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## Vascular receptor changes in ischemic stroke

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Experimental Vascular Research  
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# Vascular Receptor Changes in Ischemic Stroke

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Doctoral thesis



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

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



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**PAPER I-VI**

## 1. ORIGINAL ARTICLES

This thesis is based on the following papers:

- I. Stenman E, Malmjö M, Uddman E, Gidö G, Wieloch T, Edvinsson L. Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. *Stroke*. 2002;33:2311-2316
- II. Henriksson M, Stenman E, Edvinsson L. Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat. *Stroke*. 2003;34:1479-1483
- III. Stenman E, Edvinsson L. Cerebral ischemia enhances the vascular angiotensin AT<sub>1</sub> receptor mediated contraction in rat. *Stroke*. 2004;35:970-974
- IV. Xu CB, Stenman E, Edvinsson L. Reduction of bFGF-induced smooth muscle cell proliferation and endothelin receptor mRNA expression by mevastatin and atorvastatin. *Biochem Pharmacol*. 2002;64:497-505
- V. Stenman E, Henriksson M, Vikman P, Edvinsson L. Impact of cytokines and growth factors on contractile endothelin responses in rat cerebral arteries. *Submitted manuscript*.
- VI. Stenman E, Henriksson M, Edvinsson L. Low dose inhibition of AT<sub>1</sub> receptors decreases ischemic brain damage in rat. *Submitted manuscript*.

## 2. ABBREVIATIONS

ACE	angiotensin converting enzyme
Ang II	angiotensin II
ANOVA	analysis of variance
AP-1	activator protein-1
ARAP1	type 1 angiotensin II receptor associated protein 1
ATBP50	AT <sub>2</sub> receptor binding protein of 50 kDa
ATP	adenosine triphosphate
ATRAP	angiotensin II type 1 receptor-associated protein
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
Ca <sup>2+</sup>	calcium ion
cAMP	3',5'-cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer-binding protein
cGMP	3',5'-cyclic guanosine monophosphate
Cl <sup>-</sup>	chloride ion
CO <sub>2</sub>	carbon dioxide
C-terminal	carboxyl group terminal
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's Medium
ECE	endothelin converting enzyme
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunoabsorbant assay
ERK 1/2	extra-cellular signal regulated kinases 1 and 2
ET-1	endothelin-1
F	phenylalanine
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G-protein	guanine nucleotide binding protein
H	histidine
I	isoleucine
Il-1β	interleukin-1β

IP <sub>3</sub>	inositol triphosphate
K <sup>+</sup>	potassium ion
kDa	kilo Dalton
L	leucine
M	mol/liter
MAP	mean arterial blood pressure
MAP kinase	mitogen-activated protein kinase
MCA	middle cerebral artery
mRNA	messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
N	asparagine
Na <sup>+</sup>	sodium ion
NF-κB	nuclear factor- κB
NO	nitric oxide
N <sub>2</sub> O	nitrous oxide (laughing gas)
N-terminal	amino group terminal
O <sub>2</sub>	oxygen
P	proline
PBS	phosphate-buffered saline
pCO <sub>2</sub>	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PKC	protein kinase C
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PLC	phospholipase C
PLD	phospholipase D
pO <sub>2</sub>	partial pressure of oxygen
R	arginine
RNA	ribonucleic acid
S6c	sarafotoxin 6c
SAH	subarachnoid hemorrhage
SD	standard deviation
SEM	standard error of the mean
TNF-α	tumor necrosis factor- α
TTC	2,3,5-triphenyltetrazolium chloride
U	units
V	valine
VEGF	vascular endothelial growth factor
WHO	World Health Organization
Y	tyrosine



### 3. INTRODUCTION

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

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

#### 3.1. Pathophysiology of ischemic stroke

##### *3.1.1. Ischemic stroke and the penumbra*

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

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

### *3.1.2. Vascular pathophysiology of ischemic stroke*

The vascular pathophysiology of ischemic stroke can be divided into three separate phases. The acute phase (hours) includes a disturbed vascular tonus<sup>8</sup> along with disruption of the blood-brain barrier, partly due to generation of oxygen radicals<sup>9</sup> and an excessive production of ET-1.<sup>10-12</sup> In the subacute phase of ischemic stroke (hours to days), proinflammatory genes like tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  and transcription factors like nuclear factor (NF)- $\kappa$ B are activated, products that can stimulate expression of adhesion molecules and thereby disturb the endothelial integrity.<sup>8</sup> Moreover, factors with angiogenic properties like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) are excessively expressed in the subacute phase.<sup>13-15</sup> Finally, in the chronic phase (days to months), genes involved in the regulation of apoptosis and angiogenesis are induced.<sup>8</sup> The studies in this thesis examined vascular events mainly in the subacute phase of ischemic stroke. Therefore the impact of cytokines and growth factors on arterial receptor regulation was of particular interest and two of the included studies are dealing with this subject.

## **3.2. Endothelin**

### *3.2.1. Endothelin-1; homologous peptides and production*

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

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

The mRNA product of the gene encoding ET-1 is a precursor of ET-1 called preproendothelin (212 amino acids).<sup>20</sup> This is further converted in two steps to bigET-1, which possesses some biological activity.<sup>21</sup> BigET-1 is finally cleaved to ET-1 by endothelin converting enzymes (ECEs).<sup>22, 23</sup> ET-1 has later been shown to be produced not only by endothelial cells, but by various cell types, for example by macrophages,<sup>24</sup> vascular adventitial fibroblasts,<sup>25</sup> epithelial cells<sup>26</sup> and neurons,<sup>27</sup> and the production of ET-1 can be regulated by a number of factors such as shear stress,<sup>28</sup> thrombin<sup>29</sup> and angiotensin II (Ang II)<sup>30</sup> (interestingly, ET-1 has the ability to stimulate angiotensin converting enzyme (ACE), which produces Ang II,<sup>31</sup> suggesting a reciprocal influence between the endothelin and angiotensin systems). In addition, increased levels of ET-1 have been reported in both plasma and cerebrospinal fluid after ischemic stroke<sup>12, 32</sup> as well as in focal ischemic tissue.<sup>33</sup>

### 3.2.2. Endothelin receptors

Two endothelin receptor subtypes have been described in mammals, the endothelin ET<sub>A</sub> and ET<sub>B</sub> receptors.<sup>34</sup> In addition the existence of a combined endothelin and angiotensin receptor in rat brain has been suggested.<sup>35</sup> However, this receptor will not be further discussed in the present thesis. The ET<sub>A</sub> and ET<sub>B</sub> receptors belong to the seven transmembrane G-protein coupled receptor family,<sup>36, 37</sup> and both receptors can connect to the G<sub>q</sub>-protein.<sup>38</sup> Thereby they activate phospholipase C (PLC) which releases inositol triphosphate (IP<sub>3</sub>) with a subsequent increase of intracellular calcium. PLC also releases diacylglycerol (DAG), which in turn can activate protein kinase C (PKC).<sup>39, 40</sup> In addition, ET<sub>A</sub> receptors are suggested to stimulate production of cAMP via interaction with G<sub>s</sub>-proteins, while ET<sub>B</sub> receptors are believed to inhibit cAMP accumulation via G<sub>i</sub>-proteins.<sup>41</sup> ET-1 binds to both the ET<sub>A</sub> and ET<sub>B</sub> receptors with high affinity,<sup>42</sup> although the pharmacological effects differ between the receptors. Both receptor subtypes are expressed in vascular smooth muscle cells as well as in endothelial

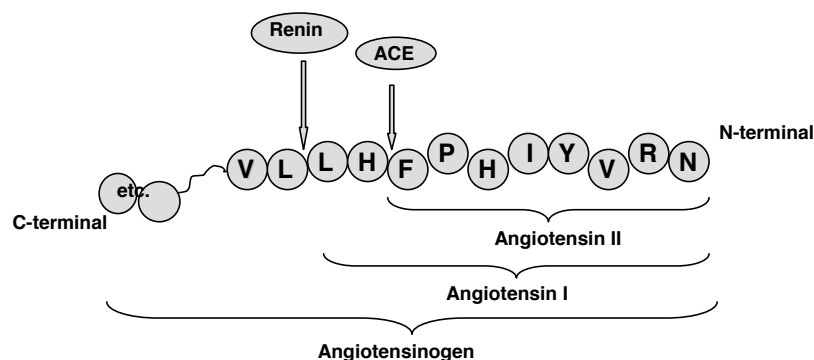
cells,<sup>43, 44</sup> though the ET<sub>A</sub> receptors are normally mediating the contractile effect of ET-1 on the arterial side of the circulation via release of intracellular calcium and activation of PKC,<sup>39, 45-47</sup> while ET<sub>B</sub> receptors located on arterial endothelial cells mediate vasodilatation via release of nitric oxide (NO).<sup>48</sup> However, an upregulation of contractile ET<sub>B</sub> receptors has previously been reported in pathological conditions like atherosclerosis<sup>49</sup> and experimental SAH.<sup>50</sup> In the present thesis, we show that experimental ischemic stroke induces an ET<sub>B</sub>-receptor mediated contraction as well (paper I). An established model for studying ET<sub>A</sub> and ET<sub>B</sub> receptor regulation is organ culture (described in the methodology chapter). Organ culture of human cerebral arteries has been shown to increase the ET<sub>A</sub> receptor mediated contraction,<sup>51</sup> while an upregulation of contractile ET<sub>B</sub> receptors after organ culture has been demonstrated in many other arterial systems like human omental,<sup>52</sup> rat mesenteric<sup>53</sup> and rat basilar arteries.<sup>54</sup> A number of factors have been suggested to affect the endothelin receptor regulation. Glucocorticoids can for example decrease the expression of ET<sub>A</sub> receptors.<sup>55</sup> Some cytokines have in turn been shown to increase the ET<sub>B</sub> receptor mediated contraction<sup>56</sup> and shear stress can increase the production of ET<sub>B</sub> receptor mRNA.<sup>57</sup> In addition, autocrine ET-1 has the ability to downregulate its own receptors,<sup>58</sup> which may reflect a negative feedback system in the cell. Intracellular factors involved in the expression of endothelin receptors are among others, PKC and extra-cellular signal regulated kinases 1 and 2 (ERK1/2) which are crucial for the upregulation of ET<sub>B</sub> receptors in organ culture models.<sup>59, 60</sup> Although much work has been performed on the subject of endothelin receptor regulation, the phenomenon remains elusive and requires further research.

### 3.3. Angiotensin

#### 3.3.1. Angiotensin II, production

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

route of mechanisms; angiotensinogen is cleaved by the enzyme renin to form a decapeptide called angiotensin I (Ang I). Ang I is in turn converted to Ang II by ACE (Figure 3.1).<sup>65</sup> However, there are alternative pathways for the synthesis of Ang II. Ang I can also be converted to Ang II by a chymotrypsin-like serine protease (chymase)<sup>63</sup> and chymostatine-sensitive Ang II-generating enzyme (CAGE).<sup>66</sup> Such alternative pathways are believed to cause the phenomenon “escape”, which can moderate the physiological effects of ACE-inhibitors.<sup>67</sup> A local production of Ang II is believed to occur throughout the body. Tissue ACE has been found in several major organs, such as heart,<sup>68</sup> brain,<sup>69</sup> blood vessels<sup>70</sup> and kidneys,<sup>71</sup> and previous studies suggest that locally produced Ang II is of great importance for the arterial contraction, possibly even greater than circulating Ang II.<sup>72, 73</sup> Factors known to affect the local renin-angiotensin system activity are among others oxidative stress, which activates tissue ACE,<sup>74</sup> hypercholesterolemia, which increases the expression of ACE and the angiotensin AT<sub>1</sub> and AT<sub>2</sub> receptors<sup>75</sup> and shear stress, which reduces the local ACE activity.<sup>76</sup> As can be seen in the present thesis, paper III, experimental focal ischemia increased the vascular ACE mRNA levels locally, which may suggest an increased production of Ang II after ischemic stroke.



**Figure 3.1** Formation of Ang II.<sup>77</sup>

### 3.3.2. Angiotensin receptors

The effects of Ang II are mainly mediated by two different membrane-bound receptors in mammals, the angiotensin AT<sub>1</sub> and AT<sub>2</sub> receptors.<sup>78</sup> Two additional receptors have been described, AT<sub>3</sub> and AT<sub>4</sub>. The AT<sub>3</sub> receptor recognizes angiotensin II and has so far only been observed in cell lines and AT<sub>4</sub> binds the angiotensin II-derived fragment angiotensin IV.<sup>79, 80</sup> The AT<sub>3</sub> and AT<sub>4</sub> subtypes will not be further discussed in this thesis. In humans a single AT<sub>1</sub>

receptor type has been found, whereas two subtypes have been described in rodents, AT<sub>1a</sub> and AT<sub>1b</sub>, with more than 95 % homology and similar pharmacological properties.<sup>81</sup> Regarding the AT<sub>2</sub> receptor, no subtypes have been described.<sup>65</sup> AT<sub>1</sub> and AT<sub>2</sub> are, like the endothelin receptors, of the seven transmembrane G-protein coupled receptor type<sup>82, 83</sup> exhibiting almost opposing effects. The AT<sub>1</sub> receptor mediates vasoconstriction and cell proliferation, stimulates drinking behaviour and induces vasopressin release, while the AT<sub>2</sub> receptor mediates vasodilatation and apoptosis, inhibits cell growth and prevents thirst.<sup>84-87</sup> Thus, the AT<sub>1</sub> receptor is mediating the “classical” actions of angiotensin II<sup>88</sup> and stimulates a number of intracellular pathways. Via interaction with G<sub>q</sub>-proteins it activates PLC with subsequent release of IP<sub>3</sub>, which mobilizes calcium from intracellular stores. This increase in intracellular calcium can result in vascular smooth muscle cell contraction. PLC also stimulates DAG, which in turn activates PKC.<sup>64, 89</sup> In addition, AT<sub>1</sub> stimulates the phospholipases PLD and PLA<sub>2</sub>, which results in activation of PKC, release of arachidonic acid, which is metabolized to eicosanoids, and activation of mitogen-activated protein (MAP) kinases.<sup>64, 90</sup> Furthermore, AT<sub>1</sub> activates receptor tyrosine kinases and small G-proteins.<sup>64</sup> While the AT<sub>2</sub> receptor is the dominating angiotensin receptor type in fetal tissues, its expression in adults is limited to certain cells and tissues like vascular endothelial cells and specialized nuclei in the brain.<sup>88, 91</sup> The intracellular signalling pathways of the AT<sub>2</sub> receptors are in many ways different from the AT<sub>1</sub>-induced pathways.<sup>88</sup> The phosphorylation cascades induced by factors like Ang II via AT<sub>1</sub> are counteracted by phosphatase activation mediated by AT<sub>2</sub>, and inactivation of the MAP kinases ERK1/2 seems to be one key event in the AT<sub>2</sub> signaling.<sup>88, 92</sup> AT<sub>2</sub> is also believed to signal via activation of the NO/cGMP system and via stimulation of PLA<sub>2</sub>.<sup>93</sup> The expression of angiotensin receptors can be affected by a number of factors. Ang II has been shown to exert negative feedback by downregulating AT<sub>1</sub> receptor mRNA and protein in vascular smooth muscle cells.<sup>94</sup> Furthermore, an increased amount of AT<sub>1</sub> receptors has been observed in the neointima formed after arterial injury,<sup>95</sup> and elevated mRNA levels for vascular AT<sub>1</sub> and AT<sub>2</sub> receptors have been demonstrated in spontaneously hypertensive rats as compared to normotensive animals.<sup>96</sup> In addition, endothelin seems to be involved in the increase in AT<sub>1</sub> receptor mRNA seen in experimental cardiac heart failure.<sup>97</sup>

### 3.3.3. Angiotensin receptor associated proteins

After activation, the AT<sub>1</sub> receptors are internalized by the angiotensin II type 1 receptor-associated protein (ATRAP).<sup>98</sup> The receptors are thereafter recycled by another protein called

type 1 angiotensin II receptor associated protein 1 (ARAP1).<sup>99</sup> Recently, a corresponding AT<sub>2</sub> receptor associated protein, ATBP50 (for AT<sub>2</sub> receptor binding protein of 50 kDa), was discovered and shown to be necessary for AT<sub>2</sub> cell surface expression.<sup>100</sup> In an ongoing study we are examining how experimental ischemic stroke affects the mRNA levels for the two AT<sub>1</sub> receptor associated proteins and the preliminary results will be described in the results and comments section below.

#### **4. AIMS OF THE THESIS**

- To examine how experimental ischemic stroke affects expression and function of vascular endothelin and angiotensin receptors in rat cerebral arteries.
- To study the time course of endothelin receptor regulation in rat cerebral arteries after organ culture.
- To examine the impact of growth factors and cytokines on endothelin receptor regulation in cultured rat cerebral arteries and vascular smooth muscle cells from rat brain.
- To examine the effect of AT<sub>1</sub> receptor inhibition in the acute phase of experimental ischemic stroke.



## 5. GENERAL METHODS

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

### 5.1. Induction of transient focal cerebral ischemia in rat

Transient focal cerebral ischemia was induced in rats by an intraluminal filament technique, originally described by Koizumi *et al.*,<sup>101</sup> and later modified by Memezawa *et al.*<sup>102</sup> In the model, the entrance of the right MCA is occluded by a filament as described below. Male Wistar rats were housed under controlled temperature and humidity with free access to water and food. In paper I and II, the animals were fasted overnight with access to water immediately before operation. Anesthesia was induced using 4.5 % halothane in N<sub>2</sub>O:O<sub>2</sub> (70 %:30 %). Thereafter, the rats were kept anesthetized by artificial inhalation (paper I) or through a mask (paper II and VI) with 1.5 % halothane. A polyethylene catheter was inserted into a tail artery for measurements of mean arterial blood pressure (MAP), pH, pO<sub>2</sub>, pCO<sub>2</sub>, and plasma glucose. A rectal temperature probe connected to a homeothermal blanket was inserted for maintenance of a body temperature about 37° C during the operational procedure. A skin incision was made in the midline of the neck and the right common, external and internal carotid arteries were exposed. The common and external carotid arteries were permanently ligated by sutures. A filament was inserted into the internal carotid artery via an incision in the common carotid artery, and further advanced until the rounded tip reached the entrance of the right MCA. In paper VI, the MCA occlusion was verified by laser-Doppler flowmetry. In this case, a probe was fixed on the skull (1 mm posterior to the bregma and 6 mm from the midline on the right side), measuring regional blood flow in an area supplied by the right MCA (Perimed, Sweden). Then the resulting occlusion was made visible by a

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

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

**5.2. Neurological evaluation**

The animals were examined neurologically by an established scoring system described in the table below.<sup>103, 104</sup>

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

**5.3. Morphological evaluation of brain damage**

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

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

#### **5.4. Organ culture procedure**

The use of organ culture for studying arterial receptor changes has previously been described by Adner and colleagues.<sup>106</sup> Male Wistar rats were anesthetized with CO<sub>2</sub>, and decapitated. The brains were removed and immediately chilled in cold bicarbonate buffer solution. The right and left MCAs were removed and incubated in Dulbecco's modified Eagle's Medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 µl/ml) and amphotericin B (25 µg/ml) at 37° C in humidified 5 % CO<sub>2</sub> in air. In experiments with cytokines, growth factors or inhibitors of intracellular factors, these substances were added to the medium in the beginning of the culture.

#### **5.5. Cell culture procedure**

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

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

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

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

## **5.6. Functional studies by myographs**

Mulvany-Halpern myographs were used for measuring contractile properties of cerebral arteries.<sup>107, 108</sup> The arteries were cut into cylindrical segments and the endothelium was removed mechanically by rubbing the luminal side with a thread. Two thin wires were then inserted into the segments for mounting in temperature controlled (37° C) myographs containing a bicarbonate buffer solution. One wire was connected to a force transducer attached to an analogue-digital converter unit. The other wire was attached to a movable displacement device to allow for fine adjustments. The experiments were recorded on a computer by the software program Chart<sup>TM</sup>. The arterial segments were given an initial tension of 1.2 mN, and were allowed to stabilize at this tension for one hour. Thereafter, the contractile capacity was determined by exposure to a 63.5 mM K<sup>+</sup> buffer solution, which causes smooth muscle cell contraction via membrane depolarization and subsequent Ca<sup>2+</sup> entry via voltage-operated Ca<sup>2+</sup>-channels.<sup>109</sup> This K<sup>+</sup>-evoked contraction was used as reference in the myograph experiments.

Concentration-response curves were obtained by addition of cumulative concentrations of the agonists studied. In order to measure the ET<sub>A</sub> receptor mediated response specifically, the response to ET-1 was recorded 30 minutes after the response to S6c in the same segments. The segments were rinsed thoroughly between the substances. In this way S6c desensitized the ET<sub>B</sub> receptors and the following contraction towards ET-1 was mediated entirely by ET<sub>A</sub> receptors.<sup>110</sup> The vascular contractile responses are expressed as percentage of the K<sup>+</sup>-elicited response. E<sub>max</sub> represents the maximum contraction induced by an agonist and the pEC<sub>50</sub> value refers to the negative logarithm of the concentration eliciting half the maximal response.

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

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

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

The amount of mRNA in each sample was calculated relative to the amount of mRNA for the endogenous standard in the same sample by the formula  $X_0/R_0 = 2^{CtR-CtX}$ , where X<sub>0</sub> is the original amount of target mRNA, R<sub>0</sub> is the original amount of mRNA for the endogenous standard, CtX is the C<sub>T</sub> value for the target and CtR is the C<sub>T</sub> value for the endogenous standard. The C<sub>T</sub> values refer to the number of PCR cycles performed for each PCR product in a sample at a specific point of time.

### **5.8. Immunohistochemistry**

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

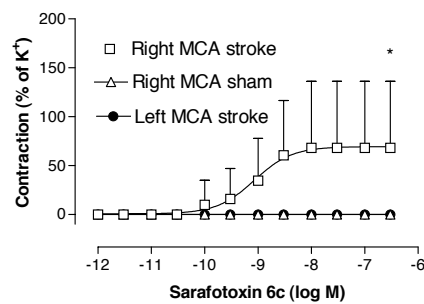
### **5.9. Statistics**

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

6. RESULTS AND COMMENTS

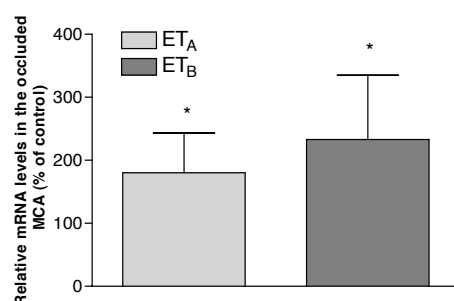
6.1. The endothelin system

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



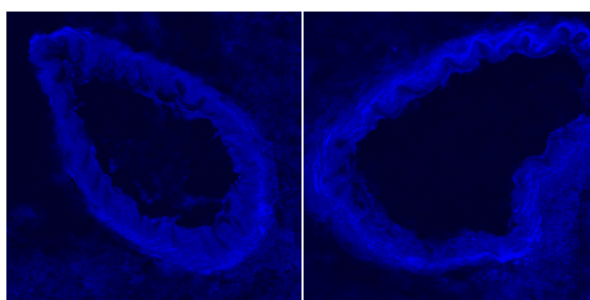
**Figure 6.1** Ischemic stroke induced a contractile ET<sub>B</sub> receptor mediated response in the occluded MCA. Data are expressed as mean ± SD.

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



**Figure 6.2** The ET<sub>A</sub> and ET<sub>B</sub> receptor mRNA levels were increased in the right occluded MCA as compared to the left MCA (control) 48 hours after onset of ischemic stroke. Data are expressed as mean  $\pm$  SD.

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

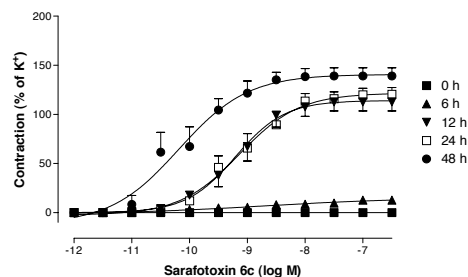


**Figure 6.3** ET<sub>B</sub> receptor expression (blue) in cross sections of the left non-occluded (left) and right occluded (right) MCA 48 hours after MCA occlusion.



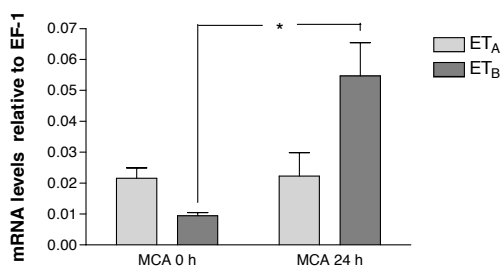
Increased circulating and tissue ET-1 levels have previously been reported after ischemic stroke.<sup>12, 32, 33</sup> These findings together with the present results suggest that ischemic stroke induces a general activation of the endothelin system. The question whether an upregulation of ET<sub>B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

To further analyze the manner of ET<sub>B</sub> receptor upregulation in rat cerebral arteries, we employed the organ culture model in paper II. The aim was to study the time course of endothelin receptor regulation *in vitro* and to examine intracellular mechanisms responsible for the regulation. As mentioned above, the ET<sub>B</sub> receptor agonist S6c did not evoke any contractile responses in fresh MCAs. However, already after 6 hours organ culture, a slight ET<sub>B</sub> receptor mediated contraction was detected, and after 12 hours culture S6c induced a potent contraction. After 48 hours culture the dose-response curve was further shifted to the left with a significantly increased pEC<sub>50</sub> value (Figure 6.4). The E<sub>max</sub> for ET-1 was not significantly affected by the organ culture, although the pEC<sub>50</sub> value was increased after 48 hours culture as compared to fresh arteries. A similar leftward shift for the ET-1 dose-response curve after 48 hours culture, has previously been described in the rat basilar artery, although the shift was even more pronounced.<sup>117</sup>



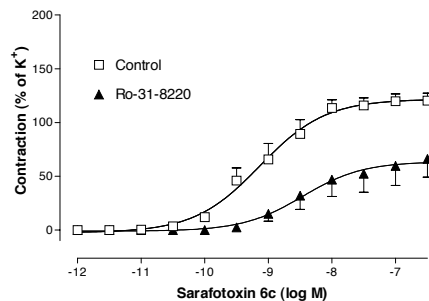
**Figure 6.4** Contractile responses to S6c after different incubation periods.

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



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

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

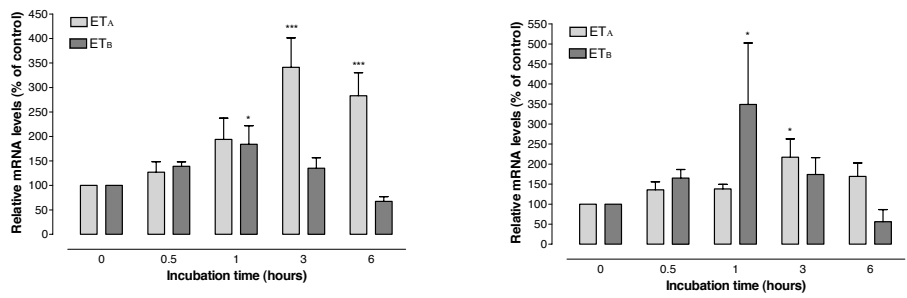


**Figure 6.6** Incubation with the PKC inhibitor Ro-31-8220 attenuated the ET<sub>B</sub> receptor mediated contraction.

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

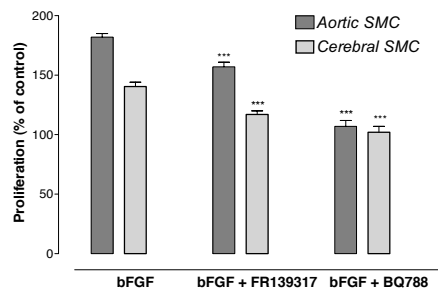
In paper IV, we aimed to analyze the impact of the growth factor bFGF and lipid-lowering statins on cell proliferation and endothelin receptor regulation in rat vascular smooth muscle cells. The main purpose was to elucidate possible mechanisms involved in atherosclerosis. However, the subject is also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

smooth muscle cells than in cerebral, whereas the effect on the ET<sub>B</sub> receptors was the opposite (Figure 7).



**Figure 6.7** Incubation with bFGF increased the mRNA levels for ET<sub>A</sub> and ET<sub>B</sub> in aortic (left) and cerebral (right) smooth muscle cells.

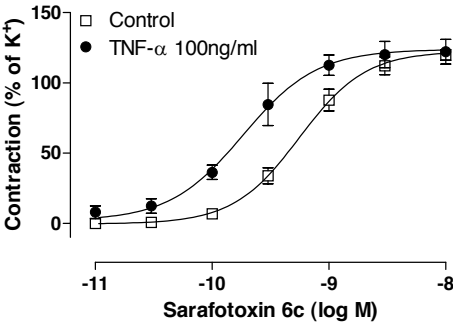
The increase in endothelin receptor mRNA was attenuated by statins. However the effect did not reach significance in cerebral smooth muscle cells. In addition to the effect of bFGF on endothelin receptor mRNA expression, the endothelin receptors turned out to be involved in bFGF-induced smooth muscle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



**Figure 6.8** Both the ET<sub>A</sub> receptor antagonist FR139317 and the ET<sub>B</sub> receptor antagonist BQ788 attenuated bFGF-induced proliferation (SMC; smooth muscle cells).

The results of paper IV regarding bFGF-induced endothelin receptor expression, together with previous findings that inflammatory cytokines<sup>54, 56</sup> can increase the contractile ET<sub>B</sub> receptor mediated response aroused our curiosity about the impact of cytokines and growth factors on

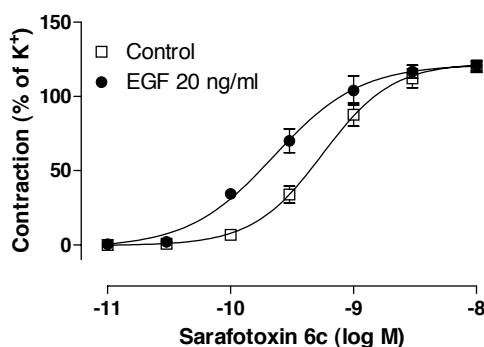
endothelin receptor regulation in the rat MCA. Since both inflammation and angiogenesis are common features of cerebral ischemia,<sup>3, 127</sup> we hoped to find possible mechanisms for the ischemia-induced increase in endothelin receptor expression and function seen in paper I. The aim of paper V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



**Figure 6.9** Organ culture with TNF- $\alpha$  significantly potentiated the ET<sub>B</sub> receptor mediated contractile response in the rat MCA.

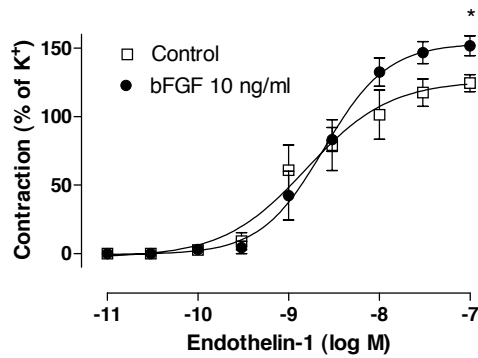
Incubation with EGF yielded similar results as incubation with TNF- $\alpha$ . EGF

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



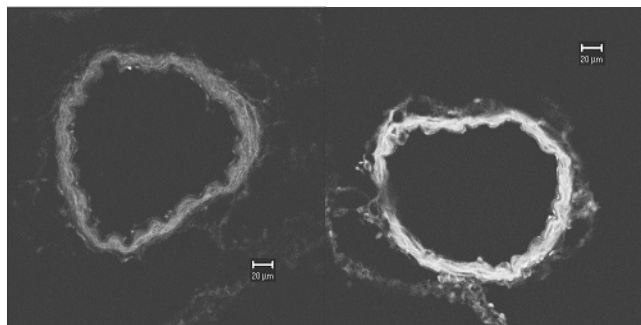
**Figure 6.10** Organ culture with EGF significantly potentiated the  $ET_B$  receptor mediated contraction in the rat MCA.

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



**Figure 6.11** Organ culture with bFGF increased the maximal contraction mediated by ET<sub>A</sub> receptors in the rat MCA.

Interestingly, the ET<sub>A</sub> receptor mRNA levels were not affected by bFGF, whereas the ET<sub>B</sub> receptor mRNA levels and protein expression were increased (Figure 6.12).



**Figure 6.12** Immunohistochemistry photographs showing ET<sub>B</sub> receptors in cross sections of middle cerebral arteries. Left: MCA control, right: MCA incubated with bFGF.

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

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

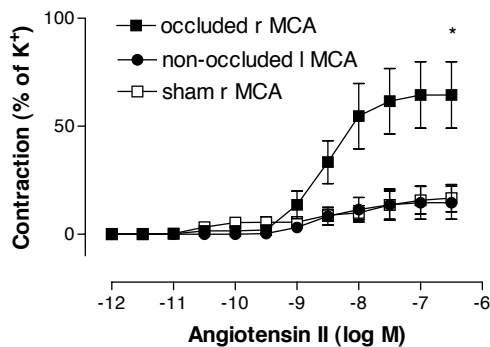
Since growth factors and cytokines are released in the subacute phase of cerebral ischemia,<sup>8, 13-15</sup> it is possible that they contribute to the induction of contractile ET<sub>B</sub> receptors seen 48 hours after MCA occlusion. Thus, let us continue to draw the picture over possible mechanisms for induction of contractile ET<sub>B</sub> receptors after ischemic stroke: In paper II, we demonstrated that the organ culture induced upregulation of ET<sub>B</sub> receptors was due to *de novo* transcription of the receptors, and that PKC contributed to the upregulation. It is likely that a *de novo* production of ET<sub>B</sub> receptors occurs in ischemic stroke as well (paper I), considering the increased mRNA levels. As stated above, the transcription of ET<sub>B</sub> receptors is controlled by the transcription factors AP-1, C/EBP and GATA-2.<sup>119</sup> Ang II can activate AP-1 by a PKC-dependent signaling mechanism in rat vascular smooth muscle cells,<sup>120</sup> and this is interesting since ischemic stroke increased the ACE mRNA levels in the ipsilateral MCA, which may involve an enhanced production of Ang II (paper III). Ang II has in turn the ability to stimulate the expression of bFGF,<sup>136</sup> a growth factor which can increase the endothelin receptor mRNA levels as demonstrated in paper IV and V. In addition, TNF- $\alpha$ , which is induced after stroke<sup>137</sup> and EGF, which is excessively expressed after vascular injury,<sup>138</sup> may contribute to the ET<sub>B</sub> receptor mediated contraction seen after ischemic stroke, according to paper V, possibly via a potentiated release of intracellular calcium. It is appealing to try to describe the reality in this way. However, it is important to remember that organ and cell culture can never be directly compared to the *in vivo* situation, but should be regarded as models for studying possible interactions between signaling pathways.

## 6.2. The angiotensin system

In paper III, we aimed to examine the impact of experimental ischemic stroke on the cerebrovascular angiotensin system. The subject is of current interest since recent experimental and clinical studies have demonstrated beneficial effects of AT<sub>1</sub> receptor

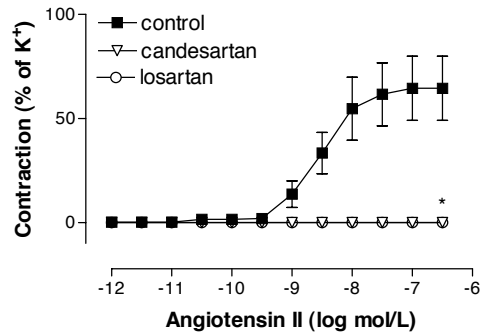


blockade in ischemic stroke.<sup>139, 140</sup> In addition, a major clinical trial examining the effect of AT<sub>1</sub> receptor blockade on survival and disability after stroke has recently been initiated.<sup>141</sup> To study vascular changes after ischemic stroke, transient focal ischemia was induced in rats as described above. 24 hours after MCA occlusion Ang II only elicited a negligible contraction, and there was no difference between the right and left MCA. However, 48 hours after occlusion, a potent contractile response to Ang II was observed in the right, occluded MCA, which differed significantly from the response in the left MCA and MCAs from sham-operated rats (Figure 6.13).



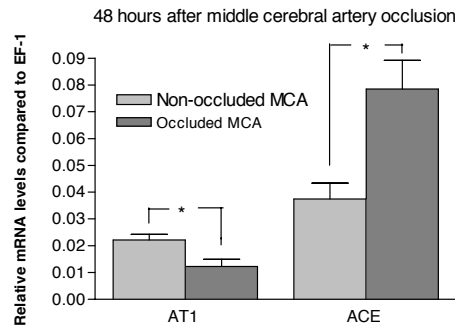
**Figure 6.13** Ischemic stroke induced an increased contraction to Ang II in the right (r) occluded MCA 48 hours after MCA occlusion, but not in the left (l) non-occluded MCA or in sham.

The contractile response could be completely blocked *in vitro* by the selective AT<sub>1</sub> receptor inhibitors candesartan and losartan, while the AT<sub>2</sub> receptor inhibitor PD123319 had no such effect. This suggests that AT<sub>1</sub> receptors were mediating the increased responses to Ang II (Figure 6.14).



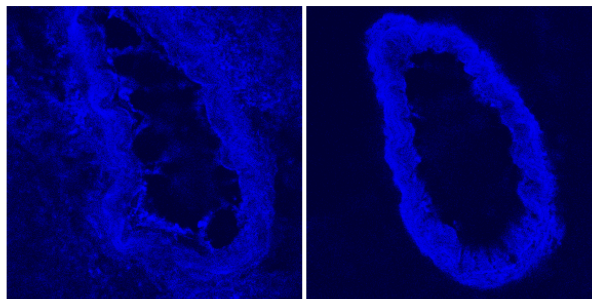
**Figure 6.14** The increased contraction to Ang II was completely abolished by the AT<sub>1</sub> receptor inhibitors candesartan and losartan.

The mRNA levels for AT<sub>1</sub>, AT<sub>2</sub>, ACE and the transcription factor NF- $\kappa$ B were studied by real time PCR. The results showed that 24 hours after MCA occlusion there was no difference between the right and left MCA in the amount of mRNA for the genes studied. Surprisingly, 48 hours after occlusion the mRNA levels for AT<sub>1</sub> were significantly lower in the right occluded MCA as compared to the left (Figure 15). However, similar results have been demonstrated in renal afferent arterioles, where oxidative stress enhanced the contraction to Ang II with a concomitant downregulation of AT<sub>1</sub> mRNA.<sup>142</sup> The downregulation of AT<sub>1</sub> receptor mRNA after ischemic stroke may reflect an increased production of AT<sub>1</sub> receptors, without a corresponding increase in transcription of the AT<sub>1</sub> receptor gene. It may also be due to a negative feedback mechanism, in which Ang II inhibits expression of its own receptor.<sup>94</sup> Supporting that idea is the fact that the mRNA levels for ACE were increased in the right occluded MCA as compared to the left 48 hours after operation, which may suggest an increased production of Ang II after ischemic stroke (Figure 6.15).



**Figure 6.15** mRNA levels for AT<sub>1</sub> and ACE 48 hours after MCA occlusion.

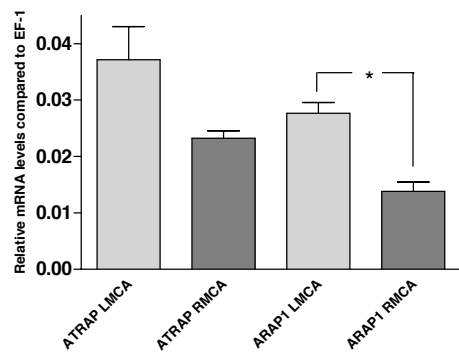
To further analyze the AT<sub>1</sub> receptor regulation after ischemic stroke, we have recently started to examine the protein expression of AT<sub>1</sub>, using immunohistochemistry. Ongoing studies suggest that there are more AT<sub>1</sub> receptors in the right occluded MCA as compared to the left MCA 48 hours after MCA occlusion (Figure 6.16; unpublished), a fact that supports our results in paper III.



**Figure 6.16** AT<sub>1</sub> receptor expression (blue) in cross sections of the left non-occluded (left) and right occluded (right) MCA 48 hours after MCA occlusion.

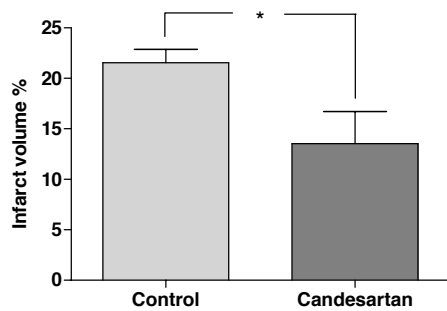
We have also studied the mRNA levels for the AT<sub>1</sub> receptor associated proteins ATRAP and ARAP1, which are responsible for internalization and recycling of the AT<sub>1</sub> receptors. The results showed that both the ATRAP and ARAP1 mRNA levels were lower in the right occluded MCA as compared to the left non-occluded MCA 48 hours after MCA occlusion; in the case of ARAP1 this difference was significant (unpublished data; Figure 6.17). The fact that the mRNA profiles of the two AT<sub>1</sub> receptor associated proteins were similar to the

mRNA profile of the AT<sub>1</sub> receptors may suggest a coordinated expression, which is a well known phenomenon between interrelated genes.<sup>143</sup>



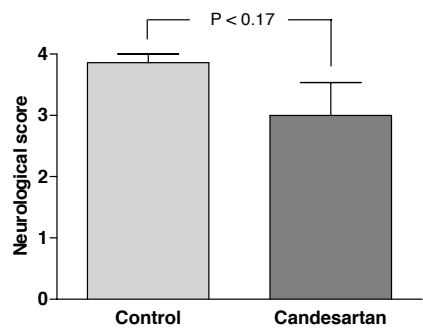
**Figure 6.17** The mRNA levels for ATRAP and ARAP1 in MCA 48 hours after induction of ischemic stroke.

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



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

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



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

The physiological parameters did not differ between the groups (table in paper VI). The mean arterial blood pressure (MAP) was measured in anesthetized animals before they were sacrificed, and it was not different to the MAP measured during operation suggesting that the candesartan dose used did not have any long-term effect on MAP. Beneficial effects of candesartan treatment in the acute phase of ischemic stroke has been shown before.<sup>139</sup> However, in the present study the dose was 10 times lower and given intraperitoneally instead of intravenously. PCR-experiments revealed significantly increased mRNA levels for TNF- $\alpha$  and IL-1 $\beta$  in the right occluded MCA after ischemic stroke compared to the left MCA. However, the candesartan dose used did not significantly affect the increased mRNA levels. There are many possible explanations for the beneficial effects of candesartan in ischemic stroke. According to the results in paper III, our suggestion is an attenuation of the ischemia-induced increase in AT<sub>1</sub> receptor mediated contraction. However, the MAP was not affected in the present study, which may point towards involvement of other mechanisms. Blockade of central AT<sub>1</sub> receptors in ischemic stroke has been shown to prevent expression of the transcription factors c-Fos and c-Jun, which are implicated in neuronal apoptosis.<sup>147</sup> Other possible mechanisms are an enhanced stimulation of protective brain AT<sub>2</sub> receptors<sup>148</sup> or reduced production of free radicals.<sup>149</sup> It is not impossible that the beneficial effects of AT<sub>1</sub> receptor blockade in cerebral ischemia are due to a combination of many factors. However, it will be interesting to see the results of the clinical SCAST study,<sup>141</sup> in which the effects of AT<sub>1</sub> receptor blockade in the acute phase of cerebral ischemia will be thoroughly analyzed.

### **6.3. Additional comment**

I have many times been asked if I think that ischemic stroke induces some kind of general increase in the contractile machinery, considering the enhanced ET<sub>B</sub> and AT<sub>1</sub> receptor mediated responses. However, the contraction elicited by 63.5 mM K<sup>+</sup> do not differ between the right and left MCA after ischemic stroke. In addition, the contractions to 5-hydroxytryptamine (5-HT) or 5-carboxamidotryptamine (5-CT) are not increased by the ischemia (unpublished data). Therefore, in my opinion the number of cerebrovascular contractile mediators affected by ischemic stroke seems to be restricted.

## 7. MAJOR CONCLUSIONS

- Focal cerebral ischemia in rat induces an enhanced contractile ET<sub>B</sub> receptor mediated response in the ipsilateral MCA 48 hours after MCA occlusion. The phenomenon is accompanied by increased ET<sub>B</sub> receptor mRNA levels. Together with preliminary data showing increased ET<sub>B</sub> receptor protein expression as well, these data suggest an upregulation of contractile ET<sub>B</sub> receptors.
- Organ culture of rat MCA induces a time-dependent upregulation of contractile ET<sub>B</sub> receptors. The upregulation is due to *de novo* transcription of the receptors and is partly dependent on PKC.
- The growth factor bFGF increases the proliferation of rat aortic and cerebral smooth muscle cells in a time- and concentration dependent manner, which can be blocked by lipid-lowering statins. bFGF also has the ability to increase the mRNA expression of ET<sub>A</sub> and ET<sub>B</sub> receptors in both cell types. Statins can attenuate this effect in aortic smooth muscle cells. In addition, the bFGF-induced cell proliferation can be blocked by specific ET<sub>A</sub> or ET<sub>B</sub> receptor inhibitors suggesting a reciprocal effect between the growth factor and the endothelin receptors.
- The cytokine TNF- $\alpha$  and the growth factor EGF can potentiate the ET<sub>B</sub> receptor mediated contraction in rat MCAs. Neither of the compounds increases mRNA or protein levels for ET<sub>B</sub> significantly, suggesting that the effect may be due to other factors like potentiated release of intracellular calcium. Incubation with bFGF enhances the maximal ET<sub>A</sub> receptor mediated contraction and increases the mRNA and protein levels for the ET<sub>B</sub> receptors in rat MCAs.
- Focal cerebral ischemia in rat increases the contractile response to angiotensin II in the ipsilateral MCA. This phenomenon occurs between 24 and 48 hours after the MCA occlusion, and is mediated by AT<sub>1</sub> receptors. The increased AT<sub>1</sub> receptor mediated contraction is accompanied by increased AT<sub>1</sub> receptor protein density, but not mRNA levels, suggesting an enhanced production of receptors without a concomitant increase in transcription. It may also reflect a negative feedback-system, in which angiotensin II prevents expression of its own receptors. Supporting this idea is the fact that the

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

- A low dose of the AT<sub>1</sub> receptor blocker candesartan decreases the brain damage after focal cerebral ischemia in rat. In addition, the vascular TNF- $\alpha$  and IL-1 $\beta$  mRNA levels are increased in the ipsilateral MCA.



## 8. SVENSK SAMMANFATTNING (SWEDISH SUMMARY)

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

### 8.1. Endotelinreceptor-reglering

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

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

## **8.2. Angiotensinreceptor-reglering**

Liksom endotelin binder angiotensin huvudsakligen till två receptortyper, AT<sub>1</sub> och AT<sub>2</sub>. De två receptortyperna utövar i stort sett motsatta effekter; Till exempel kontraherar AT<sub>1</sub>-receptorerna blodkärl, orsakar celltillväxt och stimulerar törst, medan AT<sub>2</sub>-receptorerna orsakar en utvidgning av blodkärl, inducerar celldöd och motverkar törst. AT<sub>1</sub>-receptorblockad vid hjärninfarkt har diskuterats livligt de senaste åren och studier både på djur och människa har visat att en sådan blockad kan ha gynnsamma effekter. Vid de flesta av dessa studier har dock AT<sub>1</sub>-receptorinhibitorer givits preventivt under lång tid före hjärninfarkten. Studier angående effekten av akut behandling med AT<sub>1</sub>-inhibitorer vid hjärninfarkt har däremot varit motsägande. Likaså diskuteras det flitigt vad som är orsaken till att AT<sub>1</sub>-inhibitorer fungerar skyddande. En möjlig mekanism presenterar vi i denna avhandling. Vi upptäckte att hjärninfarkt i råttan orsakar en ökad kontraktion mot angiotensin i den högra, drabbade mediaartären. Den här kontraktionen förmedlades just av AT<sub>1</sub>-receptorer, och detta skulle enligt vår hypotes kunna orsaka ett sämre blodflöde i den redan påverkade hjärnhalvan. Här har vi alltså en möjlig förklaring till varför AT<sub>1</sub>-receptorinhibitorer kan skydda vid hjärninfarkt. Dessutom visade det sig att genuttrycket av angiotensin convertering enzyme, ett protein som producerar angiotensin, ökar i den högra mediaartären efter hjärninfarkt, vilket kan innebära att hjärninfarkt orsakar en ökad produktion av angiotensin. Slutligen lyckades vi, i den sista inkluderade studien, bekräfta att blockad av AT<sub>1</sub>-receptorer (i en låg dos) är gynnsamt vid hjärninfarkt i råttan. Hjärnskadan i de behandlade råttorna var betydligt mindre än i de obehandlade 48 timmar efter operationen, och dessutom visade de behandlade råttorna en tendens till färre neurologiska symptom.

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## 10. REFERENCES

1. Thorvaldsen P, Kuulasmaa K, Rajakangas AM, Rastenyte D, Sarti C, Wilhelmsen L. Stroke trends in the WHO MONICA project. *Stroke*. 1997;28:500-506
2. American Heart Association. Heart disease and stroke statistics-2005 update. 2005
3. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: An integrated view. *Trends Neurosci*. 1999;22:391-397
4. Martin RL, Lloyd HG, Cowan AI. The early events of oxygen and glucose deprivation: Setting the scene for neuronal death? *Trends Neurosci*. 1994;17:251-257
5. Wieloch T. Molecular mechanisms of ischemic brain damage. In: Edvinsson L, Krause D, eds. *Cerebral blood flow and metabolism*. Philadelphia, USA: Lippincott Williams & Wilkins; 2002:423-451.
6. Siesjo BK. Pathophysiology and treatment of focal cerebral ischemia. Part I: Pathophysiology. *J Neurosurg*. 1992;77:169-184
7. van Lookeren Campagne M, Gill R. Ultrastructural morphological changes are not characteristic of apoptotic cell death following focal cerebral ischaemia in the rat. *Neurosci Lett*. 1996;213:111-114
8. Fagan SC, Hess DC, Hohnadel EJ, Pollock DM, Ergul A. Targets for vascular protection after acute ischemic stroke. *Stroke*. 2004;35:2220-2225
9. Nelson CW, Wei EP, Povlishock JT, Kontos HA, Moskowitz MA. Oxygen radicals in cerebral ischemia. *Am J Physiol*. 1992;263:H1356-1362
10. Kontos HA. Oxygen radicals in cerebral ischemia: The 2001 Willis lecture. *Stroke*. 2001;32:2712-2716
11. Matsuo Y, Mihara S, Ninomiya M, Fujimoto M. Protective effect of endothelin type A receptor antagonist on brain edema and injury after transient middle cerebral artery occlusion in rats. *Stroke*. 2001;32:2143-2148
12. Ziv I, Fleminger G, Djaldetti R, Achiron A, Melamed E, Sokolovsky M. Increased plasma endothelin-1 in acute ischemic stroke. *Stroke*. 1992;23:1014-1016
13. Plate KH, Beck H, Danner S, Allegrini PR, Wiessner C. Cell type specific upregulation of vascular endothelial growth factor in an MCA-occlusion model of cerebral infarct. *J Neuropathol Exp Neurol*. 1999;58:654-666
14. Renner O, Tsimpas A, Kostin S, Valable S, Petit E, Schaper W, Marti HH. Time- and cell type-specific induction of platelet-derived growth factor receptor-beta during cerebral ischemia. *Brain Res Mol Brain Res*. 2003;113:44-51

15. Lin TN, Te J, Lee M, Sun GY, Hsu CY. Induction of basic fibroblast growth factor (bFGF) expression following focal cerebral ischemia. *Brain Res Mol Brain Res*. 1997;49:255-265
16. Hickey KA, Rubanyi G, Paul RJ, Highsmith RF. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am J Physiol*. 1985;248:C550-556
17. Yanagisawa M, Kurihara H, Kimura S, Goto K, Masaki T. A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle  $Ca^{2+}$  channels. *J Hypertens Suppl*. 1988;6:S188-191
18. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T. The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci U S A*. 1989;86:2863-2867
19. Kloog Y, Ambar I, Sokolovsky M, Kochva E, Wollberg Z, Bdolah A. Sarafotoxin, a novel vasoconstrictor peptide: Phosphoinositide hydrolysis in rat heart and brain. *Science*. 1988;242:268-270
20. Itoh Y, Yanagisawa M, Ohkubo S, Kimura C, Kosaka T, Inoue A, Ishida N, Mitsui Y, Onda H, Fujino M, et al. Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: Identity of human and porcine endothelin. *FEBS Lett*. 1988;231:440-444
21. Kashiwabara T, Inagaki Y, Ohta H, Iwamatsu A, Nomizu M, Morita A, Nishikori K. Putative precursors of endothelin have less vasoconstrictor activity in vitro but a potent pressor effect in vivo. *FEBS Lett*. 1989;247:73-76
22. Schmidt M, Kroger B, Jacob E, Seiberger H, Subkowski T, Otter R, Meyer T, Schmalzing G, Hillen H. Molecular characterization of human and bovine endothelin converting enzyme (ECE-1). *FEBS Lett*. 1994;356:238-243
23. Emoto N, Yanagisawa M. Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. *J Biol Chem*. 1995;270:15262-15268
24. Ehrenreich H, Anderson RW, Fox CH, Rieckmann P, Hoffman GS, Travis WD, Coligan JE, Kehrl JH, Fauci AS. Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. *J Exp Med*. 1990;172:1741-1748
25. An SJ, Boyd R, Wang Y, Qiu X, Wang HD. Endothelin-1 expression in vascular adventitial fibroblasts. *Am J Physiol Heart Circ Physiol*. 2005
26. Vittori E, Marini M, Fasoli A, De Franchis R, Mattoli S. Increased expression of endothelin in bronchial epithelial cells of asthmatic patients and effect of corticosteroids. *Am Rev Respir Dis*. 1992;146:1320-1325

27. Naidoo V, Naidoo S, Mahabeer R, Raidoo DM. Cellular distribution of the endothelin system in the human brain. *J Chem Neuroanat.* 2004;27:87-98
28. Malek AM, Greene AL, Izumo S. Regulation of endothelin 1 gene by fluid shear stress is transcriptionally mediated and independent of protein kinase C and cAMP. *Proc Natl Acad Sci U S A.* 1993;90:5999-6003
29. Shrestha B, Hidai C, Ikeda H, Okada-Ohno M, Kasanuki H, Kawana M. Endothelin-1 gene expression in endothelial cells is potently inhibited by a vasodilator, dilazep. *Hypertens Res.* 2004;27:409-415
30. Emori T, Hirata Y, Ohta K, Kanno K, Eguchi S, Imai T, Shichiri M, Marumo F. Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. *Hypertension.* 1991;18:165-170
31. Kawaguchi H, Sawa H, Yasuda H. Effect of endothelin on angiotensin converting enzyme activity in cultured pulmonary artery endothelial cells. *J Hypertens.* 1991;9:171-174
32. 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
33. Barone FC, Globus MY, Price WJ, White RF, Storer BL, Feuerstein GZ, Busto R, Ohlstein EH. Endothelin levels increase in rat focal and global ischemia. *J Cereb Blood Flow Metab.* 1994;14:337-342
34. Masaki T, Vane JR, Vanhoutte PM. International union of pharmacology nomenclature of endothelin receptors. *Pharmacol Rev.* 1994;46:137-142
35. Ruiz-Opazo N, Hirayama K, Akimoto K, Herrera VL. Molecular characterization of a dual endothelin-1/angiotensin II receptor. *Mol Med.* 1998;4:96-108
36. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature.* 1990;348:730-732
37. Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature.* 1990;348:732-735
38. Hersch E, Huang J, Grider JR, Murthy KS. Gq/g13 signaling by ET-1 in smooth muscle: Mypt1 phosphorylation via eta and cpi-17 dephosphorylation via etb. *Am J Physiol Cell Physiol.* 2004;287:C1209-1218
39. Danthuluri NR, Brock TA. Endothelin receptor-coupling mechanisms in vascular smooth muscle: A role for protein kinase C. *J Pharmacol Exp Ther.* 1990;254:393-399
40. Endoh M, Fujita S, Yang HT, Talukder MA, Maruya J, Norota I. Endothelin: Receptor subtypes, signal transduction, regulation of Ca<sup>2+</sup> transients and contractility in rabbit ventricular myocardium. *Life Sci.* 1998;62:1485-1489

41. Eguchi S, Hirata Y, Imai T, Marumo F. Endothelin receptor subtypes are coupled to adenylate cyclase via different guanyl nucleotide-binding proteins in vasculature. *Endocrinology*. 1993;132:524-529
42. Adner M. Altered expression of contractile endothelin receptors in the vascular bed. *Department of internal medicine*. 1998
43. Nilsson T, Cantera L, Adner M, Edvinsson L. Presence of contractile endothelin-A and dilatory endothelin-B receptors in human cerebral arteries. *Neurosurgery*. 1997;40:346-351; discussion 351-343
44. Klipper E, Gilboa T, Levy N, Kisliouk T, Spanel-Borowski K, Meidan R. Characterization of endothelin-1 and nitric oxide generating systems in corpus luteum-derived endothelial cells. *Reproduction*. 2004;128:463-473
45. Moreland S, McMullen D, Abboa-Offei B, Seymour A. Evidence for a differential location of vasoconstrictor endothelin receptors in the vasculature. *Br J Pharmacol*. 1994;112:704-708
46. Sumner MJ, Cannon TR, Munding JW, White DG, Watts IS. Endothelin ETA and ETB receptors mediate vascular smooth muscle contraction. *Br J Pharmacol*. 1992;107:858-860
47. Sudjarwo SA, Hori M, Tanaka T, Matsuda Y, Karaki H. Coupling of the endothelin ETA and ETB receptors to  $Ca^{2+}$  mobilization and  $Ca^{2+}$  sensitization in vascular smooth muscle. *Eur J Pharmacol*. 1995;289:197-204
48. Szok D, Hansen-Schwartz J, Edvinsson L. In depth pharmacological characterization of endothelin b receptors in the rat middle cerebral artery. *Neurosci Lett*. 2001;314:69-72
49. Dagassan PH, Breu V, Clozel M, Kunzli A, Vogt P, Turina M, Kiowski W, Clozel JP. Up-regulation of endothelin-B receptors in atherosclerotic human coronary arteries. *J Cardiovasc Pharmacol*. 1996;27:147-153
50. Hansen-Schwartz J, Hoel NL, Zhou M, Xu CB, Svendgaard NA, Edvinsson L. Subarachnoid hemorrhage enhances endothelin receptor expression and function in rat cerebral arteries. *Neurosurgery*. 2003;52:1188-1194; 1194-1185
51. Hansen-Schwartz J, Nordstrom CH, Edvinsson L. Human endothelin subtype A receptor enhancement during tissue culture via de novo transcription. *Neurosurgery*. 2002;50:127-133; discussion 133-125
52. 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
53. Adner M, Uddman E, Cardell LO, Edvinsson L. Regional variation in appearance of vascular contractile endothelin-B receptors following organ culture. *Cardiovasc Res*. 1998;37:254-262



54. 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
55. Nambi P, Pullen M, Wu HL, Nuthulaganti P, Elshourbagy N, Kumar C. Dexamethasone down-regulates the expression of endothelin receptors in vascular smooth muscle cells. *J Biol Chem*. 1992;267:19555-19559
56. Uddman E, Moller S, Adner M, Edvinsson L. Cytokines induce increased endothelin ET(B) receptor-mediated contraction. *Eur J Pharmacol*. 1999;376:223-232
57. Morawietz H, Talanow R, Szibor M, Rueckschloss U, Schubert A, Bartling B, Darmer D, Holtz J. Regulation of the endothelin system by shear stress in human endothelial cells. *J Physiol*. 2000;525 Pt 3:761-770
58. Clozel M, Loffler BM, Breu V, Hilfiger L, Maire JP, Butscha B. Downregulation of endothelin receptors by autocrine production of endothelin-1. *Am J Physiol*. 1993;265:C188-192
59. Uddman E, Adner M, Edvinsson L. Protein kinase c inhibitors decrease endothelin ET(B) receptor mRNA expression and contraction during organ culture of rat mesenteric artery. *Eur J Pharmacol*. 2002;452:215-222
60. 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
61. Tigerstedt R, Bergman P. Niere und kreislauf. *Skand Arch Physiol*. 1898;8:223-271
62. Basso N, Terragno NA. History about the discovery of the renin-angiotensin system. *Hypertension*. 2001;38:1246-1249
63. Touyz RM, Schiffrin EL. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev*. 2000;52:639-672
64. Touyz RM, Berry C. Recent advances in angiotensin II signaling. *Braz J Med Biol Res*. 2002;35:1001-1015
65. Kim S, Iwao H. Molecular and cellular mechanisms of angiotensin II-mediated cardiovascular and renal diseases. *Pharmacol Rev*. 2000;59:395-412
66. Shiota N, Okunishi H, Fukamizu A, Sakonjo H, Kikumori M, Nishimura T, Nakagawa T, Murakami K, Miyazaki M. Activation of two angiotensin-generating systems in the balloon-injured artery. *FEBS Lett*. 1993;323:239-242
67. Roig E, Perez-Villa F, Morales M, Jimenez W, Orus J, Heras M, Sanz G. Clinical implications of increased plasma angiotensin II despite ace inhibitor therapy in patients with congestive heart failure. *Eur Heart J*. 2000;21:53-57

68. Shirani J, Loredó ML, Eckelman WC, Jagoda EM, Dilsizian V. Imaging the renin-angiotensin-aldosterone system in the heart. *Curr Heart Fail Rep.* 2005;2:78-86
69. Francis J, Wei SG, Weiss RM, Felder RB. Brain angiotensin-converting enzyme activity and autonomic regulation in heart failure. *Am J Physiol Heart Circ Physiol.* 2004;287:H2138-2146
70. Morishita R, Gibbons GH, Ellison KE, Lee W, Zhang L, Yu H, Kaneda Y, Ogihara T, Dzau VJ. Evidence for direct local effect of angiotensin in vascular hypertrophy. In vivo gene transfer of angiotensin converting enzyme. *J Clin Invest.* 1994;94:978-984
71. Sadjadi J, Kramer GL, Yu CH, Welborn MB, 3rd, Modrall JG. Angiotensin II exerts positive feedback on the intrarenal renin-angiotensin system by an angiotensin converting enzyme-dependent mechanism. *J Surg Res.* 2005
72. Saris JJ, van Dijk MA, Kroon I, Schalekamp MA, Danser AH. Functional importance of angiotensin-converting enzyme-dependent in situ angiotensin II generation in the human forearm. *Hypertension.* 2000;35:764-768
73. MaassenVanDenBrink A, de Vries R, Saxena PR, Schalekamp MA, Danser AH. Vasoconstriction by in situ formed angiotensin II: Role of ace and chymase. *Cardiovasc Res.* 1999;44:407-415
74. Usui M, Egashira K, Kitamoto S, Koyanagi M, Katoh M, Kataoka C, Shimokawa H, Takeshita A. Pathogenic role of oxidative stress in vascular angiotensin-converting enzyme activation in long-term blockade of nitric oxide synthesis in rats. *Hypertension.* 1999;34:546-551
75. Song K, Shiota N, Takai S, Takashima H, Iwasaki H, Kim S, Miyazaki M. Induction of angiotensin converting enzyme and angiotensin II receptors in the atherosclerotic aorta of high-cholesterol fed cynomolgus monkeys. *Atherosclerosis.* 1998;138:171-182
76. Rieder MJ, Carmona R, Krieger JE, Pritchard KA, Jr., Greene AS. Suppression of angiotensin-converting enzyme expression and activity by shear stress. *Circ Res.* 1997;80:312-319
77. Rang H, Dale M, Ritter J, Moore P. *Pharmacology, fifth edition.* Elsevier Science; 2003.
78. Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL, et al. Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun.* 1989;165:196-203
79. Chaki S, Inagami T. Identification and characterization of a new binding site for angiotensin II in mouse neuroblastoma Neuro-2A cells. *Biochem Biophys Res Commun.* 1992;182:388-394
80. Swanson GN, Hanesworth JM, Sardinia MF, Coleman JK, Wright JW, Hall KL, Miller-Wing AV, Stobb JW, Cook VI, Harding EC, et al. Discovery of a distinct

- binding site for angiotensin II (3-8), a putative angiotensin IV receptor. *Regul Pept.* 1992;40:409-419
81. Iwai N, Inagami T. Identification of two subtypes in the rat type I angiotensin II receptor. *FEBS Lett.* 1992;298:257-260
  82. Furuta H, Guo DF, Inagami T. Molecular cloning and sequencing of the gene encoding human angiotensin II type 1 receptor. *Biochem Biophys Res Commun.* 1992;183:8-13
  83. Tsuzuki S, Ichiki T, Nakakubo H, Kitami Y, Guo DF, Shirai H, Inagami T. Molecular cloning and expression of the gene encoding human angiotensin II type 2 receptor. *Biochem Biophys Res Commun.* 1994;200:1449-1454
  84. Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, Miyazaki M, Nozawa Y, Ozono R, Nakagawa K, Miwa T, Kawada N, Mori Y, Shibasaki Y, Tanaka Y, Fujiyama S, Koyama Y, Fujiyama A, Takahashi H, Iwasaka T. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *J Clin Invest.* 1999;104:925-935
  85. Horiuchi M, Hayashida W, Kambe T, Yamada T, Dzau VJ. Angiotensin type 2 receptor dephosphorylates BCL-2 by activating mitogen-activated protein kinase phosphatase-1 and induces apoptosis. *J Biol Chem.* 1997;272:19022-19026
  86. Gallinat S, Busche S, Raizada MK, Summers C. The angiotensin II type 2 receptor: An enigma with multiple variations. *Am J Physiol Endocrinol Metab.* 2000;278:E357-374
  87. Munzenmaier DH, Greene AS. Opposing actions of angiotensin II on microvascular growth and arterial blood pressure. *Hypertension.* 1996;27:760-765
  88. Steckelings UM, Kaschina E, Unger T. The AT2 receptor--a matter of love and hate. *Peptides.* 2005;26:1401-1409
  89. Alexander RW, Brock TA, Gimbrone MA, Jr., Rittenhouse SE. Angiotensin increases inositol trisphosphate and calcium in vascular smooth muscle. *Hypertension.* 1985;7:447-451
  90. Bonventre JV. Phospholipase A2 and signal transduction. *J Am Soc Nephrol.* 1992;3:128-150
  91. Grady EF, Sechi LA, Griffin CA, Schambelan M, Kalinyak JE. Expression of AT2 receptors in the developing rat fetus. *J Clin Invest.* 1991;88:921-933
  92. Huang XC, Richards EM, Summers C. Mitogen-activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors. *J Biol Chem.* 1996;271:15635-15641
  93. Nouet S, Nahmias C. Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol Metab.* 2000;11:1-6

94. Nickenig G, Strehlow K, Baumer AT, Baudler S, Wassmann S, Sauer H, Bohm M. Negative feedback regulation of reactive oxygen species on AT1 receptor gene expression. *Br J Pharmacol*. 2000;131:795-803
95. Viswanathan M, Stromberg C, Seltzer A, Saavedra JM. Balloon angioplasty enhances the expression of angiotensin II AT1 receptors in neointima of rat aorta. *J Clin Invest*. 1992;90:1707-1712
96. Otsuka S, Sugano M, Makino N, Sawada S, Hata T, Niho Y. Interaction of mRNAs for angiotensin II type 1 and type 2 receptors to vascular remodeling in spontaneously hypertensive rats. *Hypertension*. 1998;32:467-472
97. Sakai S, Miyauchi T, Yamaguchi I. Long-term endothelin receptor antagonist administration improves alterations in expression of various cardiac genes in failing myocardium of rats with heart failure. *Circulation*. 2000;101:2849-2853
98. Cui T, Nakagami H, Iwai M, Takeda Y, Shiuchi T, Tamura K, Daviet L, Horiuchi M. ATRAP, novel AT1 receptor associated protein, enhances internalization of AT1 receptor and inhibits vascular smooth muscle cell growth. *Biochem Biophys Res Commun*. 2000;279:938-941
99. Guo DF, Chenier I, Tardif V, Orlov SN, Inagami T. Type 1 angiotensin II receptor-associated protein ARAP1 binds and recycles the receptor to the plasma membrane. *Biochem Biophys Res Commun*. 2003;310:1254-1265
100. Wruck CJ, Funke-Kaiser H, Pufe T, Kusserow H, Menk M, Scheffe JH, Kruse ML, Stoll M, Unger T. Regulation of transport of the angiotensin AT2 receptor by a novel membrane-associated golgi protein. *Arterioscler Thromb Vasc Biol*. 2005;25:57-64
101. Koizumi J, Yoshida Y, Nakasawa T, Ooneda G. Experimental studies of ischemic brain edema, I: A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. *Jpn J Stroke*. 1986;8:1-8
102. Memezawa H, Minamisawa H, Smith M, Siesjo B. Ischemic penumbra in a model of reversible middle cerebral artery occlusion in the rat. *Exp Brain Res*. 1992;89:67-78
103. Bedersen J, Pitts L, Tsuji M. Rat middle cerebral artery occlusion: Evaluation of the model and development of a neurologic examination. *Stroke*. 1986;17:472-476
104. Menzies S, Hoff J, Betz L. Middle cerebral artery occlusion in rats: A neurological and pathological evaluation of a reproducible model. *Neurosurgery*. 1992;31:100-107
105. Bedersen J, Pitts L, Germano S, MC N, Davis R, Bartkowski H. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke*. 1986;17:1304-1308
106. Adner M, Erlinge D, Nilsson L, Edvinsson L. Upregulation of a non-ETA receptor in human arteries in vitro. *J Cardiovasc Pharmacol*. 1995;26:S314-S316

107. Mulvany M, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res.* 1977;41:19-26
108. Hogestatt E, Andersson K, Edvinsson L. Mechanical properties of rat cerebral arteries as studied by a sensitive device for recording of mechanical activity in isolated small blood vessels. *Acta Physiol Scand.* 1983;117:49-61
109. Ratz PH, Berg KM, Urban NH, Miner AS. Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *Am J Physiol Cell Physiol.* 2005;288:C769-783
110. Lodge N, Zhang R, Halaka N, Moreland S. Functional role of endothelin ETA and ETB receptors in venous and arterial smooth muscle. *Eur J Pharmacol.* 1995;287:279
111. Cannan CR, Burnett JC, Jr., Lerman A. Enhanced coronary vasoconstriction to endothelin-B-receptor activation in experimental congestive heart failure. *Circulation.* 1996;93:646-651
112. Adner M, Cantera L, Ehlert F, Nilsson L, Edvinsson L. Plasticity of contractile endothelin-B receptors in human arteries after organ culture. *Br J Pharmacol.* 1996;119:1159-1166
113. Dawson DA, Sugano H, McCarron RM, Hallenbeck JM, Spatz M. Endothelin receptor antagonist preserves microvascular perfusion and reduces ischemic brain damage following permanent focal ischemia. *Neurochem Res.* 1999;24:1499-1505
114. Fukuroda T, Fujikawa T, Ozaki S, Ishikawa K, Yano M, Nishikibe M. Clearance of circulating endothelin-1 by ETB receptors in rats. *Biochem Biophys Res Commun.* 1994;199:1461-1465
115. Patel T, Galbraith S, McAuley M, McCulloch J. Cerebrovascular significance of endothelin receptor antagonism in focal ischaemia. *J Cereb Blood Flow Metab.* 1995;15:140
116. Barone FC, White RF, Elliott JD, Feuerstein GZ, Ohlstein EH. The endothelin receptor antagonist SB 217242 reduces cerebral focal ischemic brain injury. *J Cardiovasc Pharmacol.* 1995;26 Suppl 3:S404-407
117. Hansen-Schwartz J, Edvinsson L. Increased sensitivity to ET-1 in rat cerebral arteries following organ culture. *Neuroreport.* 2000;11:649-652
118. Moller S, Edvinsson L, Adner M. Transcriptional regulated plasticity of vascular contractile endothelin ET(B) receptors after organ culture. *Eur J Pharmacol.* 1997;329:69-77
119. Wagner AH, Krzesz R, Gao D, Schroeder C, Cattaruzza M, Hecker M. Decoy oligodeoxynucleotide characterization of transcription factors controlling endothelin-B receptor expression in vascular smooth muscle cells. *Mol Pharmacol.* 2000;58:1333-1340

120. Tamura K, Nyui N, Tamura N, Fujita T, Kihara M, Toya Y, Takasaki I, Takagi N, Ishii M, Oda K, Horiuchi M, Umemura S. Mechanism of angiotensin II-mediated regulation of fibronectin gene in rat vascular smooth muscle cells. *J Biol Chem.* 1998;273:26487-26496
121. Tsushima H, Urata Y, Miyazaki Y, Fuchigami K, Kuriyama K, Kondo T, Tomonaga M. Human erythropoietin receptor increases GATA-2 and bcl-xl by a protein kinase C-dependent pathway in human erythropoietin-dependent cell line as-e2. *Cell Growth Differ.* 1997;8:1317-1328
122. Mahoney CW, Shuman J, McKnight SL, Chen HC, Huang KP. Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. *J Biol Chem.* 1992;267:19396-19403
123. Sironi L, Cimino M, Guerrini U, Calvio AM, Lodetti B, Asdente M, Balduini W, Paoletti R, Tremoli E. Treatment with statins after induction of focal ischemia in rats reduces the extent of brain damage. *Arterioscler Thromb Vasc Biol.* 2003;23:322-327
124. Negre-Aminou P, van Vliet AK, van Erck M, van Thiel GC, van Leeuwen RE, Cohen LH. Inhibition of proliferation of human smooth muscle cells by various Hmg-CoA reductase inhibitors; comparison with other human cell types. *Biochim Biophys Acta.* 1997;1345:259-268
125. Yang Z, Krasnici N, Luscher TF. Endothelin-1 potentiates human smooth muscle cell growth to PDGF: Effects of ETA and ETB receptor blockade. *Circulation.* 1999;100:5-8
126. Tsujino M, Hirata Y, Eguchi S, Watanabe T, Chatani F, Marumo F. Nonselective ETA/ETB receptor antagonist blocks proliferation of rat vascular smooth muscle cells after balloon angioplasty. *Life Sci.* 1995;56:PL449-454
127. Plate KH. Mechanisms of angiogenesis in the brain. *J Neuropathol Exp Neurol.* 1999;58:313-320
128. Amrani Y, Martinet N, Bronner C. Potentiation by tumour necrosis factor-alpha of calcium signals induced by bradykinin and carbachol in human tracheal smooth muscle cells. *Br J Pharmacol.* 1995;114:4-5
129. Reynolds AM, Holmes MD, Scicchitano R. Cytokines enhance airway smooth muscle contractility in response to acetylcholine and neurokinin A. *Respirology.* 2000;5:153-160
130. Olszewska-Pazdrak B, Ives KL, Park J, Townsend CM, Jr., Hellmich MR. Epidermal growth factor potentiates cholecystokinin/gastrin receptor-mediated Ca<sup>2+</sup> release by activation of mitogen-activated protein kinases. *J Biol Chem.* 2004;279:1853-1860
131. Beg S, Hansen-Schwartz J, Vikman P, Xu C-B, Edvinsson L. ERK1/2 inhibition attenuates cerebral blood flow reduction and abolishes ET<sub>B</sub> and 5-HT<sub>1B</sub> receptor upregulation after subarachnoid hemorrhage in rat. *J Cereb Blood Flow Metab.* 2005;0:000-000

132. Sugimori H, Speller H, Finklestein SP. Intravenous basic fibroblast growth factor produces a persistent reduction in infarct volume following permanent focal ischemia in rats. *Neurosci Lett*. 2001;300:13-16
133. Li Q, Stephenson D. Postischemic administration of basic fibroblast growth factor improves sensorimotor function and reduces infarct size following permanent focal cerebral ischemia in the rat. *Exp Neurol*. 2002;177:531-537
134. Watanabe T, Okuda Y, Nonoguchi N, Zhao MZ, Kajimoto Y, Furutama D, Yukawa H, Shibata MA, Otsuki Y, Kuroiwa T, Miyatake S. Postischemic intraventricular administration of FGF-2 expressing adenoviral vectors improves neurologic outcome and reduces infarct volume after transient focal cerebral ischemia in rats. *J Cereb Blood Flow Metab*. 2004;24:1205-1213
135. Baldauf K, Reymann KG. Influence of EGF/bFGF treatment on proliferation, early neurogenesis and infarct volume after transient focal ischemia. *Brain Res*. 2005;1056:158-167
136. Satoh C, Fukuda N, Hu WY, Nakayama M, Kishioka H, Kanmatsuse K. Role of endogenous angiotensin II in the increased expression of growth factors in vascular smooth muscle cells from spontaneously hypertensive rats. *J Cardiovasc Pharmacol*. 2001;37:108-118
137. Buttini M, Appel K, Sauter A, Gebicke-Haerter PJ, Boddeke HW. Expression of tumor necrosis factor alpha after focal cerebral ischaemia in the rat. *Neuroscience*. 1996;71:1-16
138. Hayry P, Myllarniemi M, Aavik E, Alatalo S, Aho P, Yilmaz S, Raisanen-Sokolowski A, Cozzone G, Jameson BA, Baserga R. Stable D-peptide analog of insulin-like growth factor-1 inhibits smooth muscle cell proliferation after carotid ballooning injury in the rat. *Faseb J*. 1995;9:1336-1344
139. Engelhorn T, Goerike S, Doerfler A, Okorn C, Forsting M, Heusch G, Schulz R. The angiotensin II type 1-receptor blocker candesartan increases cerebral blood flow, reduces infarct size, and improves neurologic outcome after transient cerebral ischemia in rats. *J Cereb Blood Flow Metab*. 2004;24:467-474
140. Schrader J, Luders S, Kulschewski A, Berger J, Zidek W, Treib J, Einhaupl K, Diener HC, Dominiak P. The access study: Evaluation of acute candesartan cilexetil therapy in stroke survivors. *Stroke*. 2003;34:1699-1703
141. Berge E, Aakvik R, Terént A, Boysen G. Scandinavian candesartan acute stroke trial (SCAST). *Workshop on Angiotensin II Receptor Blockade*. 2005
142. Wang D, Chen Y, Chabrashvili T, Aslam S, Borrego Conde LJ, Umans JG, Wilcox CS. Role of oxidative stress in endothelial dysfunction and enhanced responses to angiotensin II of afferent arterioles from rabbits infused with angiotensin II. *J Am Soc Nephrol*. 2003;14:2783-2789

143. Lewin B. Gene numbers. *Genes*. New York: Oxford University Press, Inc.; 1998:687-711.
144. Abraham E. NF-kappaB activation. *Crit Care Med*. 2000;28:N100-104
145. Emsley HC, Tyrrell PJ. Inflammation and infection in clinical stroke. *J Cereb Blood Flow Metab*. 2002;22:1399-1419
146. Ruiz-Ortega M, Lorenzo O, Ruperez M, Suzuki Y, Egido J. Angiotensin II activates nuclear transcription factor-kappaB in aorta of normal rats and in vascular smooth muscle cells of AT1 knockout mice. *Nephrol Dial Transplant*. 2001;16 Suppl 1:27-33
147. Dai WJ, Funk A, Herdegen T, Unger T, Culman J. Blockade of central angiotensin AT(1) receptors improves neurological outcome and reduces expression of AP-1 transcription factors after focal brain ischemia in rats. *Stroke*. 1999;30:2391-2398; discussion 2398-2399
148. Li J, Culman J, Hortnagl H, Zhao Y, Gerova N, Timm M, Blume A, Zimmermann M, Seidel K, Dirnagl U, Unger T. Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury. *FASEB J*. 2005;19:617-619
149. Sugawara T, Kinouchi H, Oda M, Shoji H, Omae T, Mizoi K. Candesartan reduces superoxide production after global cerebral ischemia. *Neuroreport*. 2005;16:325-328