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From the Division of Experimental Vascular Research, Department of Clinical Sciences,
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# Importance of MAPK and PKC in cerebrovascular endothelin receptor changes

Marie Henriksson, M.Sc

**Academic dissertation**
The public defence of this thesis for the degree Doctor of Philosophy in Medicine will, with due permission from the Faculty of Medicine, Lund University, take place in Segerfalksalen, Wallenberg Neuroscience Centre, Lund, Sweden on Saturday the 22nd of April 2006 at 10 am.

**Faculty opponent**
Professor James McCulloch
University of Edinburgh
Edinburgh, UK

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Abstract

Endothelin is a vasoactive peptide that exerts its effect through two receptors; the endothelin type A (ETA) and type B (ETB) receptor. The contractile ETA receptor is localized on smooth muscle cells in the vascular wall, while the ETB receptors are mainly situated on endothelial cells, mediating vasodilatation. The endothelin system is involved in several pathophysiological conditions, such as atherosclerosis and ischemic stroke. Previous studies have shown an upregulation of contractile ETB receptors in the ipsilateral middle cerebral artery (MCA) after experimental focal ischemia and a similar upregulation is also seen after organ culture of MCA. Furthermore, studies have shown an involvement of protein kinase C (PKC) and extracellular signal-regulated kinases (ERK) 1/2 in ischemia.

The aim of this thesis was to further elucidate the mechanisms underlying this receptor upregulation process, both in experimental ischemia and in organ culture of rat MCA.

The results from the organ culture studies (paper I-III) show that the ETB receptor upregulation is time-dependent, reaching a maximum after 24 hours of organ culture. By adding inhibitors of PKC and ERK1/2 pathways to the culture medium, the ETB receptor upregulation was attenuated.
In the experimental model of ischemia (paper IV-V), we administered PKC and ERK1/2 inhibitors to the rats in conjuction with the ischemic insult. Both inhibitors diminished the ETB receptor responses, as well as decreasing brain damage and improving neurological function.

In conclusion, the results of this thesis may provide a new perspective on possible mechanisms of actions of PKC and ERK in cerebral ischemia.



Key words: Endothelin, receptor, MCA, ischemia, PKC, MAPK

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**PAPERS I-V**

# ORIGINAL ARTICLES

This thesis is based on the following papers:

**I.** Henriksson M, Stenman E, Edvinsson L. Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat. 2003. *Stroke* 34(6):1479-83.

**II.** Henriksson M, Xu C, Edvinsson L. Importance of ERK1/2 in upregulation of endothelin type B receptors in cerebral arteries. 2004. *British journal of pharmacology* 142(7):1155-61.

**III.** Henriksson M, Vikman P, Stenman E, Beg S, Edvinsson L. The role of PKC in cerebrovascular endothelin $ET_B$ receptor upregulation. 2006. *Submitted manuscript.*

**IV.** Henriksson M, Stenman E, Vikman P, Edvinsson L. MEK1/2 inhibition attenuates vascular $ET_A$ and $ET_B$ upregulation after cerebral ischemia. 2006. *Submitted manuscript.*

**V.** Henriksson M, Stenman E, Vikman P, Edvinsson L. PKC inhibition attenuates vascular $ET_B$ receptor upregulation and decreases brain damage after cerebral ischemia. 2006. *Manuscript.*

# ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| aPKC | atypical PKC |
| ATP | adenosine triphosphate |
| bFGF | basic fibroblast growth factor |
| BMK/ERK | big mitogen-activated protein kinase/extracellular signal-regulated kinase |
| $Ca^{2+}$ | calcium ion |
| cAMP | 3',5'-cyclic adenosine monophosphate |
| cDNA | complementary deoxyribonucleic acid |
| $Cl^-$ | chloride ion |
| $CO_2$ | carbon dioxide |
| cPKC | classical PKC |
| C-terminal | carboxyl group terminal |
| DAG | 1,2-diacylglycerol |
| DMEM | Dulbecco's modified Eagle's Medium |
| DMSO | dimethyl-sulfoxide |
| ECE | endothelin converting enzyme |
| EF-1 | Elongation factor-1 |
| ERK1/2 | extra-cellular signal regulated kinases 1 and 2 |
| ET-1 | endothelin-1 |
| ET-2 | endothelin-2 |
| ET-3 | endothelin-3 |
| $ET_A$ | endothelin receptor type A |
| $ET_B$ | endothelin receptor type B |
| G-protein | guanine nucleotide binding protein |
| i.p. | intraperitoneally |
| IL-1β | interleukin-1β |
| $IP_3$ | inositol triphosphate |
| JNK | c-jun N-terminal kinase |
| $K^+$ | potassium ion |
| M | mol/liter |
| MAP | mean arterial blood pressure |
| MAPK | mitogen-activated protein kinase |
| MAPKK | mitogen-activated protein kinase kinase |
| MAPKKK | mitogen-activated protein kinase kinase kinase |
| MCA | middle cerebral artery |
| MCAO | middle cerebral artery occlusion |
| mRNA | messenger ribonucleic acid |
| $Na^+$ | sodium ion |
| NF-κB | nuclear factor- κB |
| NMDA | *N*-methyl-D-aspartate |
| NO | nitric oxide |
| $N_2O$ | nitrous oxide (laughing gas) |
| nPKC | novel PKC |
| N-terminal | amino group terminal |
| $O_2$ | oxygen |
| $pCO_2$ | partial pressure of carbon dioxide |
| PCR | polymerase chain reaction |
| $PIP_2$ | phosphatidyl inositol |
| PKC | protein kinase C |
| PLC | phospholipase C |
| $pO_2$ | partial pressure of oxygen |
| PRK | protein kinase C-related kinase |
| RNA | ribonucleic acid |
| S6c | sarafotoxin 6c |
| SAH | subarachnoid hemorrhage |
| SDS | Sodium dodecyl sulphate |
| S.E.M | standard error of the mean |
| TNF-α | tumor necrosis factor- α |
| TTC | 2,3,5-triphenyltetrazolium chloride |
| VEGF | vascular endothelial growth factor |

# INTRODUCTION

Stroke is a serious neurological disease and the third leading cause of death in the western world. In about 12% of the cases, the cause is intracranial hemorrhages, and the remaining 88% represent ischemic strokes.[1] Ischemic stroke is caused by a transient or permanent occlusion of a cerebral artery either by an embolus or by local thrombosis.[2]

When an ischemic stroke occurs, detrimental cellular alterations are induced. Many studies have aimed to restore the neuronal cells and thereby promote survival. This approach has so far not resulted in successful therapies for the patients.

In ischemic stroke, there are also modifications in the cells of the blood vessels. For example, levels of endothelin, released by the endothelial cells, are increased. Our group has focused on the vascular changes and has shown an alteration of endothelin receptors in middle cerebral arteries following an experimental ischemic stroke.[3] To be able to examine this phenomenon in detail, we have used organ culture, which produces similar changes.[4]

This thesis aims to further examine the alterations of endothelin receptor expression in middle cerebral arteries, and elucidate the intracellular signalling pathways involved. We find that both PKC and MAPK seem to play important roles in the regulation of the vascular endothelin receptors. This applies to both organ culture and experimental ischemic stroke. The results of this thesis provide new perspectives on the pathophysiology of ischemic stroke and also give a possible explanation for the beneficial effects of treatment with PKC and MAPK inhibitors.

# BACKGROUND

## Ischemic stroke

Ischemic stroke, caused by the obstruction of a cerebral artery, gives rise to a range of pathophysiological events. When the brain is deprived of oxygen and glucose, the membrane potentials of the neurons change rapidly, voltage-dependent $Ca^{2+}$ channels open and excitatory glutamate is released into the extracellular space.[5, 6] The glutamate release results in increased levels of intracellular $Na^+$, $Cl^-$ and $Ca^{2+}$. The influx of $Na^+$ and $Cl^-$ is accompanied by osmotic water uptake which consequently leads to edema. The increased levels of intracellular $Ca^{2+}$ give rise to an activation of numerous enzyme systems, for example proteolytic enzymes that degrade the cytoskeleton, as well as phospholipase $A_2$ and cyclooxygenase that generates free oxygen radicals. Beside their cell damaging characteristics, free oxygen radicals also trigger inflammation and cell death in the ischemic area.[7-10]

In the immediate vicinity of the occlusion, the ischemia results in a necrotic core. Between the ischemic core and the normal tissue is the penumbra, an area with incomplete ischemia.[11] The penumbra is characterized by low blood flow and metabolism and it is at risk of becoming incorporated in the ischemic core, or at least subjected to selective cell death. The size of the penumbra is determined by the amount of collateral blood vessels in the area. Since the neurons of the penumbra can be salvaged, this area has become a prime target for neuroprotective treatments.[11, 12] However, despite promising results from experimental animal models, clinical studies have yet failed to show significantly beneficial effects.[13-16]

## Vascular pathophysiology

The vascular pathophysiology after an ischemic stroke can be divided into three phases.[17] In the first acute phase there are hemodynamic and metabolic changes that disrupt the blood-brain barrier. This is in part due to elevated levels of endothelin-1 (ET-1).[18, 19] ET-1 along with other vasoactive factors, also affects the myogenic tone, which is diminished in the acute phase.[17, 20] Reperfusion after cerebral ischemia results in production of free oxygen radicals, for example hydrogen peroxide and superoxide. Superoxide increases the blood-brain barrier permeability and disrupts the endothelial cell membranes.[21, 22]

The following subacute phase takes place hours to days after the ischemic insult. Here, gene activation of inflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and transcription factors such as nuclear factor κB occurs as a response to the events in the acute phase.[17, 23-25] Furthermore, proteins with angiogenic and consequently protective features like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are also expressed during this phase.[26, 27]

In the final chronic phase (days to months) both apoptosis and angiogenesis occur in the vessels. Inflammatory mediators induce programmed cell death via the cascade of caspases. However, there is also a stimulation of VEGF, which in addition to its angiogenic effects, also contributes to endothelial cell survival.[17, 28]

## The endothelin system

### *Endothelin*

In 1985, Hickey and colleagues discovered a new vasoactive peptide, produced by endothelial cells.[29] Three years later, Yanagisawa and colleagues isolated the peptide and named it endothelin (ET).[30]

Subsequent studies showed three different ET genes in the human genome, all coding for peptides of 21 amino acids; the original endothelin-1 (ET-1), endothelin-2 (ET-2) which differs with two amino acids, and endothelin-3 (ET-3) which differs with six amino acids compared to ET-1.[31]

Translation of the ET-1 mRNA results in preproendothelin, a peptide consisting of 212 amino acids.[32] Preproendothelin is converted to bigET-1, which exhibits some vasoactivity.[33] BigET-1 is cleaved by endothelin converting enzymes (ECE) to the mature endothelin peptide.[34, 35] ET-1 is produced primarily in endothelial cells, but is also found in other cell types, such as neurons, epithelial cells and macrophages.[36-38]
The production and release of ET-1 is regulated by a range of different stimuli, such as thrombin, angiotensin, vasopressin and shear stress.[39-41]
Interestingly, the sarafotoxins, a group of cardiotoxic peptides isolated from the venom of the burrowing asp, resemble the structure and function of ET-1.[42, 43] They are able to activate the endothelin receptors, and sarafotoxin 6c (S6c) which we have used in the present investigations is a selective endothelin receptor type B ($ET_B$) agonist.

*Endothelin receptors*

There are two endothelin receptors in the vasculature of mammals, the endothelin receptor type A ($ET_A$) and the $ET_B$ receptor. Both are G-protein coupled receptors and both are found on the smooth muscle cells of the vasculature mediating contraction, albeit the $ET_A$ receptor in abundance.[4, 44, 45] Interestingly enough, there is an upregulation of contractile $ET_B$ receptors in several cardiovascular diseases such as atherosclerosis,[46, 47] pulmonary hypertension,[48] ischemic heart disease[49] as well as after organ culture of isolated arteries.[4]
The $ET_A$ and $ET_B$ receptors on the smooth muscle cells induce contraction through activation of phospholipase C (PLC) via $G_q$ proteins.[50] Activation of PLC hydrolyses phosphatidyl

inositol ($PIP_2$) to inositol triphosphate ($IP_3$) and 1,2-diacylglycerol (DAG). $IP_3$ causes release of $Ca^{2+}$ from intracellular stores, giving rise to a transient smooth muscle contraction. DAG can in turn activate protein kinase C (PKC).[51] In addition, $ET_A$ receptors activates $G_s$ proteins, while $ET_B$ receptors activates $G_i$ proteins.[52] These intracellular signalling events result in altered levels of cAMP, which affects the contractility of the smooth muscle cells.[53] $ET_A$ receptors also activate a Rho kinase pathway, suggested to produce the characteristic long-lasting contraction of ET-1.[50] Furthermore, most part of the $ET_B$ receptors are found on the endothelial cells, mediating vasodilatation through the release of nitric oxide (NO) and prostacyclins.[54, 55]

The $ET_A$ receptor has similar affinity for ET-1 and ET-2 and lower affinity for ET-3, while the $ET_B$ receptor shows an equal affinity for all three ligands.[56, 57]

*Endothelin and ischemic stroke*

There are several studies pointing towards a major role for the endothelin system in the pathophysiology of ischemic stroke. For instance, it has been suggested that increased endothelin-induced contractility leads to decreased perfusion of the ischemic area and subsequently an enlargement of the ischemic core.[58, 59]

After an ischemic stroke the levels of ET-1 are increased in plasma, cerebrospinal fluid and cerebral tissue.[60, 61] In addition, exogenous ET-1 is able to decrease cerebral blood flow to levels that induce ischemia.[62] However, the results concerning endothelin receptor antagonism in ischemic stroke have been contradictory. Selective $ET_A$ receptor antagonists increase cerebral perfusion and decrease the ischemic area in some studies,[63, 64] but not in others.[65, 66] Similarly, the $ET_A/ET_B$ receptor antagonist bosentan gives various results when used in models of ischemia.[67-69] Furthermore, an $ET_B$ receptor antagonist, BQ788, has actually been shown to exacerbate the ischemic damage.[70] In conclusion, the endothelin

system is activated in ischemic stroke, but the use of endothelin receptor inhibitors might not be the best way to attenuate the endothelin system alterations.

**Protein kinase C**

*PKC: isoforms and function*

PKC was first discovered in 1977 as a kinase in rat brain which could be activated by limited hydrolysis.[71] This kinase was shown to include three different isoforms, which were denominated α, β and γ.[72, 73] Since then thorough examination has revealed that PKC comprises more than ten different isoforms, all of which are serine/threonine kinases.[74-76] The isoforms are divided into three groups depending on their structure and requirements of activation. The classical PKCs (cPKC) comprise the isoforms α, $\beta_I$, $\beta_{II}$ and γ, and are activated by DAG and $Ca^{2+}$. The novel PKCs (nPKC) (δ, ε, θ, and η) require activation by DAG while the atypical PKCs (aPKC) (ζ and λ) are insensitive to both DAG and $Ca^{2+}$.[77]
Later, a fourth group, the protein kinase C-related kinases (PRKs), consisting of at least three members was found. Similar to the aPKCs, the PRKs are DAG and $Ca^{2+}$ insensitive. The PRKs have also been shown to bind and activate RhoA GTPase.[78, 79]
Members of the PKC family are single polypeptides with an N-terminal regulatory region and a C-terminal catalytic region. PKC has four conserved domains, C1-C4, each with a different function. In the cPKC isoforms there is a DAG binding site and a $Ca^{2+}$ binding site in the C1 and C2 regions, respectively. In all isoforms, the C3 domain contains an ATP binding site and the C4 a substrate binding site.[77]
PKC participate in a wide range of cellular events, such as transcription, proliferation and receptor modulations. The kinase is activated by growth factors, hormones and neurotransmitters binding to their receptors.[74, 80]

*PKC and ischemic stroke*

PKC is known to be involved in the pathophysiology of ischemic stroke, but the exact role of its involvement is unclear. Inhibition of PKC with general inhibitors such as staurosporine has been proven to protect neurons from NO-induced as well as glutamate-induced excitotoxic cell death in culture, and from ischemic damage in animal models.[81-83] However, some studies have shown that the PKC activity is abolished after ischemia.[84-86] These contradictions have been suggested to be the result of different roles for the PKC isoforms.[87, 88]

When the brain is exposed to non-lethal ischemia, neuroprotective mechanisms are launched. The mechanisms are mediated through a range of diverse events such as ion channel activations and altered gene expression, which protect the neurons of the brain against subsequent lethal attacks. It has been shown that this ischemic tolerance is dependent on PKC activation.[89, 90]

PKCγ is normally expressed only in neurons of the brain and spinal cord.[91] It is activated during ischemia in several models,[92, 93] and PKCγ knock-out mice exhibit decreased infarct size after permanent focal ischemia.[94] However, in a model of transient ischemia, the same group found that PKCγ knock-out mice did actually display larger ischemic injury.[95] This suggests that PKCγ acts in a deleterious way initially, while being beneficial in the reperfusion phase.

The role of PKCε in ischemic stroke has also been a subject of research. PKCε is required for ischemic tolerance as a response to *N*-methyl-D-aspartate (NMDA) exposure in cell culture.[89] Furthermore, a PKCε activator reduced damage in oxygen and glucose deprived neuronal cells.[96] The mechanisms underlying the beneficial effects of PKCε are still unclear.

Another isoform that has been proposed to be involved in the reperfusion injury is PKCδ. It is suggested to play a part in the reperfusion injury, as part of the apoptotic and inflammatory events.[97, 98] Treatment with PKCδ inhibitors decreases infarct size and PKCδ knock-out mice show a decreased infarct size after ischemia.[99, 100]

Taken together, the roles of PKCs in ischemic injury are truly diverse. In the present thesis we have shifted the focus towards the role of PKC in the vascular receptor alterations occurring after organ culture and ischemic stroke. This could provide new insights to the involvement of PKC in cerebral ischemia.

## MAPK

### *MAPK: structure and function*

The mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases which are evolutionary conserved in all eukaryotes. MAPKs are involved in cellular responses to external signals such as growth factors, stress and inflammatory mediators.[101-104] The MAPK family consists of four members; the extracellular signal-regulated kinase (ERK)1/2, p38, c-jun N-terminal kinase (JNK) and big mitogen-activated protein kinase/extracellular signal-regulated kinase (BMK/ERK).[105]

All MAPK pathways include a MAPK kinase kinase (MAPKKK) which upon phosphorylation becomes activated and phosphorylates a MAPK kinase (MAPKK). The MAPKK in turn phosphorylates and activates the MAPK. The initial stimulus for this cascade varies between the MAPKs. ERK1/2 is often activated by growth factors,[101] while p38 and JNK are stress-activated protein kinases, which respond to stress and cytokines.[102-104] The BMK/ERK can be activated as a response to all stimuli mentioned above (Figure 1).[106-108] Many of the substrates of MAPKs are transcription factors, such as c-fos, c-jun and elk-1.[109-111]

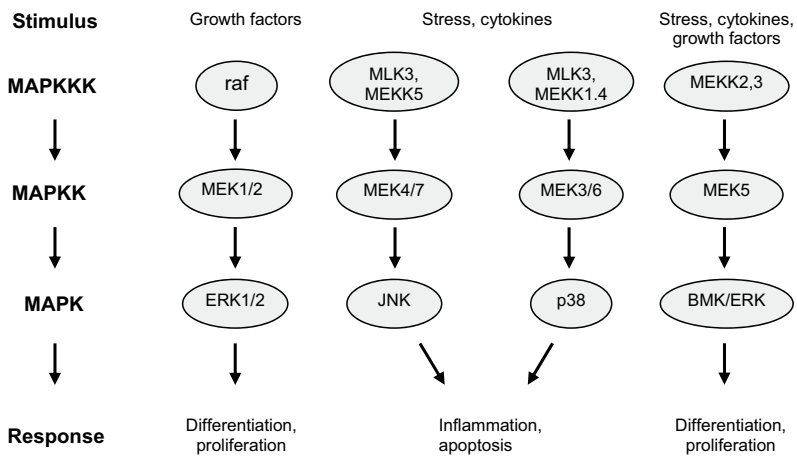


**Figure 1.** *Mitogen-activated protein kinase signalling cascades.*

*MAPK and ischemic stroke*

The importance of MAPK signalling in the pathophysiology of ischemic stroke has been widely studied. Increased ERK1/2 phosphorylation has been observed in the ischemic area after both transient and permanent middle cerebral occlusion, as well as after global ischemia.[112-116] Consequently, inhibitors of ERK1/2 and MEK1/2 (the MAPKK of ERK1/2) have been effective in decreasing the ischemic area.[113, 114]

ERK1/2 is also activated in the cerebral arteries of the ischemic brain, pointing towards a role in vascular alterations.[117]

The p38 pathway is activated by inflammatory mediators such as TNF-α and IL-1β, both of which are known to be increased after ischemic stroke.[102] Not surprisingly, there is an increased activity of p38 after cerebral ischemia,[116, 118] and inhibition of the p38 pathway has been found to decrease the infarct size.[119]

Presently, the importance of JNK in cerebral ischemia has not been investigated in detail. However, in neuronal tissue of mice subjected to permanent cerebral ischemia, JNK has been shown to be activated even earlier than both p38 and ERK1/2.[116] Lennmyr and colleagues failed to detect this activation in neurons after transient cerebral ischemia in rat, but instead showed an activation of JNK in the ipsilateral blood vessels.[117]

**PKC and MAPK in endothelin receptor regulation**

Our group has in several studies showed the involvement of both PKC and MAPK in endothelin receptor regulation in different settings. For example, in rat mesenteric artery undergoing organ culture the raf inhibitor SB386023 blocks the upregulation efficiently as do the PKC inhibitor staurosporine.[120, 121] This is also seen in human left internal mammary arteries after organ culture (Nilsson et al., 2006. Unpublished data). Furthermore, in an experimental model of SAH, both a PKC inhibitor and SB386023 blocks the $ET_B$ receptor upregulation of the MCA.[122, 123]

# AIMS OF THE THESIS

- To evaluate the time course of the $ET_B$ receptor upregulation in middle cerebral arteries after organ culture

- To examine if the $ET_B$ receptor upregulation in middle cerebral arteries after organ culture is transcriptionally induced

- To examine the involvement of MAPK and PKC in the endothelin receptor alterations during organ culture

- To study the endothelin receptor alterations in middle cerebral arteries 24 hours after middle cerebral artery occlusion

- To examine the involvement of MAPK and PKC in the endothelin receptor alterations following middle cerebral artery occlusion

# GENERAL METHODS

**Organ culture (paper I-III)**

Male Wistar rats were anesthetized with $CO_2$ and decapitated. The right and left middle cerebral arteries (MCA) were removed and dissected free from surrounding tissue. The arteries were cultured in Dulbeccos modified Eagle's medium (DMEM), supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (25 µg/ml), at 37° C in humidified 5% $CO_2$ in air. When inhibitors were used, they were added to the medium before incubation.

**Animal surgery procedure and evaluation (paper IV-V)**

*Transient middle cerebral artery occlusion*

Male Wistar rats were used for the procedure. The animals were housed under controlled temperature and humidity with free access to water and food. Transient middle cerebral artery occlusion (MCAO) was induced by an intraluminal filament technique, previously described by Memezawa et al.[124] Briefly, anesthesia was induced using 4.5% halothane in $N_2O$:$O_2$ (70%:30%). The rats were kept anesthetized by inhalation of 1.5% halothane through a mask. To confirm a proper occlusion and subsequently a proper reperfusion of the right MCA, a laser-Doppler probe was fixed on the skull (1 mm posterior to the bregma and 6 mm from the midline on the right side), measuring the blood flow in an area supplied by the right MCA. 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 at 37° C during the operation. Thereafter, an incision was made in the midline of the neck and the right common, external and internal carotid arteries were exposed. The common and external

carotid arteries were permanently ligated with sutures. A filament was inserted into the internal carotid artery via an incision in the common carotid artery, and further advanced until the rounded tip reached the entrance of the right MCA. The resulting occlusion was made visible by laser-Doppler flowmetry as an abrupt reduction of cerebral blood flow of 75-90%. Immediately after occlusion, the rats were injected intraperitoneally (i.p.) with either a kinase inhibitor dissolved in DMSO or the corresponding volume of DMSO (control). The rats were then allowed to wake up.

Two hours after occlusion the rats were reanesthetized to allow for withdrawal of the filament and thereby achieve reperfusion. Rectal temperature was measured 30 minutes before occlusion and one hour after reperfusion.

*Neurological examination*

The rats were examined neurologically before recirculation and immediately before they were sacrificed, 24 hours after MCAO, and were given a value according to the scoring system seen below.[125, 126]

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

*Brain damage evaluation*

The brains were sliced coronally in two mm thick slices, and stained with 1% 2, 3, 5-triphenyltetrazolium chloride (TTC) dissolved in physiological saline solution. The size of ischemic brain damage was calculated as percentage of the total brain volume using the software program Brain Damage Calculator 1.1.

**Myograph experiments (paper I-V)**

Myographs were used to measure the contractile properties of the arteries.[127, 128] The arteries were cut into cylindrical segments and the endothelium was removed mechanically by rubbing it off with a thread. The arteries were mounted on two 40 µm diameter stainless steel wires in a Mulvany-Halpern myograph. One of the wires was connected to a force transducer attached to an analogue-digital converter unit. The other wire was attached to a movable displacement device allowing adjustments of vascular tension by varying the distance between the wires. The experiments were recorded on a computer by use of the software program Chart™. The segments were immersed in a temperature-controlled (37° C) bicarbonate buffer. The buffer was continuously gassed with 5% $CO_2$ in $O_2$, resulting in a pH of 7.4. The arteries were given an initial tension of 1.2 mN, and were allowed to adjust to this level of tension for one hour. The contractile capacity was determined by exposure to a potassium-rich (63.5 mM) buffer, and this response was used as a reference value. Concentration-response curves for the S6c ($ET_B$ receptor agonist) and ET-1 ($ET_A$ and $ET_B$ receptor agonist) were obtained by cumulative application ($10^{-12}$-$10^{-6.5}$ M). Following S6c administration, the $ET_B$ receptors are desensitized, leaving only $ET_A$ receptors to interact with ET-1.[129] The maximum vascular contraction as response to S6c or ET-1 were calculated as percentage of the contractile response towards 63.5 mM $K^+$ and denominated as the $E_{max}$

value. The $pEC_{50}$ values represent the negative logarithm of the concentration which elicits half-maximum response. Data are expressed as mean values ± S.E.M.

**Molecular techniques**

*Real-time PCR (paper I-III)*

Total cellular RNA was extracted from each middle cerebral artery using the FastRNA Pro Green Kit following the suppliers' instructions. The resulting pellet was finally washed with ethanol, air-dried and redissolved in diethylpyrocarbonate-treated water. Reverse transcription of total RNA to cDNA was carried out using the GeneAmp RNA PCR kit in a Perkin-Elmer DNA Thermal cycler, using random hexamers as primers. Real-time PCR was performed in a GeneAmp 5700 Sequence Detection System using the GeneAmp SYBR® Green kit with the cDNA synthesized above as template. A no template control was included in all experiments. The real-time PCR consists of an optics and imaging system that is able to monitor the amount of DNA in each PCR cycle via the detection of a fluorescent dye binding to double-stranded DNA. The DNA levels of the genes of interest are compared to an endogenous standard, expressed to a constant amount. In all studies in this thesis, elongation factor-1 (EF-1) was used as an endogenous standard.

*Immunohistochemistry (paper II-V)*

Middle cerebral arteries were immersed in paraformaldehyde, frozen and subsequently sectioned into 15 µm slices (paper II) or placed onto Tissue TEK, frozen and sectioned into 10 µm slices (papers III-V). The primary antibodies against the protein of interest were added, and thereafter the secondary fluorescent antibodies. The antibodies were then detected at the appropriate wavelength in a confocal microscope. As control, only addition of secondary antibodies was used.

*ELISA (paper II)*

The arteries were homogenized in buffer with a protease inhibitor cocktail. After centrifugation, the supernatant was collected and total amount of phosphorylated ERK1/2 was measured using the BioSource International Inc. ERK1/2 [pTpY185/187] ELISA kit according to suppliers' instructions. This kit is designed to detect and quantify the level of both dual-phosphorylated ERK1 and ERK2. To quantify the activated ERK1/2 levels, total protein content in each sample was measured and the (ERK1/2)/protein ratio was used.

*Western Blot (paper III)*

The middle cerebral arteries were collected and placed on ice, homogenized in lysis-buffer with protease- and phosphatase inhibitors. After 20 minutes incubation in lysis buffer on ice, homogenates were centrifuged and the supernatant was collected. Total protein concentration was determined using a BioRad DC kit.

Lysates were dissolved in Tris-glycine SDS sample buffer and boiled for 5 minutes. Equal amounts of protein were loaded on an 8% Tris-glycine gel and separated by SDS-polyacrylamide gel electrophoresis. Molecular weight markers were loaded on each gel for protein band identification. After separation, proteins were transferred to a nitrocellulose membrane. Membranes were then incubated with primary antibodies, followed by secondary antibodies connected to horseradish peroxidase, which was detected using the Supersignal Dura kit and visualized in a Fujifilm LAS-1000 Luminiscent Image Analyzer.

**Statistics**

In paper I, data were analyzed with ANOVA and Student´s t test. In paper II-V, data were analyzed with Mann-Whitney's non-parametric test for two groups and Kruskal-Wallis test together with Dunn's post-hoc test for more than two groups.

# RESULTS AND COMMENTS

**Endothelin receptor upregulation in organ culture (paper I-III)**

*Time-course of the endothelin receptor upregulation*

In **paper I**, we examined the time-course of the endothelin receptor upregulation in cultured middle cerebral arteries. To separate the $ET_A$ and $ET_B$ receptor mediated contractions in the myographs, we first added S6c which binds to and desensitizes the $ET_B$ receptors. After thorough rinsing the artery segments were exposed to ET-1, which subsequently only interacted with the $ET_A$ receptors.

Previous studies have shown that in fresh arteries there is no $ET_B$ receptor mediated contraction. However, organ culture has been shown to induce an upregulation of contractile $ET_B$ receptors, something that was confirmed in paper I.[4, 130, 131] We demonstrated a slight contractile response to S6c already after 6 hours of organ culture, and this response was increased over time. In MCA incubated for 48 hours, the $pEC_{50}$ values were increased, pointing to a potentiation of the response (Figure 2).

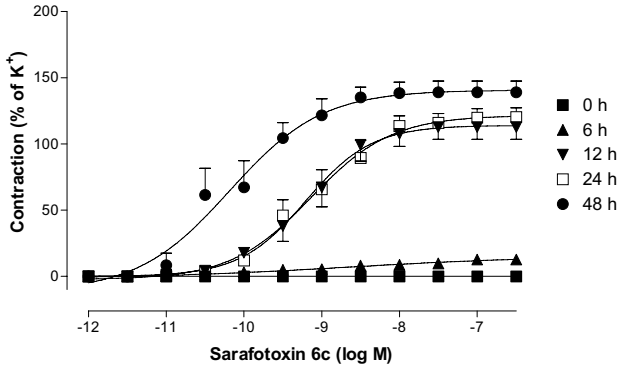


**Figure 2.** *Contractile responses to S6c after organ culture.*

The $E_{max}$ contractions induced by ET-1 did not change with organ culture. However, the $pEC_{50}$ value of the dose-response curve of MCAs cultured for 48 hours was higher. This leftward shift has been described before, although that study was conducted on basilar arteries.[130]

After 24 hours of organ culture, the $ET_B$ receptor mRNA levels were increased compared to fresh arteries, while the $ET_A$ receptor mRNA levels remained unchanged (Figure 3).

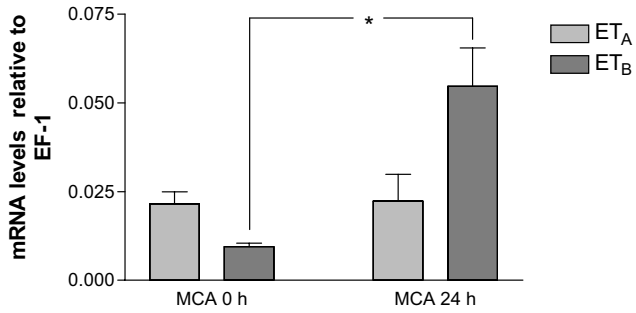


**Figure 3.** *ET receptor mRNA levels in fresh MCA and in MCA after 24 hours of organ culture.*

*Intracellular factors*

To elucidate which intracellular mechanisms that are involved in the upregulation of $ET_B$ receptors we added inhibitors to the medium before incubation. Both the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide diminished receptor upregulation considerably (paper I). Actinomycin D forms a complex with the DNA and thereby blocks the RNA polymerase from binding, while cycloheximide inhibits protein synthesis by binding to the 60S subunit of the ribosomes.[132, 133] Thus, this suggests that there is a production of new $ET_B$ receptors from gene level in organ culture, which is in accordance with previous studies performed in rat mesenteric arteries.[134]

*PKC*

The upregulation of $ET_B$ receptors in mesenteric arteries has been found to be dependent on PKC.[120] Logically, we wanted to examine if PKC was involved in the $ET_B$ receptor upregulation in the MCA as well.

In **paper I**, a general PKC inhibitor, Ro-31-8220, was added to the medium before incubation. Ro-31-8220 attenuated both the upregulated $ET_B$ receptor mRNA levels and the contractile $ET_B$ receptor mediated response seen after 24 hours of organ culture (Figure 4).

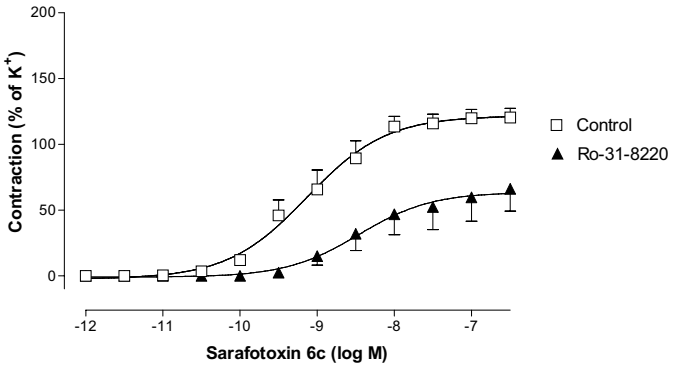


**Figure 4.** *Contractile responses to S6c after 24 hours of organ culture.*

Ro-31-8220 has been shown to inhibit not only PKC, but also other factors such as c-jun, JNK and mitogen-activated protein kinase phosphatase-1 (MKP-1).[135, 136] Consequently, there is a risk of the inhibiting effect not being solely dependent on PKC. For that reason, we decided to follow up with a more extensive study with PKC inhibitors.

In **paper III**, four different PKC inhibitors were added to the medium in which MCAs were cultured for 24 hours. The arteries were examined with myographs, immunohistochemistry, real-time PCR and Western blot. The functional responses measured in the myographs showed that in MCAs incubated with the PKC inhibitors bisindolylmaleimide I, Ro-32-0432 and PKC inhibitor 20-28 (a peptide mimicking an inactivating part of PKC[137]) the $ET_B$

receptor mediated contractions were diminished (Figure 5). The PKC inhibitors did not affect the $ET_A$ receptor mediated contractions.

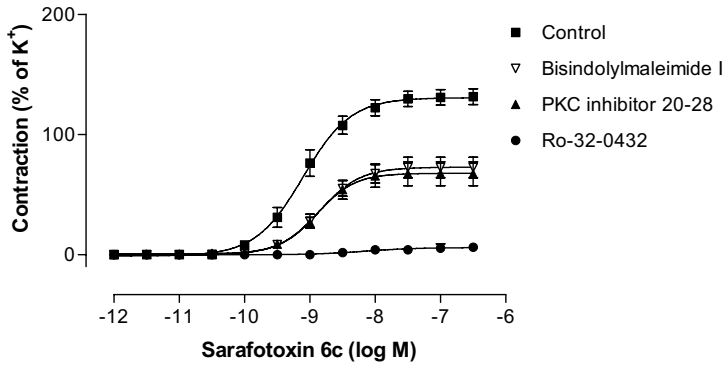


**Figure 5.** *Contractile responses to S6c after 24 hours of organ culture.*

Real-time PCR showed that Ro-32-0432 decreased both the $ET_A$ and $ET_B$ receptor mRNA levels compared to control. Bisindolylmaleimide I decreased the $ET_B$ receptor mRNA levels, and showed a tendency to decrease the $ET_A$ receptor mRNA levels, while PKC inhibitor 20-28 had no effect on the ET receptor mRNA.

On the other hand, PKC inhibitor 20-28 was shown to be the most efficient inhibitor when measuring the amount of $ET_B$ receptor protein in the arteries with immunohistochemistry (Figure 6).

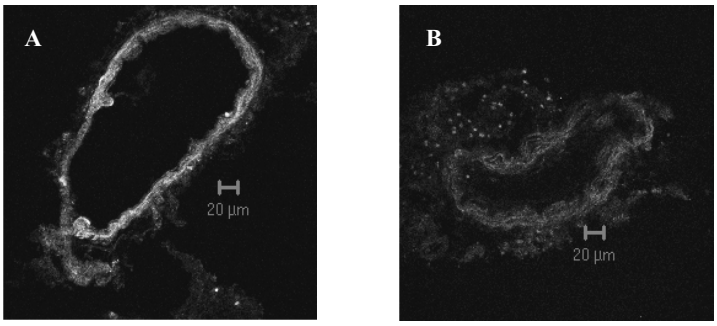


**Figure 6.** *Expression of $ET_B$ receptor protein in MCA incubated for 24 hours (A) and MCA incubated for 24 hours with PKC inhibitor 20-28 (B).*

The discrepancy between the effects of the PKC inhibitors on the mRNA level compared to protein levels can be explained in several ways. Firstly, Ro-32-0432 and bisindolylmaleimide I might exert their inhibitory effects on PKC early in the production of new contractile $ET_B$ receptors, which causes the decrease in $ET_B$ receptor mRNA. In contrast, PKC inhibitor 20-28 might not come into play until the $ET_B$ receptor production reaches the protein levels. Bisindolylmaleimide I and Ro-32-0432 did affect the $ET_B$ receptor protein levels as well, albeit marginally. However, this small decrease in receptor density in the arteries could be sufficient to diminish the functional response.

Another way to explain the discrepancy is that the effect of PKC inhibitor 20-28 is more transient than for the two other inhibitors, and after 24 hours the $ET_B$ receptor mRNA might be restored to its original levels.

Furthermore, these PKC inhibitors exhibit differences in the affinity for the PKC isoforms. Bisindolylmaleimide I inhibits the cPKCs ($\alpha$, $\beta_I$, $\beta_{II}$, $\gamma$) effectively and PKC$\delta$ and PKC$\varepsilon$ to a lesser extent.[138, 139] Ro-32-0432 primarily inhibits PKC$\alpha$, but in high concentrations Ro-32-0432 also inhibits the other isoforms of the cPKCs.[140] The PKC inhibitor 20-28 is based on a motif from the PKC$\alpha$ and $\beta$ isoforms.[137] In paper III, Western blot shows that PKC inhibitor

20-28 is able to decrease the protein amount of not only the PKCα and β isoforms, but of all five subtypes tested (α, $\beta_I$, γ, δ and ε). The decrease was most prominent in the PKCγ and δ isoforms.

Taken together, the differences in affinity could be important if there are several PKC isoforms playing a part in the $ET_B$ receptor upregulation after organ culture.

As shown in paper I, the $ET_A$ receptor mRNA levels are not altered during organ culture. Nevertheless, Ro-32-0432 diminished these levels after organ culture, without affecting the functional $ET_A$ receptor response.

*MAPK*

As mentioned above, MAPKs are activated by a range of extracellular stimuli, such as growth factors and cytokines. A previous study performed in our laboratory showed that cytokines, which also activate the p38 MAPK, are able to increase the $ET_B$ receptor mediated contractions in arteries that have undergone organ culture.[141] Furthermore, PKC is known to activate the ERK1/2 MAPK pathway.[142, 143] Thus, the involvement of MAPK in the upregulation of $ET_B$ receptors after organ culture seemed very plausible.

In **paper II** we investigated the involvement of ERK1/2 and p38 MAPK pathways in the upregulation of contractile $ET_B$ receptors after 24 hours of organ culture. The inhibitors selected for the ERK1/2 pathway were U0126, which inhibits MEK1/2, and SB386023, which inhibits raf. To inhibit p38, SB239063 was added to the culture medium.

The functional responses measured with myographs showed that in MCA incubated with the ERK1/2 pathway inhibitors, the $ET_B$ receptor upregulation was diminished (Figure 7). This was not obtained with the p38 inhibitor.

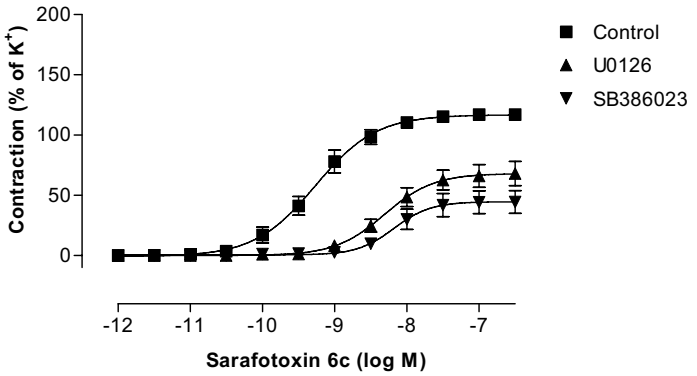


**Figure 7.** *Contractile responses to S6c after 24 hours of organ culture.*

Interestingly, the raf inhibitor SB386023 and the p38 inhibitor SB239063 enhanced the contractile responses to ET-1, pointing towards a possible functional upregulation of $ET_A$ receptors.

The real-time PCR partly confirmed our contractile experiments. U0126 and SB386023 diminished the $ET_B$ mRNA levels (although not significantly in the case of U0126). In addition SB386023 and SB239063 showed a tendency to elevate the $ET_A$ receptors mRNA levels (Figure 8). The difference between the functional $ET_A$ receptor responses and mRNA levels could simply be due to the fact that the mRNA levels were increased at an earlier stage of the organ culture and at the time point chosen had returned to their original levels.

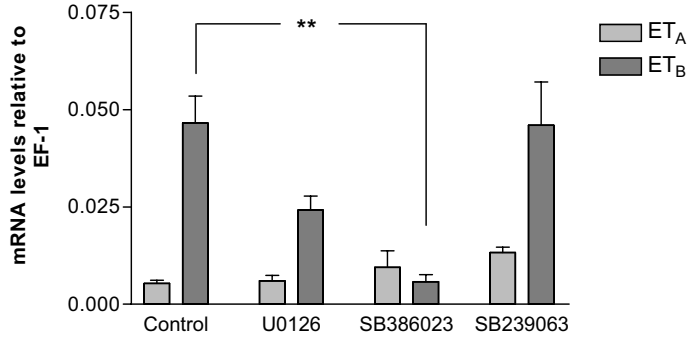


**Figure 8.** *mRNA levels of ET receptors in MCA after 24 hours of organ culture.*

The raf inhibitor SB386023 also affected the expression of $ET_B$ receptor protein on smooth muscle cells, which was established with immunohistochemistry (Figure 9).

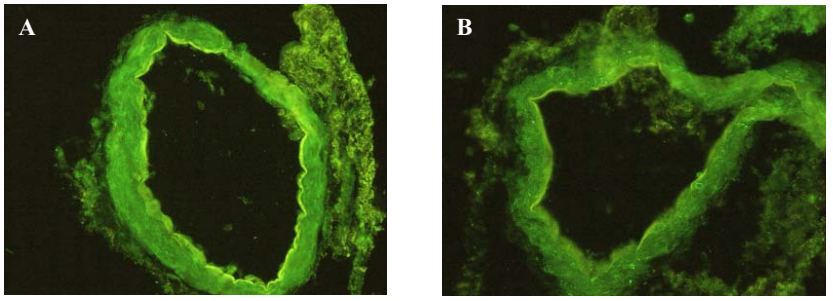


**Figure 9.** *Expression of $ET_B$ receptor protein in MCA incubated for 24 hours (A) and MCA incubated for 24 hours with raf inhibitor SB386023 (B).*

**Endothelin receptor upregulation in ischemic stroke (paper IV-V)**

*Endothelin receptor alterations*

Previous studies in our group have shown that 48 hours after transient MCAO in rat, there is an upregulation of contractile $ET_B$ receptors in the ipsilateral MCA similar to the one seen in

organ culture, although not as prominent.[3] There is also a potentiation of the angiotensin II contraction mediated by angiotensin I receptors.[144] We now wanted to examine the endothelin receptor mediated contractions 24 hours after transient MCAO.

In **paper IV**, when examining the control group, there was indeed an upregulation of contractile $ET_B$ receptors 24 hours after MCAO. The ipsilateral and contralateral MCAs were examined in myographs, and the $ET_B$ receptor mediated response in the contralateral MCA was 8% of the potassium-induced reference contractions. In the right, ipsilateral MCA, this value was 40%. In **paper V**, where the control group was treated identically to the one in paper IV, these percentages were 3% and 21%, respectively.

In paper IV, there was also an upregulation of the $ET_A$ receptor mediated contractions when compared the ipsilateral MCA and the contralateral MCA of the control group. However, this difference was not seen in the control group of paper V. The discrepancies in the endothelin receptor mediated responses between these studies may be due to relatively small groups (n = 6) and further investigations may show that there is indeed an $ET_A$ receptor upregulation, however not as pronounced as in the case of the $ET_B$ receptors. This scenario is strengthened by the study in which contractile endothelin responses were analyzed 48 hours after the occlusion. In that study, there was a tendency towards upregulation of $ET_A$ receptors in the right occluded MCA. ET-1 induced contractions for right MCA was 203% and for left MCA 160% compared to the potassium-induced reference contractions. However, this difference was not significant.[3]

*PKC*

**In paper V**, we examined the effect of the PKC inhibitor Ro-32-0432 on the endothelin receptor alterations 24 hours after transient middle cerebral artery occlusion. Ro-32-0432 was injected i.p. in conjuction with the occlusion, and as in organ culture (paper III), this inhibitor was able to diminish the upregulation of contractile $ET_B$ receptors (Figure 10). The contractile results were confirmed by immunohistochemistry, showing a lower density of $ET_B$ receptors in the right MCA of the Ro-32-0432 treated rats (Figure 11). This was accompanied by a decrease in brain damage (9% of brain volume compared to 25% in the control group) and an improvement of neurological status of the rats.

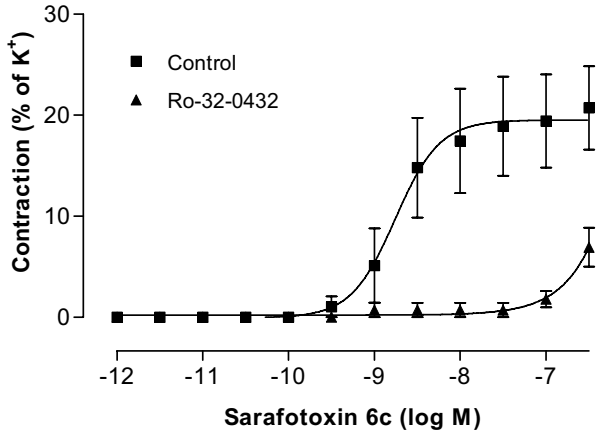


**Figure 10.** *Contractile responses to S6c in RMCA of control rats and Ro-32-0432 treated rats.*

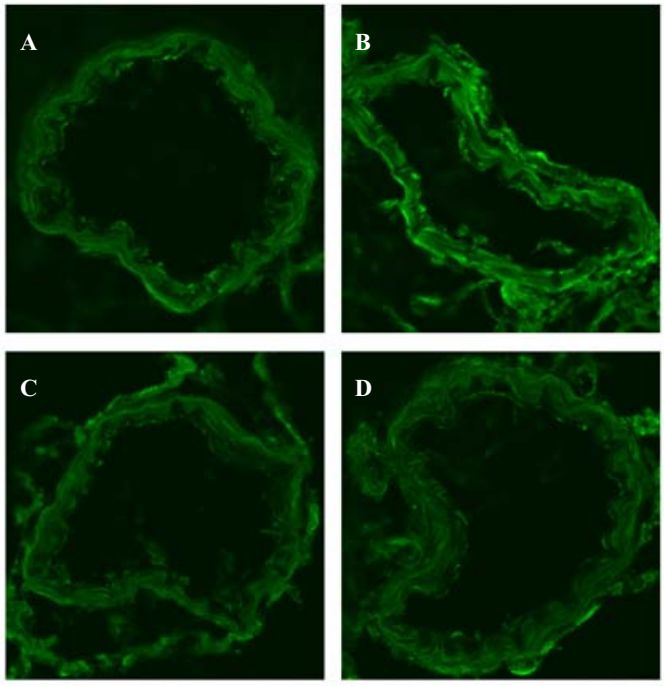


**Figure 11.** *$ET_B$ receptor protein in (A) Ro-32-0432 RMCA, (B) control RMCA, (C) Ro-32-0432 LMCA and (D) control LMCA.*

*MAPK*

In **paper IV**, we examined the effect of the MEK1/2 inhibitor U0126 in rats subjected to transient middle cerebral artery occlusion. U0126 was injected i.p in conjuction with the occlusion, and similarly to the PKC inhibitor in paper V, this inhibitor was able to diminish the upregulation of contractile $ET_B$ receptors. It also decreased the brain damage (11% of brain volume compared to 25% in the control group) and improved the neurological status of the rats significantly. Immunohistochemistry revealed a fainter staining of phosphorylated ERK1/2 protein in the U0126 treated rats compared to control rats (Figure 12).

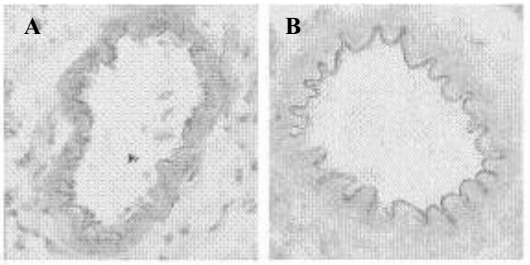


**Figure 12.** *pERK1/2 protein in (A) control RMCA and (B) U0126 RMCA.*

# MAJOR CONCLUSIONS

- Organ culture induces a time-dependent $ET_B$ receptor upregulation in middle cerebral arteries and this upregulation is due to production of new $ET_B$ receptors.

- The $ET_B$ receptor upregulation in middle cerebral arteries after 24 hours of organ culture involves both ERK1/2 MAPK and PKC.

- 24 hours after transient middle cerebral artery occlusion $ET_B$ receptors are upregulated in the ipsilateral middle cerebral artery.

- The $ET_B$ receptor upregulation 24 hours after transient middle cerebral artery occlusion is attenuated by treatment with a MEK1/2 MAPK inhibitor. The treatment also results in improved neurological status and diminished brain damage area.

- The $ET_B$ receptor upregulation 24 hours after transient middle cerebral artery occlusion is attenuated by treatment with a PKC inhibitor. The treatment also results in improved neurological status and diminished brain damage area.

# SVENSK SAMMANFATTNING (SWEDISH SUMMARY)

**Bakgrund**

Stroke är en av våra största folksjukdomar och den tredje vanligaste dödsorsaken efter hjärtinfarkt och cancer i Sverige. Ungefär 90% av alla stroke-fall beror på hjärninfarkt, det vill säga en blodpropp som täpper till ett blodkärl i hjärnan. I det mest centrala området av infarkten dör hjärnans celler på grund av syrebrist. Runtom kärnan i infarkten finns ett område kallat penumbran där syrebrist råder, dock inte lika påtaglig som i kärnan. Hjärncellerna i penumbran kan därmed under gynnsamma omständigheter överleva. Mycket tyder på att blodkärl i närheten av infarkten drar ihop sig, vilket försämrar syretillförseln till penumbran ytterligare.

Kroppens blodkärl har till uppgift att transportera syre och näring till kroppens alla celler, samt forsla bort slaggprodukter. Blodkärlen kan ändra diameter genom att dra ihop sig eller vidgas och därmed påverka blodtryck och flöde. Detta är ett effektivt sätt för kroppen att reglera blodflödet ut till de minsta kärlen, kapillärerna, där näringsutbytet äger rum. Blodkärl består av tre lager; det yttre bindvävslagret som ger kärlet stadga, innanför det ett lager med muskulatur och längst in ett lager av celler som fungerar som en barriär och transportör av ämnen mellan blodet och vävnaden. Musklerna i blodkärlen kan inte kontrolleras viljemässigt, utan styrs av nervsignaler samt cirkulerande substanser i blodet som binder till mottagarstrukturer på muskelcellernas yta. Dessa mottagare, så kallade receptorer, är specifika för varje substans och antalet kan förändras vid sjukdom.

En av dessa cirkulerande substanser är endotelin-1. Vi vet att i stroke stiger nivåerna av endotelin-1 i blodet. Endotelin-1 bildas i endotelcellerna i blodkärl och substansen binder till två receptorer, $ET_A$ och $ET_B$, på muskelcellerna vilket resulterar i en långvarig och stark sammandragning av kärlet. I normala fall medieras kontraktion av hjärnans artärer av $ET_A$-receptorer, men vi har tidigare visat att efter stroke hos råttor finns det även $ET_B$- receptorer på muskelcellerna, vilket skulle kunna ge upphov till en mer potent sammandragning av blodkärlet och därmed sämre syretillförsel till hjärnan.

**Syfte med avhandlingen**

Syftet med min avhandling är att klargöra vissa av de sjukliga förändringar i hjärnans blodkärl efter stroke. Avhandlingen består av fem delstudier, där vi har undersökt uppkomsten av $ET_B$-receptorer på muskelcellerna i hjärnans blodkärl. Vi har också försökt utröna de bakomliggande orsakerna till denna ökning av $ET_B$-receptorer.
En ökad förståelse för bakomliggande mekanismer till denna förändring i hjärnans blodkärl kan leda till nya sätt att behandla strokepatienter.

**Metoder**

För att undersöka vad som ligger bakom uppkomsten av $ET_B$-receptorer på muskelcellerna har vi använt en metod där blodkärl odlas i 37° C, varvid bl.a. förändringar i receptoruppsättning sker. Vi har också inducerat stroke i råttor för att undersöka detta.

**Resultat**

I **studie I** odlades blodkärl i 37° C, varefter deras kontraktila (sammandragande) förmåga undersöktes. Vi såg att precis som vid stroke ökar antalet $ET_B$-receptorer i hjärnans artärer efter organkultur i minst 12 timmar. Denna uppreglering sker på gennivå, dvs. genen för $ET_B$-receptorn aktiveras. Exakt hur cellsignaleringen som leder till produktion av receptorer går till vet man inte, men i studie I redovisar vi resultat som pekar på att en speciell typ av enzym, sk proteinkinas C, är involverat i signalkaskaden. I **studie III** bekräftade vi involveringen av proteinkinas C genom att tillsätta specifika hämmare mot proteinkinas C till blodkärlen innan de odlades i 24 timmar. Detta resulterade i starkt hämmade $ET_B$-receptorkontraktioner i kärlen. I **studie V** visade vi att proteinkinas C-blockad ger en minskad hjärnskada hos råttor där stroke inducerats. Även här är $ET_B$-receptorkontraktionerna i kärlen minskade.

Proteinkinas C kan aktivera en annan molekyl i cellerna, sk mitogen-activated protein kinase, förkortat MAPK. I **studie II** undersöktes om blockad av MAPK gav lägre $ET_B$-receptorkontraktioner i odlade kärl, vilket det visade sig göra. Slutligen i **studie IV** injicerades råttor med en MAPK-hämmare i samband med att stroke inducerades. Detta minskade både hjärnskadorna och $ET_B$-receptorkontraktionerna hos djuren.

**Slutsats**

Eftersom liknande $ET_B$-receptorförändringar sker vid såväl organkultur som vid stroke kan denna avhandling ge ledtrådar till vilka bakomliggande faktorer som orsakar förändringarna även vid stroke hos människa. Avhandlingen visar att organkultur såväl som stroke kan orsaka en ökning av kontraktila $ET_B$-receptorer i hjärnans blodkärl. Vi har också funnit signalvägar som är involverade i detta skeende.

Resultaten kan på lång sikt leda till nya behandlingsmetoder, med läkemedel som motverkar orsakerna till den ökade nivån av dessa receptorer. Det skulle förhoppningsvis minska syrebristen och göra att fler hjärnceller överlever efter stroke.

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