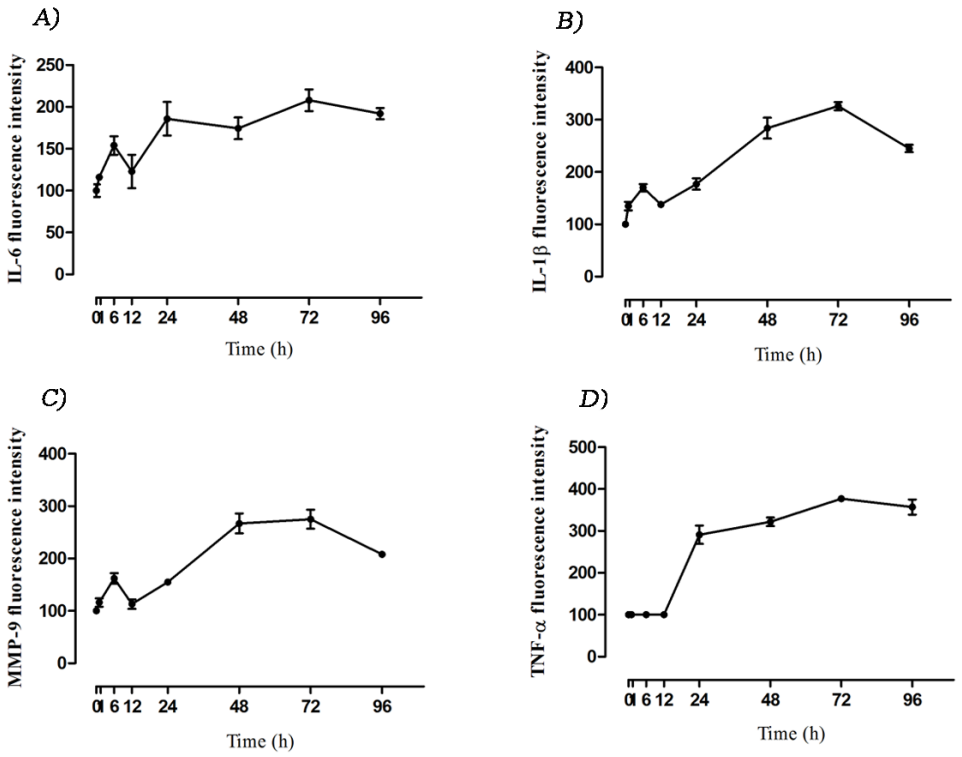

Figure 10. Immunohistochemical evaluation of IL-6, IL-1ß, MMP-9 and TNF-α in the wall of MCA following SAH. Densitometry analysis showed that there was an early increase of IL-6 **(A)** and IL-1ß **(B)** protein in the wall of MCA around 6 hours, followed by a slight decrease at about 12 hours and a later increase during 24-96 hours with maximum peak at 72 hours. MMP-9 protein level showed a slight increase at 6 hours and a second gradual increase during the period of 24-96 hours with maximum increase at 48-72 hours **(C)**. Expression of TNF-α protein started during 12-24 hours and increased with time until 96 hours **(D)**.

The early increase in MMP-9 protein expression in the wall of cerebral arteries at 6 hours was followed by a further increase for up to 72 hours is consistent with previous studies that demonstrated upregulation of MMP-9 mRNA at 6, 24 and 48 hours after organ culture, and *in vivo* MCAO and SAH [9]. On the other hand, there was very weak expression of MMP-9 in the brain tissue at all time-points, which is in agreement with previous work [241]. We therefore suggest that the upregulation of MMP-9 is a response to SAH specific for the cerebral vasculature, and that the upregulated MMP-9 may play a role in the complex vasculopathy following SAH.

At early time points (1-24 hours) following SAH, there was a marked expression of TNF-α in the brain parenchyma (Figure 11). Interestingly, there was no significant expression of IL-6

and IL-1ß. The reason for this is not clear but the early production of TNF-α in the brain tissue during ischemia has been reported earlier [242].

The TNF-α expression was co-localized with GFAP around the vessels and in the brain tissue **(Figure 11)**. This agrees with the observations of TNF-α mRNA and protein expression at an initial peak around 1-3 hours and a second peak at 12-24 hours in neurons [105], microglia and astrocytes [243] in the brain cortex after MCAO in rat. Further, activation of microglia and astrocytes leading to release of cytotoxic substances like nitric oxide and TNF-α, have been reported in response to early brain injury after cerebral ischemia [244].

There was significant expression of TNF-α in SMCs of the MCA at 2-4 days post SAH **(paper IV)**. Interestingly, there was no TNF-α expression at the early time-points (during 0-12 hours) in the wall of cerebral arteries. On the other hand, there was a moderate expression at 12-24 hours that continued to increase over time during 48-96 hours. This is in line with a previous study that showed that TNF-α mRNA was elevated at 24-48 hours in the wall of cerebral arteries after SAH [240]. Another investigator also reported on TNF-α expression in the wall of the basilar artery (BA) at 2-5 days post SAH in mice [245]. These results together with our data may suggest that SAH in rats results in early elevated expression of TNF-α protein in neurons, astrocytes and glial cells in brain tissue. Then the expression of TNF-α in neurons and astrocytes appears to facilitate the change in vascular wall to farther expression of this protein and exacerbate tissue damage in cerebral ischemia. In addition, there is evidence to indicate that direct administration of TNF-α into the brain produces a dramatic increase in leukocyte adhesion to vascular walls and an infiltration of these inflammatory cells into tissue that occurs after focal stroke [105].

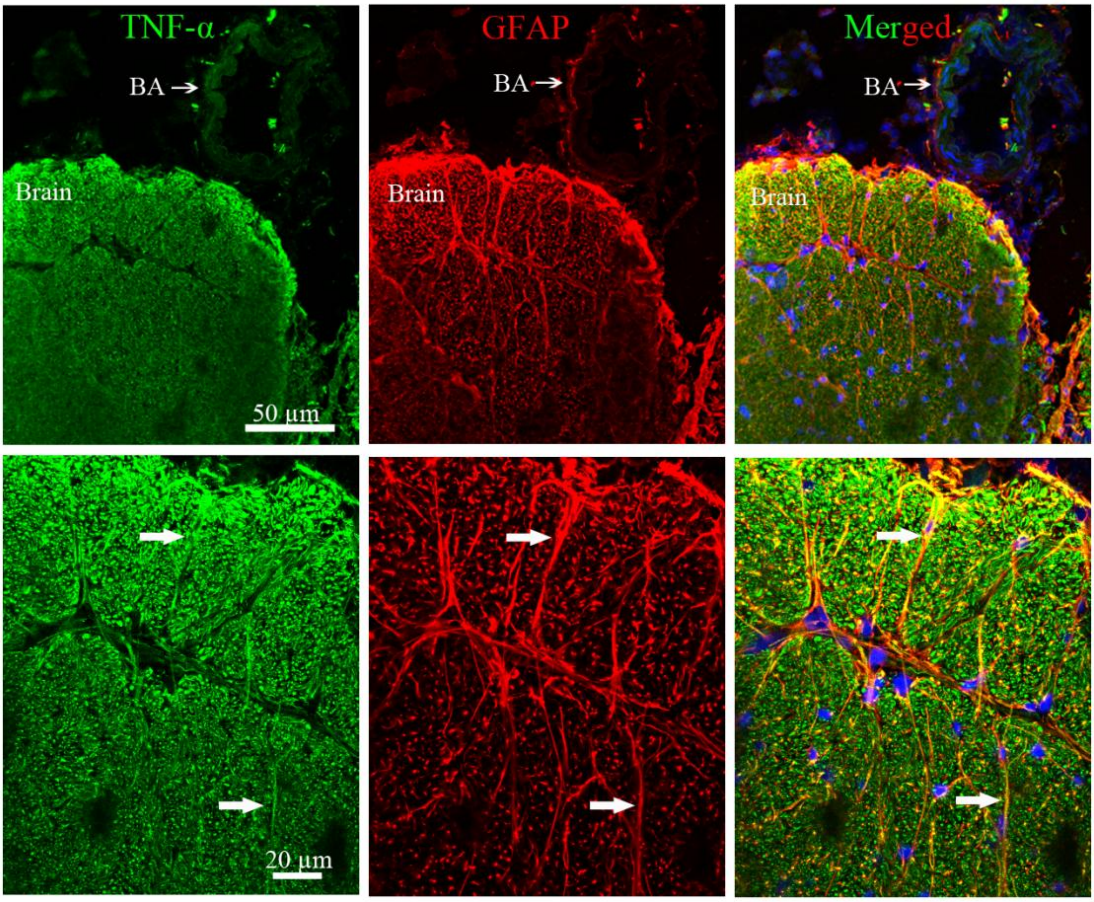

Figure 11. Double immunofluorescence staining for TNF-α (green) and GFAP (red); in the wall of BA and in surrounding brain tissue at 0-24 hours post SAH. There was no expression of TNF-α in the wall of the BA at 0-24 h post SAH, but an enhancement in TNF-α immunoreactivity was seen in the brain tissue. This was co-localized with GFAP (white arrows in merged picture). GFAP expression was observed in astrocytes around BA and in the surrounding brain tissue.

We noted that there was a modest early expression of IL-6 and IL-1ß proteins in the wall of cerebral arteries at the early time point (6 hours), which further increased over time to reach maximum at 72 hours. The findings are consistent with other investigations, which have demonstrated that IL-6 and IL-1ß mRNAs and proteins were increased at an early time point between 1-6 hours and at later time points at 24-48 hours in cerebral vessels following transient global ischemia [246] and SAH [240].

We did not find any expression of TNF-α, IL-6 and IL-1ß in the fresh group or in the 0 hour group, which indicates that production and secretion of the studied cytokines correlates with brain injury after induced SAH.

Raf-MEK-ERK1/2 pathway regulating MMP-9 and cytokines upregulation after SAH

Lack of significant beneficial effect of anti-inflammatory drugs and corticosteroids on patients with late cerebral ischemia after SAH suggests that this is a multifactorial disease with several mechanisms probably involved at different stages of the disease and with significant interplay between them. Treatments targeting a signaling pathway common to all mechanisms might be a beneficial way forward.

Several studies have shown involvement of the MEK/ERK/MAPK pathway in the regulation of CVS after experimental SAH [191, 192, 247]. We observed a significant increase in protein expression of phosphorylated ERK1/2 in the SMCs of the wall of cerebral arteries at 2 and 3 days following SAH as compared with the sham group. Notably, this activation was also seen in microvessels but not in the adjacent brain tissue. Interestingly, quantitative western blot analysis showed that the expression started already at the early time point, 1 hour. This was in line with a previous report from Ansar and co-workers which indicated that only the pERK1/2 pathway was activated at this early time point after SAH, while pp38 or pJNK were activated at 48 h after SAH [71]. JNK and p38 activation may relate to later effects involving further inflammatory mediators and apoptosis.

Administration of a specific Raf inhibitor, SB386023-b, **(Figure 12)** or a MEK1/2 inhibitor, U0126, starting at 6 hours after SAH prevented the ERK1/2 activation in cerebral vessels. Importantly, treatment with SB386023-b, which also started at 6 hours after the SAH, prevented the reduction in CBF after SAH **(Figure 13)**. In addition, we observed similar results when SB386023-b or U0126 were administrated at 0 hour after SAH, but there was no significant effect on reduction of pERK1/2 and associated CBF when SB386023-b was given at 12 hours after SAH. Treatment with U0126 and/or SB386023-b in selected dose did not induce any change in the ICP, CBF or physiological parameters and was well tolerated by the animals.

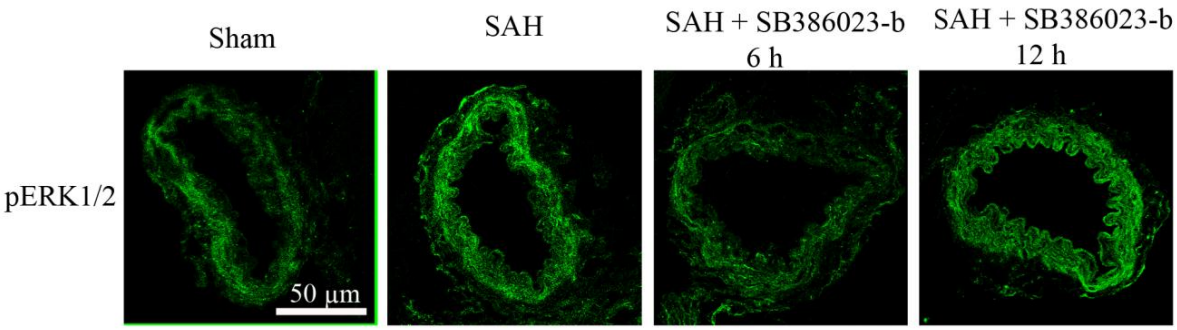

Figure 12. Cerebral artery showing phosphorylated ERK1/2, immunoreactivity in the SMC layer in sham, SAH, SAH treated with SB386023-b starting at 6 hours and SAH treated with SB386023-b starting at 12 hours. There was increased activation of pERK1/2 after SAH as compared to sham operated rats. Treatment with SB386023-b given at 6 hours after SAH prevented the increased protein expression. Data were obtained with confocal microscopy.

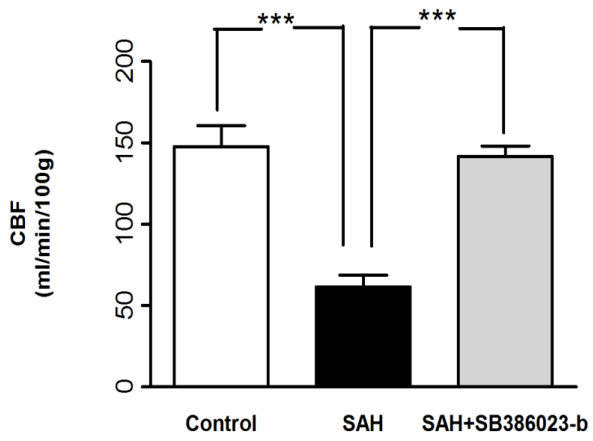

Figure 13. Effect of treatment with SB386023-b on the CBF after induced SAH. There is a reduction in the global CBF in the SAH compared to the control rats but treatment with SB386023-b inhibited this reduction (here shown administration that started 6 hours after SAH). Measured with the auto-radiographic method of Sakurada. Data are expressed as mean ± S.E.M. (n = 5-6), values, ***P≤ 0.001.

In a dose finding approach we have examined the MEK1/2 inhibitor U0126 both in the MCAO model [68, 72] and in the SAH model [202]. Contrary to the dose used in previous study in SAH [248] we used a higher dose to obtain pERK1/2 inhibition in cells. In addition, we administrated the drug in the acute phase, while previous study [248] where no effect was obtained administrated the drug in the late phase (3 days) after SAH. This might be an explanation to the lack of effects in previous study [248]. Thus, it appears important to validate the inhibition of signal transduction in the experimental condition between a firm negative conclusion can be drown.

Furthermore, we revealed that the inhibition of the ERK1/2 pathway upstream of MEK1/2 with SB386023-b or U0126 (start of administration at 0 hour or 6 hours after SAH) prevented the increased expression of cytokines, iNOS and MMP-9 in the cerebral vascular walls at 48 and 72 hours post SAH. Interestingly, treatment with U0126 showed similar effects irrespective of whether (A) we administrated it at 6, 12, 24, 36 h and terminated the experiment at 48 hours after SAH or if (B) we administrated it at 6, 12 and 24 hours and sacrificed the animals at 72 hours after SAH. The reason for treatment regimen B (3 days survival) was to test if U0126 would be active if it was only given in the early phase (first 24 h) after SAH. This treatment protocol shows that it not critical for the effect of U0126 whether the animals are left untreated from 24 h to 72 h, as long as they are properly treated in the critical period at 6 to 24 h. We suggest that early activation of ERK1/2 pathway participates as a "switch-on mechanism" for the pro-inflammatory mediators' upregulation and the associated brain damage. Inhibition of this pathway with U0126 when given at 6 hours after SAH induction is effectively inhibited ERK1/2 activity and improve outcome after SAH.

Other significant observations in this study include the fact that inhibition of the ERK1/2 signaling pathway by U0126 improved behavior scores at 3 days post SAH, including locomotors function and coordination, and spontaneous activity **(Figure 14).** This is in contrast to the recent Clazosentan studies that were positive in reducing vessels diameter but failed to improve neurological outcome [249, 250]. In addition, an earlier study by Larsen and co-workers [251] showed that the effects of U0126 treatment on neurological function by using rotating pole test at 2 days post SAH was comparable to the effects shown here. Thus, inhibition of early MEK-ERK1/2 signaling after SAH provides a novel therapeutic target which can be administered within a clinically relevant time window and has the potential of improving outcome after SAH.

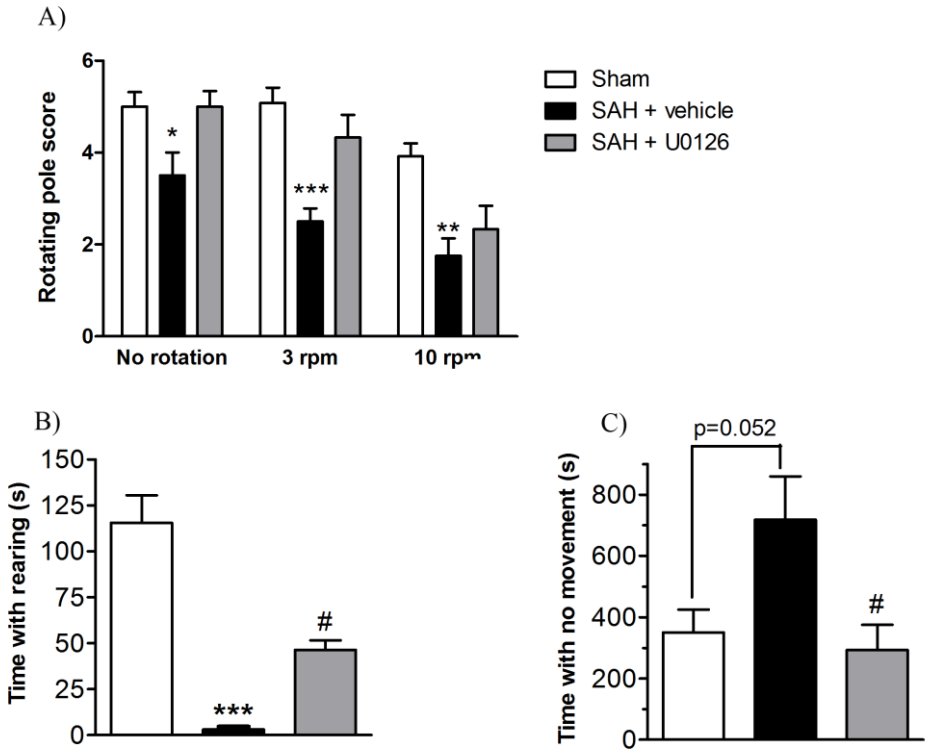

Figure 14. Neurological function for sham, SAH+ vehicle and SAH + U0126 given at 6 hours, 12 hours and 24 hours after SAH, determined by the rotating pole test **(A)** and spontaneous behavior **(B and C)**. Scores were obtained at day 3 after SAH. **(B)** Time spent with rearing. **(C))** Time spent sitting or lying at the same place (no movement). Data are expressed as mean ± S.E.M., n= 5 in each group.* indicates significant differences as compared to sham-operated rats, # indicates significant differences as compared to the SAH group. * and $^{\#}$ $p<0.05$, ** and $^{\#\#}$ $p<0.01$ and *** $p<0.001$.

Taken together, the present results show that SAH induces activation of the ERK/MAPK pathway at the early time points in SMCs of the cerebral artery leading to production and upregulation of pro-inflammatory cytokines and MMP-9 in cerebral vascular walls. Inhibition of this pathway as late as 6 hours after SAH prevented the activation of pro-inflammatory mediators, normalized CBF and improved neurological function. Importantly, SB386023-b and U0126 were equally effective within a clinically relevant time frame. They were effective not only when administered at the start of the insult but also when initiating treatment at 6 hours after the insult.

Expression of TNF-α and its receptors in cerebral ischemia and in organ culture (paper V)

Organ culture has been used to induce stress to isolated vessels segments and it has been observed that the expression of some GPCRs is upregulated in this model [252]. For example it has been demonstrated that the increased ET_B receptor-mediated contractile response is associated with an increased in VSMCs ET_B protein and mRNA levels at 24 hours after both organ culture and experimental focal ischemia, suggesting that this upregulation is a phenomenon characteristic for the response to cerebral arteries to situations of drops in intraluminal pressure and vascular wall tension [253].

As described and discussed previously in this thesis, we have shown that there is correlation between upregulation of MMP-9, cytokines and iNOS and activation of the ERK1/2 signaling pathway in MCAO and SAH. Here, we examined whether the *in vitro* system organ culture could replicate the changes in protein expression found in the *in vivo* models, in particular those engaged in the activation and regulation of pro-inflammatory factors. Therefore, the aims of **paper V** were first to investigate if there is an altered expression of TNF-α and of its receptors (TNF-R1 and TNF-R2) in cerebral artery walls following global (SAH) or focal ischemia (MCAO), and after organ culture of isolated artery segments. Secondly, we asked what intracellular signaling events are involved in regulating the expression of these molecules.

TNF-α was interesting given its high and rapid upregulation in the brain after injury following cerebral ischemia. TNF-α is a pleiotropic cytokine which participates in the regulation of the BBB, inflammation, angiogenesis, increased expression of other cytokines, vasoconstrictor substances and vascular changes associated with brain injury [104, 105] and utilizes its two receptors to initiate target cell responses [254].

The results showed that the levels of TNF-α, TNF-R1 and TNF-R2 proteins were increased in the cerebral arteries at 48 hours following MCAO, SAH and organ culture **(Figure 15)**. These results are in agreement with previous studies that have reported increases in TNF-α, TNF-R1 and TNF-R2 mRNA and protein levels in the brain [134, 255] and in retinal arteries [256] after ischemia.

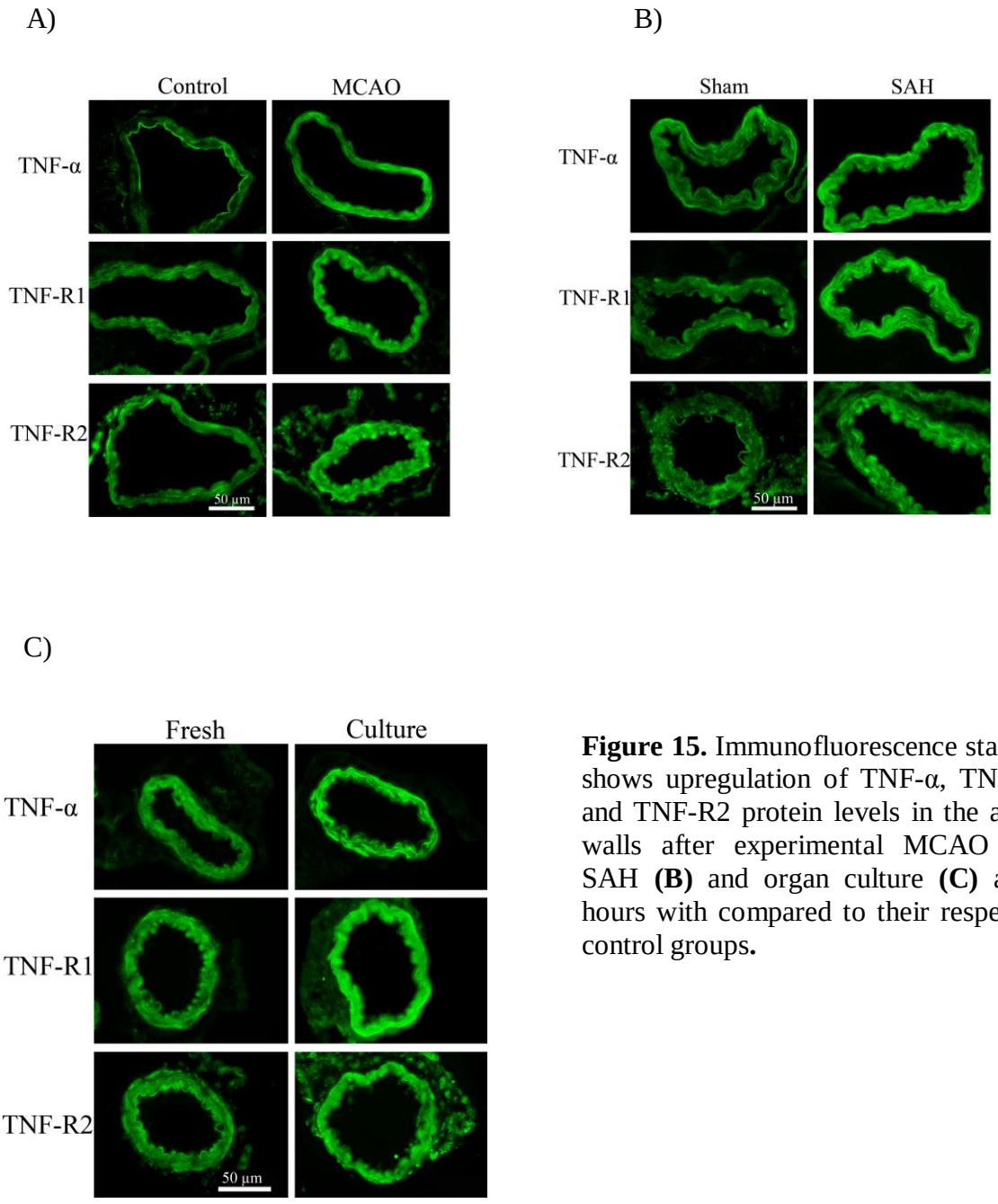

Figure 15. Immunofluorescence staining shows upregulation of TNF-α, TNF-R1 and TNF-R2 protein levels in the artery walls after experimental MCAO **(A)**, SAH **(B)** and organ culture **(C)** at 48 hours with compared to their respective control groups**.**

Importantly, there is a similarity between the upregulation of the TNF-α and its receptors in the cerebral arteries after cerebral ischemia and organ culture. We found that expression of TNF-α and TNF-R1 are located mostly in the cytoplasm and cell membrane of SMCs in the medial layer while the expression of TNF-R2 is located in the cell membrane of both SMCs and endothelial cells **(paper V)**. Further, we observed enhanced expression of TNF-R1 and

TNF-R2 in cerebral microvessels following experimental MCAO and SAH, which is in line with a previous report [257].

Organ culture is well suited for testing the effect of various inhibitors in a controlled fashion. To further analyze intracellular mechanisms involved in the upregulation of TNF-α, TNF-R1 and TNF-R2, we added the inhibitors SB386023-b, U0126 or IMD-0354 (a NF-κB blocker) to the organ culture medium before incubation. We observed that all three inhibitors significantly decreased expression of TNF-α **(Figure 16)** and TNF-R1 expression at 48 hours incubation, while the increased expression of TNF-R2 was significantly prevented only by U0126 treatment **(paper V)**. These observations demonstrate independent regulation of TNF receptors in cerebral arteries. One possible explanation for the difference might be that TNF-R1 contains a death domain in its cytoplasmic region whereas TNF-R2 lacks this [258]. Activation of TNF-R1 may lead to activation of the death domain, which activates the Ras and Raf kinases and thereafter phosphorylated pERK1/2 promotes activation of NF-κB by degradation of IκB. Therefore, blockade of phosphorylation and activation of this pathway can potentially inhibit the expression of TNF-R1, which correlates with suppression and inhibition of TNF-α expression. In addition, previous studies have demonstrated that TNF-R1 mediated cell death uses apoptosis signal-regulating MAPK to activate downstream signaling. In contrast, TNF-R2 mediated cell survival and angiogenesis use endothelial/epithelial tyrosine kinases to promote cell adhesion, migration and proliferation [257, 258]. Our results could therefore suggest that inhibition of the Raf-MEK-ERK1/2 pathway results in decreased expression of TNF-R1 and TNF-α, which result in attenuation of the secondary ischemic damage.

Since changes in vascular TNF-α and its receptors after *in vitro* organ culture show a striking similarity to the changes observed in animal models of ischemic and hemorrhagic stroke, we hypothesize that one major factor behind this is the change in shear stress, which *in vivo* is caused by the rise in intracranial pressure and a reduction in wall tension in SAH or MCAO, and *in vitro* by the removal of the intraluminal pressure during the organ culture procedure. We therefore suggest that organ culture can be used as a convenient *in vitro* method to study the pharmacological characteristics and underlying cellular and molecular mechanisms of cerebrovascular pro-inflammatory alterations.

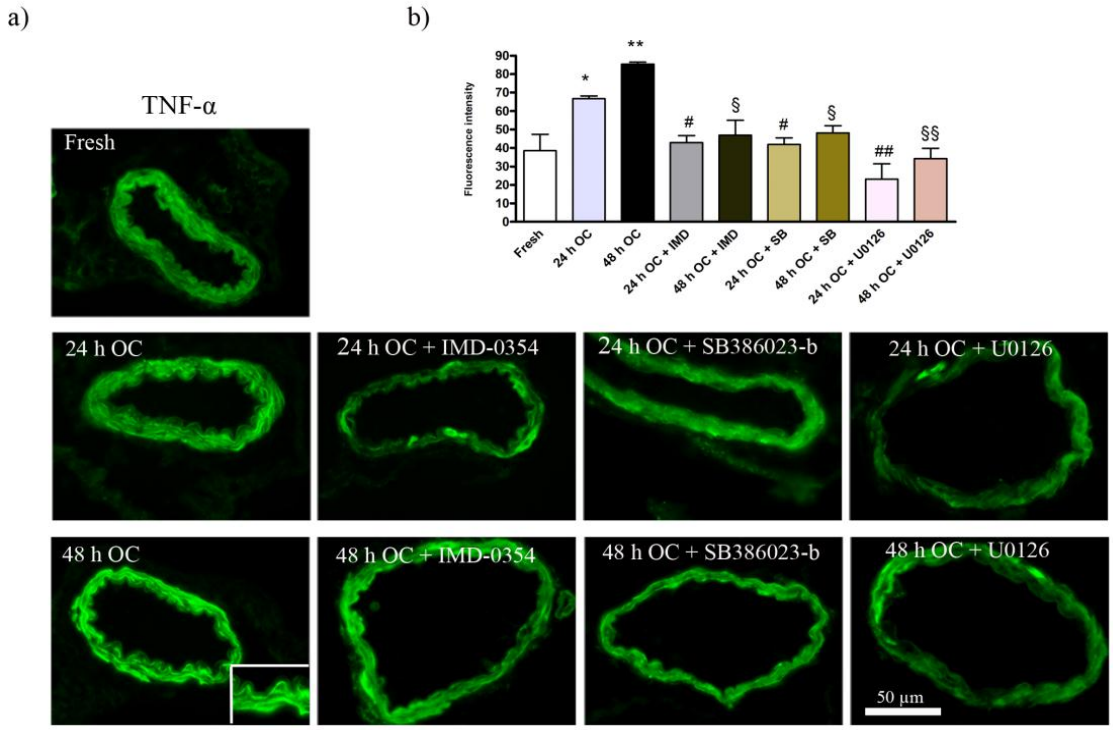

Figure 16. a) Immunofluorescence staining for TNF-α in fresh and organ cultured MCA at 24 and 48 hours after incubation in the presence and absence of IMD-0354, SB386023-b and U012648 inhibitors. There was a clear increase in TNF-α protein level in the SMC layer after 24 and 48 hours culture as compared to fresh vessels. All three inhibitors significantly prevented the enhanced expression of TNF-α in culture for 24 and 48 hours. **b)** Bar graph demonstrating the fluorescence intensity for TNF-α. The results are expressed as mean values ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***} P < 0.001$ significant difference between fresh and culture after 24 and 48 hours. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, significant difference between 48 hours organ culture and treatment with 48 hours culture + (IMD-0354, SB386023-b and U0126). #P < 0.05,##P < 0.01, significant difference between 24 hours organ culture and treatment with 24 hours culture + (IMD-0354, SB386023-b and U0126).

Finally, the results of this thesis indicate that cerebral artery activity participates in the inflammatory process and production of pro-inflammatory mediators following focal and global ischemia as well as organ culture and that the MEK-ERK 1/2 signaling pathway may be responsible for the early transcriptional regulation that occurs. Therefore, we suggest that inhibition of Raf-MEK-ERK1/2 signaling may represent a potential target for the development of novel anti-inflammatory therapy approaches to alleviate the tissue damage associated with cerebral ischemia.

MAJOR CONCLUSIONS

The work described in this thesis highlights the fact that cerebral arteries and brain parenchyma microvessels participate actively in the inflammatory response and production of pro-inflammatory mediators following focal and global ischemia. The process involves activation of the ERK1/2 MAPK pathway at early time-points in the SMC of the cerebral vessels. This in turn activates the transcription of inflammatory genes such as cytokines, iNOS and MMPs as wells as genes for specific vasoconstrictor receptors in the wall of cerebral vessels **(Figure 17)**. Each of these factors by direct or indirect effects on cerebral arteries may lead to more damage and deleterious events which result in a rapid decrease of CBF, further exacerbating secondary damage and worsening of cerebral ischemia.

Because of the synergistic nature of these processes, which involve several gene groups, a single factor approach to the treatment of cerebral ischemia seems unlikely to work completely. Since not only vasoconstrictor receptors are involved but also production of genes involved in inflammation, apoptosis and blood brain barrier modulation a multifactorial approach is needed.

Therefore, blockade of MAPK-ERK1/2 pathway upstream with either a MEK1/2 inhibitor or a Raf inhibitor given at the onset of a cerebral ischemia insult or as late as 6 hours afterwards may result in reduction of the enhanced expression of vascular pro-inflammatory cytokines, MMPs and iNOS, decreased infarct volume and improved CBF and neurological function. Further, inhibition of the Raf/MEK/ERK pathway indirectly targets other transcriptional mechanisms activated following a stroke and prevents many of the deleterious events that are induced. Thus, we suggest that modulation of Raf-MEK-ERK1/2 signaling may therefore be used to improve the outcome after stroke and may represent a potential target for the development of novel ant-inflammatory therapy.

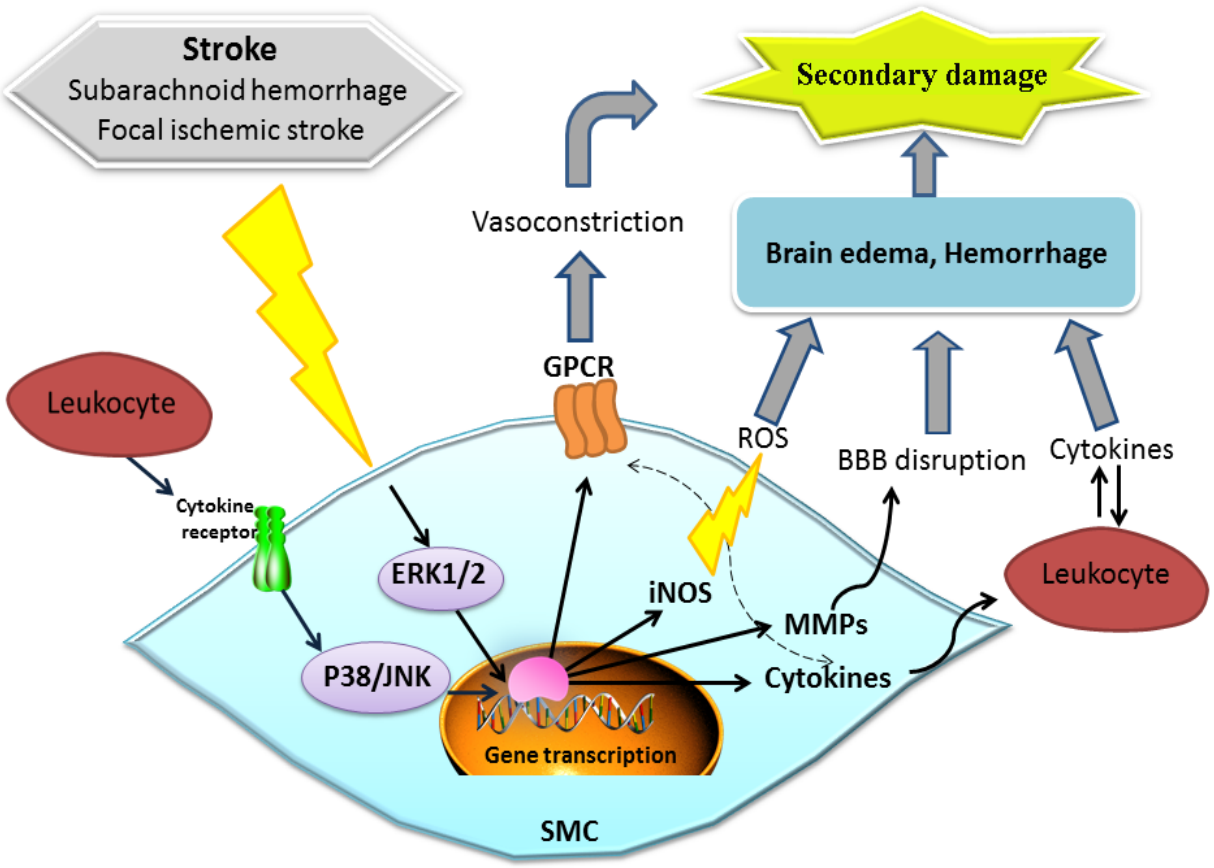

Figure 17. Schematic illustration of the main conclusions in this thesis. Generation of oxidative stress after stroke activates the ERK1/2 pathway in the early phase, which in turn activates the transcription of inflammatory cytokines, MMPs, iNOS and vasoconstrictor receptor genes in the SMCs of cerebral arteries. Each of this factor leads to more damage and deleterious events which result in secondary damage and worsening of cerebral ischemia. By blocking this pathway cerebral infarct volume after ischemic stroke and delayed cerebral ischemia after SAH can be considerably reduced.

SWEDISH SUMMARY

Bakgrund

Stroke är en av våra största folksjukdomar och den tredje vanligaste dödsorsaken efter hjärtinfarkt och cancer i Sverige. Det finns två typer av stroke, de som beror på en blödning och de som beror på en propp. Både dessa typer orsaker en minskning av blodflödet i hjärnan. Eftersom hjärnan är extremt beroende av kontinuerlig tillförsel av syre och näringämnen leder tillståndet snabbt till hjärnskada. Detta gör att det viktigaste efter en stroke är ett återfå ett så normalt blodflödet som möjligt. Trots den intensiva forskning som finns idag för att återfå flödet och begränsa skador finns få effektiva metoder för behandling, och de har inte alltid den positiva effekt som man önskar.

Det har visat sig att inflammation utvecklas inom några timmar efter en stroke, och spelar en viktig roll i patologin efter en stroke. Vi har fokuserat våra studier kring det som sker i den cerebrala cirkulationen. Det sker ett förändrat receptoruttryck i blodkärlens glatta muskelceller samt en uppreglering av inflammatoriska faktorer i cerebrala blodkärl vid en stroke. Graden av uppreglering tycks korrelera med den slutliga skadas omfattning.

Syftet med avhandlingen

Målet med denna avhandlingen är att undersöka förändringar av inflammatoriska faktorer i hjärnans blodkärl vid global och fokal ischemi samt utforska de molekylära mekanismer som är involverade. Ökad kunskap inom detta området och för bakomliggande mekanismer till förändringar i hjärnans blodkärl leder förhoppningsvis till nya terapier för behandling av cerebral ischemi i kliniken.

Metoder

För att undersökas uppreglering av inflammatoriska faktorer i hjärnans blodkärl samt bakomliggande mechanismer har vi använt två stroke modeller hos råtta och en kärl odlings modell på isolerade blodkärl från hjärnan. Vi har mätt infarktvolym, neurologiska funktioner och blodflödet i hjärnan. Kärlen har undersökts med molekylära metoder för att påvisa förändringar.

Resultat

I delarbete I och II undersöktes uppreglering av inflammatoriska faktorer (MMP-9 och TIMP-1, TNF-α, IL-6, IL-1β och iNOS proteiner) i hjärnans blodkärl från råtta efter fokal ischemi (MCAO) 48 timmar efter skadan. Vi observerade att uppreglering av alla ovanstående proteiner i hjärnans blodkärl var associerad med infarktvolym, försämrad neurologisk funktion och ödembildning. I kärlväggen var detta associerat med att MAPK MEK-ERK1/2 signalvägen var aktiverad. Vi visade att normaliserat cerebralt blodflöde och minskat uttryck av inflammatoriska faktorer i hjärnans blodkärl kunde vara associerat med reducerad infarktvolymen och förbättrad neurologiska funktion. I det tredje delarbetet har vi undersökt om det finns samma uppreglering av inflammatoriska faktorer och om ERK1/2 signalvägen är aktiverad i blodkärl från råtta 48 timmar efter global ischemi (SAH). Liksom vid MCAO fann vi att uppreglering av inflammatoriska faktorer i hjärnans blodkärl var associerad med minskning av cerebralt blodflöde. Dessutom visade vi att behandling med en specifik Raf-MEK-ERK1/2 hämmare normaliserade cerebralt blodflöde samt minskade uttrycket av inflammatoriska faktorer i hjärnans blodkärl.

För att undersöka hur dessa förändringar äger rum, beslutade vi att genomföra en studie kring ifall uppreglering av inflammatoriska förändringar i kärl väggen efter SAH är associerad med MEK-ERK1/2 vägen. I delarbete IV fann vi att SAH inducerar aktivering av ERK/MAPK vägen också vid de tidiga tidpunkterna i hjärnans kärl, vilket leder till utveckling av cerebral kärlsammandragning och därefter i fördröjd cerebral ischemi vilket är associerat med uppreglering av inflammatoriska faktorer. Behandling med transkriptionshämmare av denna signal väg, så sent som 6 timmar efter skadan, minskade

ökningen av inflammatoriska faktorer, normaliserade CBF och den neurologiska funktionen. Dessa resultat kan ha stor betydelse i kliniska sammanhang eftersom denna signalväg är effektiv i en kliniskt relevant tidsram. Den var effektiv inte bara när det gavs vid initieringen av stroke, men också 6 timmar efter att skadan inträffat.

I det sista delarbetet visade vi att det finns likheter i uppreglering av TNF-α, TNF receptorer och ERK1/2 signalvägen i cerebrala artärer efter fokal/global ischemi och organkultur. Detta tyder på att organkultur kan användas som en *in vitro* metod för att studera de farmakologiska egenskaperna, och underliggande cellulära och molekylära mekanismerna för cerebrovaskulära inflammatoriska förändringar.

Slutsats

Vi har funnit att såväl organ kultur av isolerade segment av hjärnkärl som vid två experimentella cerebrala ischemi modeller (MCAO och SAH) sker en förändring och uppreglering av inflammatoriska mediatorer i hjärnas blodkärl. Mitogen aktiverade protein kinas (MAPK) signal vägen är aktiverad i hjärnas blodkärl. Behandling av försöksdjuret med en MAPK hämmare förbättrade de neurologiska symptomen, normaliserade uttryck av kontraktila receptorer och inflammatoriska faktorer samt minskade infarkt volymen.

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REFERENCES

1. Donnan GA, Fisher M, Macleod M, Davis SM: **Stroke.** *Lancet* 2008, **371:**1612-1623.
2. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, et al: **Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association.** *Circulation* 2010, **121:**948-954.
3. Doyle KP, Simon RP, Stenzel-Poore MP: **Mechanisms of ischemic brain damage.** *Neuropharmacology* 2008, **55:**310-318.
4. Dirnagl U, Iadecola C, Moskowitz MA: **Pathobiology of ischaemic stroke: an integrated view.** *Trends Neurosci* 1999, **22:**391-397.
5. Kleinig TJ, Vink R: **Suppression of inflammation in ischemic and hemorrhagic stroke: therapeutic options.** *Curr Opin Neurol* 2009, **22:**294-301.
6. Jin R, Yang G, Li G: **Inflammatory mechanisms in ischemic stroke: role of inflammatory cells.** *J Leukoc Biol* 2010, **87:**779-789.
7. Moskowitz MA, Lo EH, Iadecola C: **The science of stroke: mechanisms in search of treatments.** *Neuron* 2010, **67:**181-198.
8. Vikman P, Beg S, Khurana T, Hansen-Schwartz J, Edvinsson L: **Gene expression and molecular changes in cerebral arteries following subarachnoid hemorrhage in the rat.** *J Neurosurg* 2006, **105:**438-444.
9. Vikman P, Ansar S, Henriksson M, Stenman E, Edvinsson L: **Cerebral ischemia induces transcription of inflammatory and extracellular-matrix-related genes in rat cerebral arteries.** *Exp Brain Res* 2007, **183:**499-510.
10. Stapf C, Mohr JP: **Ischemic stroke therapy.** *Annu Rev Med* 2002, **53:**453-475.
11. Feigin VL: **Stroke epidemiology in the developing world.** *Lancet* 2005, **365:**2160-2161.
12. Hossmann KA: **The two pathophysiologies of focal brain ischemia: implications for translational stroke research.** *J Cereb Blood Flow Metab* 2012.
13. Lo EH: **A new penumbra: transitioning from injury into repair after stroke.** *Nat Med* 2008, **14:**497-500.
14. Siesjo BK: **Pathophysiology and treatment of focal cerebral ischemia. Part II: Mechanisms of damage and treatment.** *J Neurosurg* 1992, **77:**337-354.
15. Hossmann KA: **Viability thresholds and the penumbra of focal ischemia.** *Ann Neurol* 1994, **36:**557-565.
16. Mehta SL, Manhas N, Raghubir R: **Molecular targets in cerebral ischemia for developing novel therapeutics.** *Brain Res Rev* 2007, **54:**34-66.
17. Edvinsson L: **Cerebrovascular endothelin receptor upregulation in cerebral ischemia.** *Curr Vasc Pharmacol* 2009, **7:**26-33.

18. Rothwell NJ, Hopkins SJ: **Cytokines and the nervous system II: Actions and mechanisms of action.** *Trends Neurosci* 1995, **18:**130-136.

19. Rothwell NJ, Strijbos PJ: **Cytokines in neurodegeneration and repair.** *Int J Dev Neurosci* 1995, **13:**179-185.

20. Fagan SC, Hess DC, Hohnadel EJ, Pollock DM, Ergul A: **Targets for vascular protection after acute ischemic stroke.** *Stroke* 2004, **35:**2220-2225.

21. Vikman P, Edvinsson L: **Gene expression profiling in the human middle cerebral artery after cerebral ischemia.** *Eur J Neurol* 2006, **13:**1324-1332.

22. Lyden P: **Thrombolytic therapy for acute stroke--not a moment to lose.** *N Engl J Med* 2008, **359:**1393-1395.

23. Khurana D, Bal S: **Neuroprotection in stroke.** *J Indian Med Assoc* 2009, **107:**378-382, 391.

24. Stemer A, Lyden P: **Evolution of the thrombolytic treatment window for acute ischemic stroke.** *Curr Neurol Neurosci Rep* 2010, **10:**29-33.

25. Shobha N, Buchan AM, Hill MD: **Thrombolysis at 3-4.5 hours after acute ischemic stroke onset--evidence from the Canadian Alteplase for Stroke Effectiveness Study (CASES) registry.** *Cerebrovasc Dis* 2011, **31:**223-228.

26. Wang X, Lee SR, Arai K, Tsuji K, Rebeck GW, Lo EH: **Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator.** *Nat Med* 2003, **9:**1313-1317.

27. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW: **1,026 experimental treatments in acute stroke.** *Ann Neurol* 2006, **59:**467-477.

28. Toni D, Chamorro A, Kaste M, Lees K, Wahlgren NG, Hacke W: **Acute treatment of ischaemic stroke. European Stroke Initiative.** *Cerebrovasc Dis* 2004, **17 Suppl 2:**30-46.

29. Dubow J, Fink ME: **Impact of hypertension on stroke.** *Curr Atheroscler Rep* 2011, **13:**298-305.

30. Dorhout Mees SM, Rinkel GJ, Feigin VL, Algra A, van den Bergh WM, Vermeulen M, van Gijn J: **Calcium Antagonists for Aneurysmal Subarachnoid Hemorrhage.** *Stroke* 2008.

31. Edvinsson L, N.Krause D: ***Cerebral blood flow and metabolism***. Philadelphia: Lippincot williams and Wilkins; 2002.

32. **Epidemiology of aneurysmal subarachnoid hemorrhage in Australia and New Zealand: incidence and case fatality from the Australasian Cooperative Research on Subarachnoid Hemorrhage Study (ACROSS).** *Stroke* 2000, **31:**1843-1850.

33. Rose MJ: **Aneurysmal subarachnoid hemorrhage: an update on the medical complications and treatments strategies seen in these patients.** *Curr Opin Anaesthesiol* 2011, **24:**500-507.

34. Jackowski A, Crockard A, Burnstock G, Russell RR, Kristek F: **The time course of intracranial pathophysiological changes following experimental subarachnoid haemorrhage in the rat.** *J Cereb Blood Flow Metab* 1990, **10:**835-849.

35. Delgado TJ, Brismar J, Svendgaard NA: **Subarachnoid haemorrhage in the rat: angiography and fluorescence microscopy of the major cerebral arteries.** *Stroke* 1985, **16:**595-602.

36. Keyrouz SG, Diringer MN: **Clinical review: Prevention and therapy of vasospasm in subarachnoid hemorrhage.** *Crit Care* 2007, **11:**220.

37. Zemke D, Farooq MU, Mohammed Yahia A, Majid A: **Delayed ischemia after subarachnoid hemorrhage: result of vasospasm alone or a broader vasculopathy?** *Vasc Med* 2007, **12:**243-249.

38. Harrod CG, Bendok BR, Batjer HH: **Prediction of cerebral vasospasm in patients presenting with aneurysmal subarachnoid hemorrhage: a review.** *Neurosurgery* 2005, **56:**633-654; discussion 633-654.

39. Kassell NF, Sasaki T, Colohan AR, Nazar G: **Cerebral vasospasm following aneurysmal subarachnoid hemorrhage.** *Stroke* 1985, **16:**562-572.

40. MacDonald RL: **Evidence-based treatment of subarachnoid hemorrhage: current status and future possibilities.** *Clin Neurosurg* 2006, **53:**257-266.

41. Chen G, Zhang S, Shi J, Ai J, Hang C: **Effects of recombinant human erythropoietin (rhEPO) on JAK2/STAT3 pathway and endothelial apoptosis in the rabbit basilar artery after subarachnoid hemorrhage.** *Cytokine* 2009, **45:**162-168.

42. Friedrich V, Flores R, Sehba FA: **Cell death starts early after subarachnoid hemorrhage.** *Neurosci Lett* 2012, **512:**6-11.

43. Findlay JM, Weir BK, Kanamaru K, Espinosa F: **Arterial wall changes in cerebral vasospasm.** *Neurosurgery* 1989, **25:**736-745; discussion 745-736.

44. Mayberg MR, Okada T, Bark DH: **The significance of morphological changes in cerebral arteries after subarachnoid hemorrhage.** *J Neurosurg* 1990, **72:**626-633.

45. Asano T: **Oxyhemoglobin as the principal cause of cerebral vasospasm: a holistic view of its actions.** *Critical Reviews in Neurosurgery* 1999, **9:**303-318.

46. Macdonald RL, Marton LS, Andrus PK, Hall ED, Johns L, Sajdak M: **Time course of production of hydroxyl free radical after subarachnoid hemorrhage in dogs.** *Life Sci* 2004, **75:**979-989.

47. Nozaki K, Okamoto S, Yanamoto H, Kikuchi H: **Red blood cells are essential for late vasospasm following experimentally induced subarachnoid hemorrhage in dogs.** *Neurol Med Chir (Tokyo)* 1990, **30:**10-15.

48. Zimmermann M, Seifert V: **Endothelin and subarachnoid hemorrhage: an overview.** *Neurosurgery* 1998, **43:**863-875; discussion 875-866.

49. Ansar S, Maddahi A, Edvinsson L: **Inhibition of cerebrovascular raf activation attenuates cerebral blood flow and prevents upregulation of contractile receptors after subarachnoid hemorrhage.** *BMC Neurosci* 2011, **12:**107.

50. Dumont AS, Dumont RJ, Chow MM, Lin CL, Calisaneller T, Ley KF, Kassell NF, Lee KS: **Cerebral vasospasm after subarachnoid hemorrhage: putative role of inflammation.** *Neurosurgery* 2003, **53:**123-133; discussion 133-125.

51. Prunell GF, Svendgaard NA, Alkass K, Mathiesen T: **Inflammation in the brain after experimental subarachnoid hemorrhage.** *Neurosurgery* 2005, **56:**1082-1092; discussion 1082-1092.

52. Sercombe R, Dinh YR, Gomis P: **Cerebrovascular inflammation following subarachnoid hemorrhage.** *Jpn J Pharmacol* 2002, **88:**227-249.

53. Pluta RM, Hansen-Schwartz J, Dreier J, Vajkoczy P, Macdonald RL, Nishizawa S, Kasuya H, Wellman G, Keller E, Zauner A, et al: **Cerebral vasospasm following subarachnoid hemorrhage: time for a new world of thought.** *Neurol Res* 2009, **31:**151-158.

54. Tomasello F, Albanese V, Picozzi P, Spadaro A, Conforti P: **Relation of cerebral vasospasm to operative findings of subarachnoid blood around ruptured aneurysms.** *Acta Neurochir (Wien)* 1982, **60:**55-62.

55. Loch Macdonald R: **Management of cerebral vasospasm.** *Neurosurg Rev* 2006, **29:**179-193.

56. Macdonald RL, Weir BK: **A review of hemoglobin and the pathogenesis of cerebral vasospasm.** *Stroke* 1991, **22:**971-982.

57. McFaul SJ, Bowman PD, Villa VM: **Hemoglobin stimulates the release of proinflammatory cytokines from leukocytes in whole blood.** *J Lab Clin Med* 2000, **135:**263-269.

58. Shishido T, Suzuki R, Qian L, Hirakawa K: **The role of superoxide anions in the pathogenesis of cerebral vasospasm.** *Stroke* 1994, **25:**864-868.

59. Okada H, Endo S, Kamiyama K, Suzuki J: **[Oxyhemoglobin-induced cerebral vasospasm and sequential changes of vascular ultrastructure (author's transl)].** *Neurol Med Chir (Tokyo)* 1980, **20:**573-582.

60. Martin W, Villani GM, Jothianandan D, Furchgott RF: **Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta.** *J Pharmacol Exp Ther* 1985, **232:**708-716.

61. Schulz E, Jansen T, Wenzel P, Daiber A, Munzel T: **Nitric oxide, tetrahydrobiopterin, oxidative stress, and endothelial dysfunction in hypertension.** *Antioxid Redox Signal* 2008, **10:**1115-1126.

62. Misra HP, Fridovich I: **The generation of superoxide radical during the autoxidation of hemoglobin.** *J Biol Chem* 1972, **247:**6960-6962.

63. Monteiro HP, Abdalla DS, Faljoni-Alario A, Bechara EJ: **Generation of active oxygen species during coupled autoxidation of oxyhemoglobin and delta-aminolevulinic acid.** *Biochim Biophys Acta* 1986, **881:**100-106.

64. Edvinsson LI, Povlsen GK: **Vascular plasticity in cerebrovascular disorders.** *J Cereb Blood Flow Metab* 2011, **31:**1554-1571.

65. Masaki T, Vane JR, Vanhoutte PM: **International Union of Pharmacology nomenclature of endothelin receptors.** *Pharmacol Rev* 1994, **46:**137-142.

66. Alexander SP, Mathie A, Peters JA: **Guide to Receptors and Channels (GRAC), 2nd edition (2007 Revision).** *Br J Pharmacol* 2007, **150 Suppl 1:**S1-168.

67. Touyz RM, Berry C: **Recent advances in angiotensin II signaling.** *Braz J Med Biol Res* 2002, **35:**1001-1015.

68. Maddahi A, Edvinsson L: **Enhanced expressions of microvascular smooth muscle receptors after focal cerebral ischemia occur via the MAPK MEK/ERK pathway.** *BMC Neurosci* 2008, **9:**85.

69. Ansar S, Vikman P, Nielsen M, Edvinsson L: **Cerebrovascular ETB, 5-HT1B and AT1 receptor upregulation correlates with reduction in regional CBF after subarachnoid hemorrhage.** *Am J Physiol Heart Circ Physiol* 2008, **294:**H3750-H3758.

70. Ahnstedt H, Saveland H, Nilsson O, Edvinsson L: **Human cerebrovascular contractile receptors are upregulated via a B-Raf/MEK/ERK-sensitive signaling pathway.** *BMC Neurosci* 2011, **12:**5.

71. Ansar S, Edvinsson L: **Subtype activation and interaction of protein kinase C and mitogen-activated protein kinase controlling receptor expression in cerebral arteries and microvessels after subarachnoid hemorrhage.** *Stroke* 2008, **39:**185-190.

72. Henriksson M, Stenman E, Vikman P, Edvinsson L: **MEK1/2 inhibition attenuates vascular ETA and ETB receptor alterations after cerebral ischaemia.** *Exp Brain Res* 2007, **178:**470-476.

73. Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE: **Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation.** *Clin Exp Immunol* 2007, **147:**227-235.

74. Whiteley W, Jackson C, Lewis S, Lowe G, Rumley A, Sandercock P, Wardlaw J, Dennis M, Sudlow C: **Inflammatory markers and poor outcome after stroke: a prospective cohort study and systematic review of interleukin-6.** *PLoS Med* 2009, **6:**e1000145.

75. Chamorro A, Hallenbeck J: **The harms and benefits of inflammatory and immune responses in vascular disease.** *Stroke* 2006, **37:**291-293.

76. Wang Q, Tang XN, Yenari MA: **The inflammatory response in stroke.** *J Neuroimmunol* 2007, **184:**53-68.

77. Palasik W, Fiszer U, Lechowicz W, Czartoryska B, Krzesiewicz M, Lugowska A: **Assessment of relations between clinical outcome of ischemic stroke and activity of inflammatory processes in the acute phase based on examination of selected parameters.** *Eur Neurol* 2005, **53:**188-193.

78. Iadecola C, Anrather J: **The immunology of stroke: from mechanisms to translation.** *Nat Med* 2011, **17:**796-808.

79. Amantea D, Nappi G, Bernardi G, Bagetta G, Corasaniti MT: **Post-ischemic brain damage: pathophysiology and role of inflammatory mediators.** *FEBS J* 2009, **276:**13-26.

80. Barone FC, Feuerstein GZ: **Inflammatory mediators and stroke: new opportunities for novel therapeutics.** *J Cereb Blood Flow Metab* 1999, **19:**819-834.

81. del Zoppo G, Ginis I, Hallenbeck JM, Iadecola C, Wang X, Feuerstein GZ: **Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia.** *Brain Pathol* 2000, **10:**95-112.

82. Kriz J: **Inflammation in ischemic brain injury: timing is important.** *Crit Rev Neurobiol* 2006, **18:**145-157.

83. Rosenberg GA: **Ischemic brain edema.** *Prog Cardiovasc Dis* 1999, **42:**209-216.

84. Nilupul Perera M, Ma HK, Arakawa S, Howells DW, Markus R, Rowe CC, Donnan GA: **Inflammation following stroke.** *J Clin Neurosci* 2006, **13:**1-8.

85. Lindsberg PJ, Strbian D, Karjalainen-Lindsberg ML: **Mast cells as early responders in the regulation of acute blood-brain barrier changes after cerebral ischemia and hemorrhage.** *J Cereb Blood Flow Metab* 2010, **30:**689-702.

86. Dimitrijevic OB, Stamatovic SM, Keep RF, Andjelkovic AV: **Effects of the chemokine CCL2 on blood-brain barrier permeability during ischemia-reperfusion injury.** *J Cereb Blood Flow Metab* 2006, **26:**797-810.

87. Ceulemans AG, Zgavc T, Kooijman R, Hachimi-Idrissi S, Sarre S, Michotte Y: **The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia.** *J Neuroinflammation* 2010, **7:**74.

88. Yong VW: **Metalloproteinases: mediators of pathology and regeneration in the CNS.** *Nat Rev Neurosci* 2005, **6:**931-944.

89. Dityatev A, Schachner M: **Extracellular matrix molecules and synaptic plasticity.** *Nat Rev Neurosci* 2003, **4:**456-468.

90. Danton GH, Dietrich WD: **Inflammatory mechanisms after ischemia and stroke.** *J Neuropathol Exp Neurol* 2003, **62:**127-136.

91. Han HS, Yenari MA: **Cellular targets of brain inflammation in stroke.** *Curr Opin Investig Drugs* 2003, **4:**522-529.

92. Becker K, Kindrick D, Relton J, Harlan J, Winn R: **Antibody to the alpha4 integrin decreases infarct size in transient focal cerebral ischemia in rats.** *Stroke* 2001, **32:**206-211.

93. Sughrue ME, Mehra A, Connolly ES, Jr., D'Ambrosio AL: **Anti-adhesion molecule strategies as potential neuroprotective agents in cerebral ischemia: a critical review of the literature.** *Inflamm Res* 2004, **53:**497-508.

94. Zhao B, Schwartz JP: **Involvement of cytokines in normal CNS development and neurological diseases: recent progress and perspectives.** *J Neurosci Res* 1998, **52:**7-16.

95. Feuerstein GZ, Liu T, Barone FC: **Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha.** *Cerebrovasc Brain Metab Rev* 1994, **6:**341-360.

96. Huang J, Upadhyay UM, Tamargo RJ: **Inflammation in stroke and focal cerebral ischemia.** *Surg Neurol* 2006, **66:**232-245.

97. Rothwell NJ: **Annual review prize lecture cytokines - killers in the brain?** *J Physiol* 1999, **514 (Pt 1):**3-17.

98. Fassbender K, Hodapp B, Rossol S, Bertsch T, Schmeck J, Schutt S, Fritzinger M, Horn P, Vajkoczy P, Kreisel S, et al: **Inflammatory cytokines in subarachnoid haemorrhage: association with abnormal blood flow velocities in basal cerebral arteries.** *J Neurol Neurosurg Psychiatry* 2001, **70:**534-537.

99. Kikuchi T, Okuda Y, Kaito N, Abe T: **Cytokine production in cerebrospinal fluid after subarachnoid haemorrhage.** *Neurol Res* 1995, **17:**106-108.

100. Jean WC, Spellman SR, Nussbaum ES, Low WC: **Reperfusion injury after focal cerebral ischemia: the role of inflammation and the therapeutic horizon.** *Neurosurgery* 1998, **43:**1382-1396; discussion 1396-1387.

101. Pannu H, Kim DH, Guo D, King TM, Van Ginhoven G, Chin T, Chang K, Qi Y, Shete S, Milewicz DM: **The role of MMP-2 and MMP-9 polymorphisms in sporadic intracranial aneurysms.** *J Neurosurg* 2006, **105:**418-423.

102. Feuerstein GZ, Wang X, Barone FC: **The role of cytokines in the neuropathology of stroke and neurotrauma.** *Neuroimmunomodulation* 1998, **5:**143-159.

103. Zaremba J, Losy J: **[Cytokines in clinical and experimental ischemic stroke].** *Neurol Neurochir Pol* 2004, **38:**S57-62.

104. Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN, Lysko PG, Feuerstein GZ: **Tumor necrosis factor-alpha. A mediator of focal ischemic brain injury.** *Stroke* 1997, **28:**1233-1244.

105. Liu T, Clark RK, McDonnell PC, Young PR, White RF, Barone FC, Feuerstein GZ: **Tumor necrosis factor-alpha expression in ischemic neurons.** *Stroke* 1994, **25:**1481-1488.

106. Mantovani A, Bussolino F, Dejana E: **Cytokine regulation of endothelial cell function.** *FASEB J* 1992, **6:**2591-2599.

107. Goukassian DA, Qin G, Dolan C, Murayama T, Silver M, Curry C, Eaton E, Luedemann C, Ma H, Asahara T, et al: **Tumor necrosis factor-alpha receptor p75 is required in ischemia-induced neovascularization.** *Circulation* 2007, **115:**752-762.

108. Limb GA, Chignell AH, Green W, LeRoy F, Dumonde DC: **Distribution of TNF alpha and its reactive vascular adhesion molecules in fibrovascular membranes of proliferative diabetic retinopathy.** *Br J Ophthalmol* 1996, **80:**168-173.

109. Wajant H, Pfizenmaier K, Scheurich P: **Tumor necrosis factor signaling.** *Cell Death Differ* 2003, **10:**45-65.

110. Barnes PJ, Karin M: **Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases.** *N Engl J Med* 1997, **336:**1066-1071.

111. Ge C, Zhang C, Ye J, Tang X, Wu Y: **Ginsenosides promote proliferation of chicken primordial germ cells via PKC-involved activation of NF-kappaB.** *Cell Biol Int* 2007, **31:**1251-1256.

112. Sriram K, O'Callaghan JP: **Divergent roles for tumor necrosis factor-alpha in the brain.** *J Neuroimmune Pharmacol* 2007, **2:**140-153.

113. Zou JY, Crews FT: **TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition.** *Brain Res* 2005, **1034:**11-24.

114. Chao CC, Ala TA, Hu S, Crossley KB, Sherman RE, Peterson PK, Frey WH, 2nd: **Serum cytokine levels in patients with Alzheimer's disease.** *Clin Diagn Lab Immunol* 1994, **1:**433-436.

115. Mogi M, Harada M, Riederer P, Narabayashi H, Fujita K, Nagatsu T: **Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients.** *Neurosci Lett* 1994, **165:**208-210.

116. Sharief MK, Noori MA, Ciardi M, Cirelli A, Thompson EJ: **Increased levels of circulating ICAM-1 in serum and cerebrospinal fluid of patients with active multiple sclerosis. Correlation with TNF-alpha and blood-brain barrier damage.** *J Neuroimmunol* 1993, **43:**15-21.

117. Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holtsberg FW, Mattson MP: **Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors.** *Nat Med* 1996, **2:**788-794.

118. Akira S: **IL-6-regulated transcription factors.** *Int J Biochem Cell Biol* 1997, **29:**1401-1418.

119. Kishimoto T: **The biology of interleukin-6.** *Blood* 1989, **74:**1-10.

120. Kim JS: **Cytokines and adhesion molecules in stroke and related diseases.** *J Neurol Sci* 1996, **137:**69-78.

121. Hagberg H, Gilland E, Bona E, Hanson LA, Hahin-Zoric M, Blennow M, Holst M, McRae A, Soder O: **Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats.** *Pediatr Res* 1996, **40:**603-609.

122. Osuka K, Suzuki Y, Tanazawa T, Hattori K, Yamamoto N, Takayasu M, Shibuya M, Yoshida J: **Interleukin-6 and development of vasospasm after subarachnoid haemorrhage.** *Acta Neurochir (Wien)* 1998, **140:**943-951.

123. Suzuki S, Tanaka K, Nogawa S, Nagata E, Ito D, Dembo T, Fukuuchi Y: **Temporal profile and cellular localization of interleukin-6 protein after focal cerebral ischemia in rats.** *J Cereb Blood Flow Metab* 1999, **19:**1256-1262.

124. Orion D, Schwammenthal Y, Reshef T, Schwartz R, Tsabari R, Merzeliak O, Chapman J, Mekori YA, Tanne D: **Interleukin-6 and soluble intercellular adhesion molecule-1 in acute brain ischaemia.** *Eur J Neurol* 2008, **15:**323-328.

125. Clark WM, Rinker LG, Lessov NS, Hazel K, Hill JK, Stenzel-Poore M, Eckenstein F: **Lack of interleukin-6 expression is not protective against focal central nervous system ischemia.** *Stroke* 2000, **31:**1715-1720.

126. Wang X, Barone FC, Aiyar NV, Feuerstein GZ: **Interleukin-1 receptor and receptor antagonist gene expression after focal stroke in rats.** *Stroke* 1997, **28:**155-161; discussion 161-152.

127. Touzani O, Boutin H, Chuquet J, Rothwell N: **Potential mechanisms of interleukin-1 involvement in cerebral ischaemia.** *J Neuroimmunol* 1999, **100:**203-215.

128. Dower SK, Kronheim SR, Hopp TP, Cantrell M, Deeley M, Gillis S, Henney CS, Urdal DL: **The cell surface receptors for interleukin-1 alpha and interleukin-1 beta are identical.** *Nature* 1986, **324:**266-268.

129. Allan SM, Tyrrell PJ, Rothwell NJ: **Interleukin-1 and neuronal injury.** *Nat Rev Immunol* 2005, **5:**629-640.

130. Korherr C, Hofmeister R, Wesche H, Falk W: **A critical role for interleukin-1 receptor accessory protein in interleukin-1 signaling.** *Eur J Immunol* 1997, **27:**262-267.

131. Caso JR, Moro MA, Lorenzo P, Lizasoain I, Leza JC: **Involvement of IL-1beta in acute stress-induced worsening of cerebral ischaemia in rats.** *Eur Neuropsychopharmacol* 2007, **17:**600-607.

132. Moriwaki T, Takagi Y, Sadamasa N, Aoki T, Nozaki K, Hashimoto N: **Impaired progression of cerebral aneurysms in interleukin-1beta-deficient mice.** *Stroke* 2006, **37:**900-905.

133. Saito K, Suyama K, Nishida K, Sei Y, Basile AS: **Early increases in TNF-alpha, IL-6 and IL-1 beta levels following transient cerebral ischemia in gerbil brain.** *Neurosci Lett* 1996, **206:**149-152.

134. Wang X, Yue TL, Barone FC, White RF, Gagnon RC, Feuerstein GZ: **Concomitant cortical expression of TNF-alpha and IL-1 beta mRNAs follows early response gene expression in transient focal ischemia.** *Mol Chem Neuropathol* 1994, **23:**103-114.

135. Sternlicht MD, Werb Z: **How matrix metalloproteinases regulate cell behavior.** *Annu Rev Cell Dev Biol* 2001, **17:**463-516.

136. Rosenberg GA: **Matrix metalloproteinases in neuroinflammation.** *Glia* 2002, **39:**279-291.

137. Visse R, Nagase H: **Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry.** *Circ Res* 2003, **92:**827-839.

138. Romanic AM, White RF, Arleth AJ, Ohlstein EH, Barone FC: **Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size.** *Stroke* 1998, **29:**1020-1030.

139. Pfefferkorn T, Rosenberg GA: **Closure of the blood-brain barrier by matrix metalloproteinase inhibition reduces rtPA-mediated mortality in cerebral ischemia with delayed reperfusion.** *Stroke* 2003, **34:**2025-2030.

140. Gasche Y, Fujimura M, Morita-Fujimura Y, Copin JC, Kawase M, Massengale J, Chan PH: **Early appearance of activated matrix metalloproteinase-9 after focal cerebral ischemia in mice: a possible role in blood-brain barrier dysfunction.** *J Cereb Blood Flow Metab* 1999, **19:**1020-1028.

141. Planas AM, Sole S, Justicia C: **Expression and activation of matrix metalloproteinase-2 and -9 in rat brain after transient focal cerebral ischemia.** *Neurobiol Dis* 2001, **8:**834-846.

142. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA: **Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat.** *J Cereb Blood Flow Metab* 2007, **27:**697-709.

143. Asahi M, Wang X, Mori T, Sumii T, Jung JC, Moskowitz MA, Fini ME, Lo EH: **Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia.** *J Neurosci* 2001, **21:**7724-7732.

144. Rosenberg GA, Navratil M, Barone F, Feuerstein G: **Proteolytic cascade enzymes increase in focal cerebral ischemia in rat.** *J Cereb Blood Flow Metab* 1996, **16:**360-366.

145. Zhao BQ, Wang S, Kim HY, Storrie H, Rosen BR, Mooney DJ, Wang X, Lo EH: **Role of matrix metalloproteinases in delayed cortical responses after stroke.** *Nat Med* 2006, **12:**441-445.

146. Rosenberg GA, Estrada EY, Dencoff JE: **Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain.** *Stroke* 1998, **29:**2189-2195.

147. Fujimura M, Gasche Y, Morita-Fujimura Y, Massengale J, Kawase M, Chan PH: **Early appearance of activated matrix metalloproteinase-9 and blood-brain barrier disruption in mice after focal cerebral ischemia and reperfusion.** *Brain Res* 1999, **842:**92-100.

148. Rosell A, Ortega-Aznar A, Alvarez-Sabin J, Fernandez-Cadenas I, Ribo M, Molina CA, Lo EH, Montaner J: **Increased brain expression of matrix metalloproteinase-9 after ischemic and hemorrhagic human stroke.** *Stroke* 2006, **37:**1399-1406.

149. Fukuda S, Fini CA, Mabuchi T, Koziol JA, Eggleston LL, Jr., del Zoppo GJ: **Focal cerebral ischemia induces active proteases that degrade microvascular matrix.** *Stroke* 2004, **35:**998-1004.

150. Asahi M, Asahi K, Jung JC, del Zoppo GJ, Fini ME, Lo EH: **Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94.** *J Cereb Blood Flow Metab* 2000, **20:**1681-1689.

151. Park KP, Rosell A, Foerch C, Xing C, Kim WJ, Lee S, Opdenakker G, Furie KL, Lo EH: **Plasma and brain matrix metalloproteinase-9 after acute focal cerebral ischemia in rats.** *Stroke* 2009, **40:**2836-2842.

152. Iadecola C, Alexander M: **Cerebral ischemia and inflammation.** *Curr Opin Neurol* 2001, **14:**89-94.

153. Alderton WK, Cooper CE, Knowles RG: **Nitric oxide synthases: structure, function and inhibition.** *Biochem J* 2001, **357:**593-615.

154. Gross SS, Wolin MS: **Nitric oxide: pathophysiological mechanisms.** *Annu Rev Physiol* 1995, **57:**737-769.

155. Perez-Asensio FJ, Hurtado O, Burguete MC, Moro MA, Salom JB, Lizasoain I, Torregrosa G, Leza JC, Alborch E, Castillo J, et al: **Inhibition of iNOS activity by 1400W decreases glutamate release and ameliorates stroke outcome after experimental ischemia.** *Neurobiol Dis* 2005, **18:**375-384.

156. Cui J, Holmes EH, Greene TG, Liu PK: **Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain.** *FASEB J* 2000, **14:**955-967.

157. Widenka DC, Medele RJ, Stummer W, Bise K, Steiger HJ: **Inducible nitric oxide synthase: a possible key factor in the pathogenesis of chronic vasospasm after experimental subarachnoid hemorrhage.** *J Neurosurg* 1999, **90:**1098-1104.

158. Sayama T, Suzuki S, Fukui M: **Role of inducible nitric oxide synthase in the cerebral vasospasm after subarachnoid hemorrhage in rats.** *Neurol Res* 1999, **21:**293-298.

159. Iadecola C, Zhang F, Casey R, Clark HB, Ross ME: **Inducible nitric oxide synthase gene expression in vascular cells after transient focal cerebral ischemia.** *Stroke* 1996, **27:**1373-1380.

160. Grandati M, Verrecchia C, Revaud ML, Allix M, Boulu RG, Plotkine M: **Calcium-independent NO-synthase activity and nitrites/nitrates production in transient focal cerebral ischaemia in mice.** *Br J Pharmacol* 1997, **122:**625-630.

161. Iadecola C, Zhang F, Casey R, Nagayama M, Ross ME: **Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene.** *J Neurosci* 1997, **17:**9157-9164.

162. Iadecola C, Zhang F, Xu X: **Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage.** *Am J Physiol* 1995, **268:**R286-292.

163. Parmentier S, Bohme GA, Lerouet D, Damour D, Stutzmann JM, Margaill I, Plotkine M: **Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury.** *Br J Pharmacol* 1999, **127:**546-552.

164. Tuttolomondo A, Di Raimondo D, Pecoraro R, Arnao V, Pinto A, Licata G: **Inflammation in Ischemic Stroke Subtypes.** *Curr Pharm Des* 2012.

165. Wang X, Xu L, Wang H, Young PR, Gaestel M, Feuerstein GZ: **Mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2 deficiency protects brain from ischemic injury in mice.** *J Biol Chem* 2002, **277:**43968-43972.

166. Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH: **Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion.** *Circ Res* 1996, **79:**162-173.

167. Wu DC, Ye W, Che XM, Yang GY: **Activation of mitogen-activated protein kinases after permanent cerebral artery occlusion in mouse brain.** *J Cereb Blood Flow Metab* 2000, **20:**1320-1330.

168. Roux PP, Blenis J: **ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions.** *Microbiol Mol Biol Rev* 2004, **68:**320-344.

169. Davis RJ: **The mitogen-activated protein kinase signal transduction pathway.** *J Biol Chem* 1993, **268:**14553-14556.

170. Ferrell JE, Jr.: **Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs.** *Trends Biochem Sci* 1996, **21:**460-466.

171. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR: **PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo.** *J Biol Chem* 1995, **270:**27489-27494.

172. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH: **Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.** *Endocr Rev* 2001, **22:**153-183.

173. Force T, Bonventre JV: **Growth factors and mitogen-activated protein kinases.** *Hypertension* 1998, **31:**152-161.

174. Gotoh Y, Nishida E: **[MAP kinase kinase/MAP kinase cascade].** *Tanpakushitsu Kakusan Koso* 1993, **38:**1625-1628.

175. Sugino T, Nozaki K, Takagi Y, Hattori I, Hashimoto N, Moriguchi T, Nishida E: **Activation of mitogen-activated protein kinases after transient forebrain ischemia in gerbil hippocampus.** *J Neurosci* 2000, **20:**4506-4514.

176. Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C, Cobb MH: **MAP kinases.** *Chem Rev* 2001, **101:**2449-2476.

177. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME: **Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis.** *Science* 1995, **270:**1326-1331.

178. Wang X, Feuerstein GZ: **Role of immune and inflammatory mediators in CNS injury.** *Drug News Perspect* 2000, **13:**133-140.

179. Kaminska B: **MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits.** *Biochim Biophys Acta* 2005, **1754:**253-262.

180. Wang ZQ, Wu DC, Huang FP, Yang GY: **Inhibition of MEK/ERK 1/2 pathway reduces pro-inflammatory cytokine interleukin-1 expression in focal cerebral ischemia.** *Brain Res* 2004, **996:**55-66.

181. Miller F, Fenart L, Landry V, Coisne C, Cecchelli R, Dehouck MP, Buee-Scherrer V: **The MAP kinase pathway mediates transcytosis induced by TNF-alpha in an in vitro blood-brain barrier model.** *Eur J Neurosci* 2005, **22:**835-844.

182. Takada Y, Aggarwal BB: **TNF activates Syk protein tyrosine kinase leading to TNF-induced MAPK activation, NF-kappaB activation, and apoptosis.** *J Immunol* 2004, **173:**1066-1077.

183. Saccani S, Pantano S, Natoli G: **p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment.** *Nat Immunol* 2002, **3:**69-75.

184. Beg SA, Hansen-Schwartz JA, Vikman PJ, Xu CB, Edvinsson LI: **ERK1/2 inhibition attenuates cerebral blood flow reduction and abolishes ET(B) and 5-HT(1B) receptor upregulation after subarachnoid hemorrhage in rat.** *J Cereb Blood Flow Metab* 2006, **26:**846-856.

185. Henriksson M, Xu CB, Edvinsson L: **Importance of ERK1/2 in upregulation of endothelin type B receptors in cerebral arteries.** *Br J Pharmacol* 2004, **142:**1155-1161.

186. Slevin M, Krupinski J, Slowik A, Rubio F, Szczudlik A, Gaffney J: **Activation of MAP kinase (ERK-1/ERK-2), tyrosine kinase and VEGF in the human brain following acute ischaemic stroke.** *Neuroreport* 2000, **11:**2759-2764.

187. Kawasaki H, Morooka T, Shimohama S, Kimura J, Hirano T, Gotoh Y, Nishida E: **Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells.** *J Biol Chem* 1997, **272:**18518-18521.

188. Barone FC, Irving EA, Ray AM, Lee JC, Kassis S, Kumar S, Badger AM, Legos JJ, Erhardt JA, Ohlstein EH, et al: **Inhibition of p38 mitogen-activated protein kinase provides neuroprotection in cerebral focal ischemia.** *Med Res Rev* 2001, **21:**129-145.

189. Guan QH, Pei DS, Zong YY, Xu TL, Zhang GY: **Neuroprotection against ischemic brain injury by a small peptide inhibitor of c-Jun N-terminal kinase (JNK) via nuclear and non-nuclear pathways.** *Neuroscience* 2006, **139:**609-627.

190. Sawe N, Steinberg G, Zhao H: **Dual roles of the MAPK/ERK1/2 cell signaling pathway after stroke.** *J Neurosci Res* 2008, **86:**1659-1669.

191. Satoh M, Parent AD, Zhang JH: **Inhibitory effect with antisense mitogen-activated protein kinase oligodeoxynucleotide against cerebral vasospasm in rats.** *Stroke* 2002, **33:**775-781.

192. Chen D, Chen JJ, Yin Q, Guan JH, Liu YH: **Role of ERK1/2 and vascular cell proliferation in cerebral vasospasm after experimental subarachnoid hemorrhage.** *Acta Neurochir (Wien)* 2009, **151:**1127-1134.

193. Wang H, Xu L, Venkatachalam S, Trzaskos JM, Friedman SM, Feuerstein GZ, Wang X: **Differential regulation of IL-1beta and TNF-alpha RNA expression by MEK1 inhibitor after focal cerebral ischemia in mice.** *Biochem Biophys Res Commun* 2001, **286:**869-874.

194. Alessandrini A, Namura S, Moskowitz MA, Bonventre JV: **MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia.** *Proc Natl Acad Sci U S A* 1999, **96:**12866-12869.

195. Wang X, Wang H, Xu L, Rozanski DJ, Sugawara T, Chan PH, Trzaskos JM, Feuerstein GZ: **Significant neuroprotection against ischemic brain injury by inhibition of the MEK1 protein kinase in mice: exploration of potential mechanism associated with apoptosis.** *J Pharmacol Exp Ther* 2003, **304:**172-178.

196. Han BH, Holtzman DM: **BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway.** *J Neurosci* 2000, **20:**5775-5781.

197. Memezawa H, Minamisawa H, Smith ML, Siesjo BK: **Ischemic penumbra in a model of reversible middle cerebral artery occlusion in the rat.** *Exp Brain Res* 1992, **89:**67-78.

198. Menzies SA, Hoff JT, Betz AL: **Middle cerebral artery occlusion in rats: a neurological and pathological evaluation of a reproducible model.** *Neurosurgery* 1992, **31:**100-106.

199. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H: **Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination.** *Stroke* 1986, **17:**472-476.

200. Prunell GF, Mathiesen T, Svendgaard NA: **A new experimental model in rats for study of the pathophysiology of subarachnoid hemorrhage.** *Neuroreport* 2002, **13:**2553-2556.

201. Prunell GF, Mathiesen T, Diemer NH, Svendgaard NA: **Experimental subarachnoid hemorrhage: subarachnoid blood volume, mortality rate, neuronal death, cerebral blood flow, and perfusion pressure in three different rat models.** *Neurosurgery* 2003, **52:**165-175; discussion 175-166.

202. Larsen CC, Povlsen GK, Rasmussen MN, Edvinsson L: **Improvement in neurological outcome and abolition of cerebrovascular endothelin B and 5-hydroxytryptamine 1B receptor upregulation through mitogen-activated protein kinase kinase 1/2 inhibition after subarachnoid hemorrhage in rats.** *J Neurosurg* 2011, **114:**1143-1153.

203. Hughes JP, Staton PC, Wilkinson MG, Strijbos PJ, Skaper SD, Arthur JS, Reith AD: **Mitogen and stress response kinase-1 (MSK1) mediates excitotoxic induced death of hippocampal neurones.** *J Neurochem* 2003, **86:**25-32.

204. Ohlsson AL, Johansson BB: **Environment influences functional outcome of cerebral infarction in rats.** *Stroke* 1995, **26:**644-649.

205. Sakurada O, Kennedy C, Jehle J, Brown JD, Carbin GL, Sokoloff L: **Measurement of local cerebral blood flow with iodo [14C] antipyrine.** *Am J Physiol* 1978, **234:**H59-66.

206. Gjedde A, Hansen AJ, Siemkowicz E: **Rapid simultaneous determination of regional blood flow and blood-brain glucose transfer in brain of rat.** *Acta Physiol Scand* 1980, **108:**321-330.

207. Adner M, Erlinge D, Nilsson L, Edvinsson L: **Upregulation of a non-ETA receptor in human arteries in vitro.** *J Cardiovasc Pharmacol* 1995, **26 Suppl 3:**S314-316.

208. Tanaka A, Konno M, Muto S, Kambe N, Morii E, Nakahata T, Itai A, Matsuda H: **A novel NF-kappaB inhibitor, IMD-0354, suppresses neoplastic proliferation of human mast cells with constitutively activated c-kit receptors.** *Blood* 2005, **105:**2324-2331.

209. Dong X, Song YN, Liu WG, Guo XL: **Mmp-9, a potential target for cerebral ischemic treatment.** *Curr Neuropharmacol* 2009, **7:**269-275.

210. Rosenberg GA, Yang Y: **Vasogenic edema due to tight junction disruption by matrix metalloproteinases in cerebral ischemia.** *Neurosurg Focus* 2007, **22:**E4.

211. Guo RW, Yang LX, Wang H, Liu B, Wang L: **Angiotensin II induces matrix metalloproteinase-9 expression via a nuclear factor-kappaB-dependent pathway in vascular smooth muscle cells.** *Regul Pept* 2008, **147:**37-44.

212. Wang HH, Hsieh HL, Wu CY, Yang CM: **Endothelin-1 enhances cell migration via matrix metalloproteinase-9 up-regulation in brain astrocytes.** *J Neurochem* 2010, **113:**1133-1149.

213. Hamel E: **Perivascular nerves and the regulation of cerebrovascular tone.** *J Appl Physiol* 2006, **100:**1059-1064.

214. Wu CY, Hsieh HL, Jou MJ, Yang CM: **Involvement of p42/p44 MAPK, p38 MAPK, JNK and nuclear factor-kappa B in interleukin-1beta-induced matrix metalloproteinase-9 expression in rat brain astrocytes.** *J Neurochem* 2004, **90:**1477-1488.

215. Yamasaki Y, Matsuura N, Shozuhara H, Onodera H, Itoyama Y, Kogure K: **Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats.** *Stroke* 1995, **26:**676-680; discussion 681.

216. Vila N, Castillo J, Davalos A, Chamorro A: **Proinflammatory cytokines and early neurological worsening in ischemic stroke.** *Stroke* 2000, **31:**2325-2329.

217. Ahnstedt H, Stenman E, Cao L, Henriksson M, Edvinsson L: **Cytokines and growth factors modify the upregulation of contractile endothelin ET(A) and ET(B) receptors in rat cerebral arteries after organ culture.** *Acta Physiol (Oxf)* 2011.

218. Armour KJ, Armour KE, van't Hof RJ, Reid DM, Wei XQ, Liew FY, Ralston SH: **Activation of the inducible nitric oxide synthase pathway contributes to inflammation-induced osteoporosis by suppressing bone formation and causing osteoblast apoptosis.** *Arthritis Rheum* 2001, **44:**2790-2796.

219. Lerouet D, Beray-Berthat V, Palmier B, Plotkine M, Margaill I: **Changes in oxidative stress, iNOS activity and neutrophil infiltration in severe transient focal cerebral ischemia in rats.** *Brain Res* 2002, **958:**166-175.

220. Stanimirovic D, Satoh K: **Inflammatory mediators of cerebral endothelium: a role in ischemic brain inflammation.** *Brain Pathol* 2000, **10:**113-126.

221. Iadecola C, Zhang F, Xu S, Casey R, Ross ME: **Inducible nitric oxide synthase gene expression in brain following cerebral ischemia.** *J Cereb Blood Flow Metab* 1995, **15:**378-384.

222. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, et al: **Identification of a novel inhibitor of mitogen-activated protein kinase kinase.** *J Biol Chem* 1998, **273:**18623-18632.

223. Namura S, Iihara K, Takami S, Nagata I, Kikuchi H, Matsushita K, Moskowitz MA, Bonventre JV, Alessandrini A: **Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia.** *Proc Natl Acad Sci U S A* 2001, **98:**11569-11574.

224. Lampl Y, Fleminger G, Gilad R, Galron R, Sarova-Pinhas I, Sokolovsky M: **Endothelin in cerebrospinal fluid and plasma of patients in the early stage of ischemic stroke.** *Stroke* 1997, **28:**1951-1955.

225. Fassbender K, Hodapp B, Rossol S, Bertsch T, Schmeck J, Schutt S, Fritzinger M, Horn P, Vajkoczy P, Wendel-Wellner M, et al: **Endothelin-1 in subarachnoid hemorrhage: An acute-phase reactant produced by cerebrospinal fluid leukocytes.** *Stroke* 2000, **31:**2971-2975.

226. Cheng ZJ, Vapaatalo H, Mervaala E: **Angiotensin II and vascular inflammation.** *Med Sci Monit* 2005, **11:**RA194-205.

227. Chen P, Shibata M, Zidovetzki R, Fisher M, Zlokovic BV, Hofman FM: **Endothelin-1 and monocyte chemoattractant protein-1 modulation in ischemia and human brain-derived endothelial cell cultures.** *J Neuroimmunol* 2001, **116:**62-73.

228. Patel TR, Galbraith SL, McAuley MA, Doherty AM, Graham DI, McCulloch J: **Therapeutic potential of endothelin receptor antagonists in experimental stroke.** *J Cardiovasc Pharmacol* 1995, **26 Suppl 3:**S412-415.

229. Nishimura Y, Ito T, Saavedra JM: **Angiotensin II AT(1) blockade normalizes cerebrovascular autoregulation and reduces cerebral ischemia in spontaneously hypertensive rats.** *Stroke* 2000, **31:**2478-2486.

230. Stenman E, Jamali R, Henriksson M, Maddahi A, Edvinsson L: **Cooperative effect of angiotensin AT(1) and endothelin ET(A) receptor antagonism limits the brain damage after ischemic stroke in rat.** *Eur J Pharmacol* 2007, **570:**142-148.

231. Cahill J, Calvert JW, Zhang JH: **Mechanisms of early brain injury after subarachnoid hemorrhage.** *J Cereb Blood Flow Metab* 2006, **26:**1341-1353.

232. Gallia GL, Tamargo RJ: **Leukocyte-endothelial cell interactions in chronic vasospasm after subarachnoid hemorrhage.** *Neurol Res* 2006, **28:**750-758.

233. Feiler S, Plesnila N, Thal SC, Zausinger S, Scholler K: **Contribution of matrix metalloproteinase-9 to cerebral edema and functional outcome following experimental subarachnoid hemorrhage.** *Cerebrovasc Dis* 2011, **32:**289-295.

234. Sehba FA, Mostafa G, Knopman J, Friedrich V, Jr., Bederson JB: **Acute alterations in microvascular basal lamina after subarachnoid hemorrhage.** *J Neurosurg* 2004, **101:**633-640.

235. Fujimoto M, Takagi Y, Aoki T, Hayase M, Marumo T, Gomi M, Nishimura M, Kataoka H, Hashimoto N, Nozaki K: **Tissue inhibitor of metalloproteinases protect blood-brain barrier disruption in focal cerebral ischemia.** *J Cereb Blood Flow Metab* 2008, **28:**1674-1685.

236. Svendgaard NA, Brismar J, Delgado TJ, Rosengren E: **Subarachnoid haemorrhage in the rat: effect on the development of vasospasm of selective lesions of the catecholamine systems in the lower brain stem.** *Stroke* 1985, **16:**602-608.

237. Ansar S, Edvinsson L: **Equal contribution of increased intracranial pressure and subarachnoid blood to cerebral blood flow reduction and receptor upregulation after subarachnoid hemorrhage. Laboratory investigation.** *J Neurosurg* 2009, **111:**978-987.

238. Ansar S, Vikman P, Nielsen M, Edvinsson L: **Cerebrovascular ETB, 5-HT1B, and AT1 receptor upregulation correlates with reduction in regional CBF after subarachnoid hemorrhage.** *Am J Physiol Heart Circ Physiol* 2007, **293:**H3750-3758.

239. Leseth KH, Adner M, Berg HK, White LR, Aasly J, Edvinsson L: **Cytokines increase endothelin ETB receptor contractile activity in rat cerebral artery.** *Neuroreport* 1999, **10:**2355-2359.

240. Vikman P, Ansar S, Edvinsson L: **Transcriptional regulation of inflammatory and extracellular matrix-regulating genes in cerebral arteries following experimental**

subarachnoid hemorrhage in rats. Laboratory investigation. *J Neurosurg* 2007, **107:**1015-1022.

241. Rosenberg GA, Sullivan N, Esiri MM: **White matter damage is associated with matrix metalloproteinases in vascular dementia.** *Stroke* 2001, **32:**1162-1168.

242. Wang CX, Shuaib A: **Involvement of inflammatory cytokines in central nervous system injury.** *Prog Neurobiol* 2002, **67:**161-172.

243. Uno H, Matsuyama T, Akita H, Nishimura H, Sugita M: **Induction of tumor necrosis factor-alpha in the mouse hippocampus following transient forebrain ischemia.** *J Cereb Blood Flow Metab* 1997, **17:**491-499.

244. McMillian MK, Thai L, Hong JS, O'Callaghan JP, Pennypacker KR: **Brain injury in a dish: a model for reactive gliosis.** *Trends Neurosci* 1994, **17:**138-142.

245. Vecchione C, Frati A, Di Pardo A, Cifelli G, Carnevale D, Gentile MT, Carangi R, Landolfi A, Carullo P, Bettarini U, et al: **Tumor necrosis factor-alpha mediates hemolysis-induced vasoconstriction and the cerebral vasospasm evoked by subarachnoid hemorrhage.** *Hypertension* 2009, **54:**150-156.

246. Haqqani AS, Nesic M, Preston E, Baumann E, Kelly J, Stanimirovic D: **Characterization of vascular protein expression patterns in cerebral ischemia/reperfusion using laser capture microdissection and ICAT-nanoLC-MS/MS.** *FASEB J* 2005, **19:**1809-1821.

247. Aoki K, Zubkov AY, Tibbs RE, Zhang JH: **Role of MAPK in chronic cerebral vasospasm.** *Life Sci* 2002, **70:**1901-1908.

248. Tibbs R, Zubkov A, Aoki K, Meguro T, Badr A, Parent A, Zhang J: **Effects of mitogen-activated protein kinase inhibitors on cerebral vasospasm in a double-hemorrhage model in dogs.** *J Neurosurg* 2000, **93:**1041-1047.

249. Pluta RM, Butman JA, Schatlo B, Johnson DL, Oldfield EH: **Subarachnoid hemorrhage and the distribution of drugs delivered into the cerebrospinal fluid. Laboratory investigation.** *J Neurosurg* 2009, **111:**1001-1007, 1001-1004.

250. Vajkoczy P, Meyer B, Weidauer S, Raabe A, Thome C, Ringel F, Breu V, Schmiedek P: **Clazosentan (AXV-034343), a selective endothelin A receptor antagonist, in the prevention of cerebral vasospasm following severe aneurysmal subarachnoid hemorrhage: results of a randomized, double-blind, placebo-controlled, multicenter phase IIa study.** *J Neurosurg* 2005, **103:**9-17.

251. Larsen CC, Povlsen GK, Rasmussen MN, Edvinsson L: **Improvement in neurological outcome and abolition of cerebrovascular endothelin B and 5-hydroxytryptamine 1B receptor upregulation through mitogen-activated protein kinase kinase 1/2 inhibition after subarachnoid hemorrhage in rats.** *J Neurosurg* 2010.

252. Moller S, Uddman E, Welsh N, Edvinsson L, Adner M: **Analysis of the time course for organ culture-induced endothelin ET B receptor upregulation in rat mesenteric arteries.** *Eur J Pharmacol* 2002, **454:**209-215.

253. Povlsen GK, Waldsee R, Ahnstedt H, Kristiansen KA, Johansen FF, Edvinsson L: **In vivo experimental stroke and in vitro organ culture induce similar changes in**

vasoconstrictor receptors and intracellular calcium handling in rat cerebral arteries. *Exp Brain Res* 2012.

254. Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA, Jr., Goeddel DV: **The two different receptors for tumor necrosis factor mediate distinct cellular responses.** *Proc Natl Acad Sci U S A* 1991, **88:**9292-9296.

255. Lambertsen KL, Clausen BH, Fenger C, Wulf H, Owens T, Dagnaes-Hansen F, Meldgaard M, Finsen B: **Microglia and macrophages express tumor necrosis factor receptor p75 following middle cerebral artery occlusion in mice.** *Neuroscience* 2007, **144:**934-949.

256. Gesslein B, Hakansson G, Gustafsson L, Ekstrom P, Malmsjo M: **Tumor necrosis factor and its receptors in the neuroretina and retinal vasculature after ischemia-reperfusion injury in the pig retina.** *Mol Vis* 2010, **16:**2317-2327.

257. Al-Lamki RS, Brookes AP, Wang J, Reid MJ, Parameshwar J, Goddard MJ, Tellides G, Wan T, Min W, Pober JS, Bradley JR: **TNF receptors differentially signal and are differentially expressed and regulated in the human heart.** *Am J Transplant* 2009, **9:**2679-2696.

258. Gaur U, Aggarwal BB: **Regulation of proliferation, survival and apoptosis by members of the TNF superfamily.** *Biochem Pharmacol* 2003, **66:**1403-1408.