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PhD-thesis

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The Renin-Angiotensin System and Autoregulation of Cerebral Blood Flow

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English Summary

The aim of this PhD-thesis was to elucidate the role of the renin angiotensin system (RAS) and bradykinin on autoregulation of cerebral blood flow (CBF), which keeps CBF constant during wide fluctuations in systemic blood pressure. Both experimental and clinical studies have indicated that the RAS plays a role in the modulation of this system, presumably by an effect on the larger resistance vessels.

The thesis is based on two original research papers. The studies were carried out in anesthetized Sprague-Dawley (SPRD) rats , and CBF was measured by the laser-Doppler technique. The lower limit of autoregulation was calculated by a computer program.

In the first study the effect of the angiotensin converting enzyme inhibitor (ACEi) enalaprilat, the ARB candesartan, and the bradykinin 2 (B2) receptor blocker Hoe 140 given intravenously on the lower limit of CBF autoregulation was studied. While ACEis cause systemic bradykinin accumulation, ARBs have no such effect, but may activate a local bradykinin system in the vessel wall.

The following observations were made:

1. Both the ACEi and the ARB shifted the lower limit of CBF autoregulation towards lower blood pressure levels whereas the B2 receptor blocker in itself had no effect on autoregulation.
2. The B2 receptor blocker abolished both the effect of the ACEi and the ARB on the lower limit of CBF autoregulation.

The conclusion of this study is that the effects of both ACEi and ARB on the lower limit of CBF autoregulation is dependent on bradykinin release. While ACEis cause systemic bradykinin accumulation, ARBs have no such effect, but may activate a local bradykinin system in the vessel wall.

In the second study we evaluated the effect of the ARB candesartan on the modulation of CBF autoregulation in animals fed either high or low sodium diet. In this study SPRD rats were fed with chow containing either 4% sodium or 0,004% sodium for one week. All the animals had access to water ad libitum. High sodium diet would expectedly suppress the renin levels in the rats whereas low sodium intake would expectedly stimulate renin. Both the high sodium and low sodium groups were given either the ARB candesartan intravenously, or were controls.

In this study the following observations were made:

1. The effect of the ARB candesartan on the lower limit of CBF autoregulation seen with normal sodium chow (0,25% Na^+) was

not present in animals on high or low sodium chow and there was no difference in the lower limit of CBF autoregulation between the 4 groups of animals.

2. Plasma renin concentration (PRC) as expected was raised in animals fed low sodium chow compared to the animals fed high sodium chow.

3. Under anesthesia the initial blood pressure levels surprisingly was higher in the animals fed low sodium chow than in the animals fed high sodium chow, despite signs of a reduced extracellular volume in the former group.

The conclusion of this study is that both high- and low sodium diet annuls the effect of the ARB candesartan on the lower limit of CBF autoregulation. During anesthesia the blood pressure levels were elevated in the low sodium animals. It may be speculated that in the low sodium animals, sympathetic nervous activation overrides blockade of the RAS and its modulation on the lower limit, while in the high sodium animals, the suppressed RAS is not responsive to blockade with the ARB.

A small additional study was made to demonstrate the hypotensive effect of intravenous enalaprilat and candesartan. Both drugs caused an abrupt similar fall in blood pressure below the lower limit of CBF autoregulation. This illustrates the necessity of transiently giving norepinephrine to demonstrate the autoregulatory plateau.

Based on these studies it is conclude that the RAS influences autoregulation of CBF and bradykinin is involved in both the response that the ACEis and the ARBs have on the lower limit of autoregulation. The effect of the ARB candesartan on the lower limit of CBF autoregulation is annulled in rats on both high and low sodium chow.

Dansk Resume

Formålet med denne PhD-afhandling var at belyse renin angiotensin systemets (RAS) og bradykinins rolle i autoregulationen af hjernens kredsløb (CBF). Autoregulationen holder CBF konstant indenfor vide blodtryksgrænser. Både dyreforsøg og kliniske studier har vist at RAS har en modulerende effekt på autoregulationen, formentlig ved en effekt på hjernens større modstandskar.

Afhandlingen bygger på to originale videnskabelige arbejder. Forsøgene er udført på anæsteserede Sprague-Dawley rotter (SPRD), og CBF er målt med laser-Doppler teknik. Autoregulationens nedre grænse blev beregnet af et computerprogram.

I det første arbejde blev effekten af forskellige farmaka givet intravenøst på CBF autoregulationens nedre grænser undersøgt. Følgende lægemidler blev undersøgt: Angiotensin converting enzym (ACE) hæmmeren enalaprilat, angiotensin receptor blokkeren (ARB) candesartan og bradykinin 2 (B2) receptor blokkeren Hoe 140.

Der blev gjort følgende observationer:

a. Både ACE-hæmmer og ARB flyttede CBF autoregulationen mod et lavere blodtryk mens B2 receptor blokkeren alene ikke havde effekt på autoregulationen.

b. Samtidig indgift af B2 receptor blokker med enten ACE-hæmmer eller ARB viste at B2 receptor blokkeren ophævede effekten af både ACE-hæmmer og ARB på autoregulationen. Det er kendt at ACE-hæmmere blokerer den systemiske nedbrydning af bradykinin. ARB-er har ikke denne systemiske effekt, men kan have en lignende effekt på bradykinin lokalt i karvæggen.

Konklusionen på dette studie er at den indflydelse ACE-hæmmer og ARB har på autoregulationen er afhængig af bradykinin.

I det andet arbejde blev det undersøgt hvilken påvirkning høj eller lav salt foder har på effekten af ARB på CBF autoregulationen. I dette studie blev SPRD rotter fodret med enten høj salt foder (4% Na^+) foder eller lav salt foder (0,004% Na^+) i en uge. Alle dyrene havde fri adgang til drikkevand. Høj salt foder vil forventeligt supprimere renin produktion i rotter imens lav salt forventeligt stimulerer renin. Rotter fra begge grupper fik enten ARBen candesartan eller kontrol.

Følgende observationer blev gjort:

1. Den modulerende effekt af ARBen candesartan på CBF autoregulationen ophæves hos dyr på enten høj- eller lavsalt foder.

Der var således ingen forskel mellem kontroller og candesartan behandlede dyr i, høj- og lavsalt grupperne hvad autoregulationens nedre grænse angår.

2. Plasma renin aktivitet var forhøjet i den gruppe dyr der fik lavsalt foder.

3. Det initiale blodtryk var højere i de bedøvede dyr der fik lav salt foder sammenlignet med dem der fik høj salt foder, til trods for tegn på nedsat ekstracellulærvolumen hos lavsaltdyrene.

Konklusionen på dette studie er at både høj-, og lavsalt foder ophæver effekten som ARBen candesartan har på CBF autoregulationens nedre grænse. Under bedøvelse har dyrene i lavsalt gruppen et højere blodtryk end dyrene i højsalt gruppen. En mulig forklaring på dette fund i lavsalt-gruppen er at dyrenekunne have en maksimal sympaticusaktivering, der overrider effekter af RAS, og som i øvrigt kan ses på det forhøjede blodtryk under anæstesi. En mulig forklaring på fundet i højsalt-gruppen er at reninsystemet er supprimeret, og derfor ikke responderer på forsøg på blokade med ARBen.

Et mindre studie blev udført for at vise den blodtryksnedsættende effekt af intravenøs indgift af enalaprilat og candesartan. Begge lægemidler havde en hurtigt indsættende og sammenlignelig nedsættende virkning på blodtrykket til niveauer under CBF autoregulationens nedre grænse. Dette viser nødvendigheden af kortvarigt at give dyrene noradrenalin for at påvise et plateau med intakt CBF autoregulation.

Konklusionen på afhandlingen er at renin angiotensin systemet har indflydelse på autoregulationen af CBF ved normal saltholdig kost, og at bradykinin spiller en rolle i den modulering som både ACE-hæmmer og ARB har på autoregulationen. Ved enten høj-, eller lavsalt kost ophæves indflydelsen ARBen candesartan har på CBF autoregulationen.

Preface

The author was enrolled as a PhD student at Copenhagen University - Faculty of Health Sciences. The scientific research in this thesis was done at the Neurobiology Research Unit at Copenhagen University Hospital, Rigshospitalet in Copenhagen from 2006 to 2009 during my employment as a clinical research associate at the department of Nephrology at Copenhagen University Hospital in Herlev. The project was supervised by Svend Strandgaard, MD, DMSci and Arne Høj Nielsen, MD, DMSci at the department of Nephrology, Copenhagen University Hospital in Herlev and Professor Olaf B. Paulson, MD, DMSci at Neurobiology Research Unit, Copenhagen University Hospital in Copenhagen.

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Papers

This thesis is based on the following publications:

Sigurdsson, S.T., Paulson, O.B., Nielsen, A.H. and Strandgaard, S.,*The bradykinin-antagonist Hoe 140 abolishes the effect of both candesartan and enalaprilat on the lower limit of autoregulation of cerebral blood flow*. Submitted to Sroke in February 2010.

Sigurdsson, S.T, Paulson, O.B., Bie, P., Nielsen, A.H. and Strandgaard, S., *High and low sodium diet eliminate the modulatory effect of the angiotensin receptor blocker candesartan on the lower limit of cerebral blood flow autoregulation*. To be submitted.

Acknowledgements

It is a privilege to have an opportunity to conduct academic research and doing so with the advice and guidance from some of the best researchers in the field makes it all the better. Unfortunately this fact is not always apparent when you actually are doing your research. This project has, like all research projects, had its ups and downs. Despite failed experiments and equipment that had the knack of breaking down during testing my supervisors still had faith in my project even when my own faith was fledgling. Without their guidance and support this project would never have mounted to this thesis.

Svend Strandgaard introduced me to this field of research in 2005 and I was immediately intrigued by the possibilities. He has been of paramount influence and his enthusiasm for the project has been a great driving factor. I am indebted to Olaf B. Paulson for giving me the opportunity to conduct the experiments at the Neurobiology Research Unit (NRU) at Rigshospitalet and his knowledge on the subject has been of great value. Arne Høj Nielsen's insight into the renin-angiotensin system was essential in writing this thesis. I deeply appreciate your guidance over the years and meticulous work on editing manuscripts and abstracts sent to meetings and congresses during the project period.

I thank all the staff at the Department of Nephrology at Herlev. Especially chief physician Steen Fugleberg who saw a potential in me and the project and agreed to provide funding for the cost of the PhD matriculation. He would always give good advice when asked and despite having a full schedule he always gave generously of his time whenever I knocked on his door.

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I thank Anette Jans at the department of Clinical Biochemistry at Rigshospitalet for analysis of electrolytes, urea and creatinine.

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Professor Gerald F. DiBona gave constructive criticism and offered some ideas for the interpretation of some of the more challenging data.

I owe special thanks to professor J. David Spence, Robarts Research Institute, London, Ontario, Canada for his assistance in acquiring enalaprilat for the studies.

AstraZeneca kindly provided candesartan for this project.

Without the support of my family this project would never have mounted to anything. Ole og Anette Harmsen have assisted us in every conceivable way. My brother in law, Morten Harmsen spent hours reading the manuscript for the thesis and his wife to be, Tanja Møller allowed me to borrow him when needed. I thank my boys Sverrir Þór and Snorri Marteinn for making catheters that lasted for almost a year and Anna Björk for being a patient little princess while her father was writing the last paragraphs for the thesis.

My biggest gratitude goes to my wife, Lotte Harmsen. Without her care and support this period would have been agonizing. While writing her own PhD thesis, she still managed to help me get focused and stay on course when things were not going my way.

List of Abbreviations

ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACEi	Angiotensin converting enzyme inhibitor
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
AT1	Angiotensin II receptor subtype 1
AT2	Angiotensin II receptor subtype 2
B2	Bradykinin subtype 2
CBF	Cerebral blood flow
CNS	Central nervous system
CPP	Cerebral perfusion pressure
CVR	Cerebrovascular resistance
EET	Epoxyeicosatrienoic acid
ET-1	Endothelin 1
hgb	Hemoglobin
i.v.	intravenously
ICP	Intracranial pressure
LDF	Laser Doppler flowmetry
MAP	Mean arterial blood pressure
MI	Myocardial infarction
NIRS	Near infrared spectroscopy
NO	Nitric oxide
NOS	Nitric oxide synthase
PRC	Plasma renin concentration
RAS	Renin-angiotensin system
SE	Standard error of means
SHR	Spontaneously hypertensive rat
SHSPR	Stroke-prone spontaneously hypertensive rat
SPECT	Single-photon emission computed tomography
SPRD	Sprague-Dawley rat
WKY	Wistar-Kyoto rat

Contents

I. Introduction

Cardiovascular disease is among the main causes of morbidity and mortality in the western world. Stroke is not only detrimental to the individual patient but also to the community because of high cost of rehabilitation and care. In the USA an estimated 610.000 cases of new onset stroke and 185.000 recurrent attacks occurred in 2006 and an estimation of the direct and indirect expenses regarding stroke in 2010 is 73,6 billion US $ [1]. In Denmark there are approximately 12.400 cases of new onset stroke and 3.100 cases of recurrent stroke every year [2]. In both the USA and Denmark stroke was the second largest cause of death in 2002 [3]. It appears that stroke incidence is falling in the industrialized world [4] whereas there is an increase in the developing world [5, 6]. Hypertension is a well known risk factor for stroke [7] and lowering blood pressure has been proven to be effective in preventing the disease [8].

Several controlled clinical studies indicate that not all antihypertensive agents appear to be equally effective in preventing stroke in hypertensive patients. In particular, the angiotensin receptor blockers (ARB's) have been shown to have a potential for stroke prevention beyond the effect of blood pressure lowering. Thus, a large clinical study (the LIFE study) showed that in hypertensive patients with left ventricular hypertrophy that were treated with the angiotensin receptor blocker (ARB) losartan fared better regarding stroke prevention than a similar patient-group treated with the β-blocker atenolol [9]. Another study where an ARB, eprosartan was compared to the calcium channel antagonist nitrendipine showed again that the ARB was superior regarding stroke [10].

Obviously when a patient's mean arterial blood pressure (MAP) falls below the lower limit of CBF autoregulation, even though oxygen extraction from the blood can be increased, there is a risk of development of ischemic stroke. This risk may be diminished by using blockers of the RAS in hypertension treatment, since the lower limit of autoregulation of CBF can be modulated with such agents. This would improve the tolerance to blood pressure lowering, and hence increase the potential for stroke prevention by the antihypertensive drug. Modulation of CBF autoregulation shifting its limits to lower blood pressure has been shown with the angiotensin converting enzyme inhibitors (ACEis) captopril, ceranopril and fosinopril [11, 12]. The ARBs candesartan and valsartan that block the angiotensin II subtype 1 (AT1) receptor have a similar effect on the lower limit [13, 14]. The ARB Losartan has by contrast in one study been shown to have the opposite effect and been shown to raise the limits of CBF autoregulation towards higher blood pressure levels [15]. This is a surprising finding since losartan was used with particular success in stroke prevention in the LIFE study.

We conducted two studies in Sprague-Dawley (SPRD) rats to elucidate the role that antihypertensive drugs, which functions via the RAS, have on autoregulation of CBF.

In the first study we investigated whether the effect of the ACEi enalaprilat and the ARB candesartan were mediated via bradykinin. In the second study we investigated the effect of high and low sodium diets causing respectively suppression and activation of the RAS on the lower limit of CBF autoregulation and its modulation by the ARB candesartan.

The present review affords a detailed discussion of the relationship between the RAS and the CBF autoregulation, and presents the author's studies in this field.

II. A Brief History of Research on the Renin-Angiotensin System and the Cerebral Circulation

1. The Renin-Angiotensin System

The Renin-Angiotensin System (RAS) is an integral player in the in control of homeostasis of water, balance of electrolytes and control of blood pressure in vertebrates [16]. The discovery of renin was made by Tigerstedt and Bergman in 1898 [17]. They were able to show that extracts from rabbit kidneys, when injected into other rabbits, caused a pressor effect. They suggested that the compound responsible for this effect was a protein derived from the kidney which they named renin. Contemporary scientists were unable to reproduce these findings, possibly due to problems with preventing proteolysis of renin in the extracts. It would take several years before this substance would gain attention again. Several attempts at raising blood pressure in animals had been attempted in the first part of the twentieth century. There was consensus that causing injury to the kidneys resulted in higher blood pressure levels. The problem was that the methods used such as injecting nephrotoxic agents, radiating the kidneys with Röntgen rays, occluding the renal veins or excising various amounts of kidney tissue with or without legating parts of the renal arteries, did not affect blood pressure permanently [18].

Figure 1. Robert Tigerstedt. Image provided by the National Library of Medicine.

New progress came first in 1934 when Goldblatt published his findings. He was inspired by work done by his colleague Cash. Cash had made the observation in dogs that their blood pressure rose when the dog kidney was deprived of its circulation, by ligation of the renal artery. The following had to be fulfilled to see that effect; The kidney injury should result in a 50% reduction in the kidney volume and the kidneys were left in situ to allow for absorption of the necrotic tissue. [19]. Goldblatt went further with the idea that injury to the kidney could result in

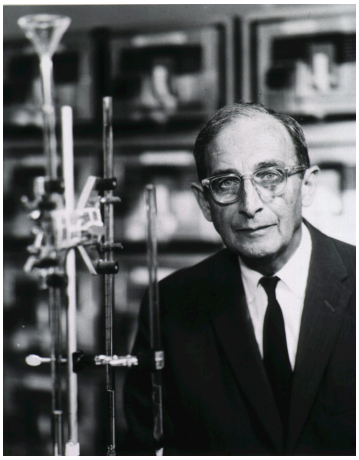

Figure 2. Harry Goldblatt. Image provided by the National Library of Medicine.

changes in blood pressure and designed a silver-clamp that fit around the renal arteries in dogs. By using this clamp Goldblatt achieved a renal artery stenosis instead of a complete occlusion of the renal arteries. In several of the animals in the study he decreased the lumen of the renal arteries stepwise over several weeks to achieve even higher blood pressure levels. He investigated the effect of clamping one or both of the renal arteries and he also tried clamping other arteries in the dogs. Only clamping of the renal arteries resulted in persistent elevated blood pressure. An examination of the kidneys from the animals did not show any sign of necrosis. Goldblatt concluded that ischemia localized to the kidneys resulted in elevated blood pressure levels. He hypothesized that there might be an accumulation of some substance, or a disturbance of chemical equilibrium between substances present in the blood which might effect a pressor action e.g. a hormone [20]. Goldblatt's technique was quite difficult and his findings were initially met with skepticism, but the results were accepted after other groups managed to reproduce his experiments.

The next discoveries came at a faster pace. In 1939 after a method to extract renin from blood was developed, further progress took place.

Figure 3. Eduardo Braun-Menéndez. Wikimedia commons

In Argentina, Braun-Menéndez took nephrectomized dogs and transplanted one of the kidneys to the neck. When the artery of the graft was compressed the blood pressure rose and this effect was attenuated the longer time the renal artery was compressed. The group also performed experiments using Starlings' heart-lung preparation [21]. Here they found that when injecting venous blood from a kidney where the blood flow had been reduced, blood pressure rose in the recipient animal whereas injection from a kidney that had normal blood flow did not cause increased blood pressure. Braun-Menéndez continued his experiments and found that citrated blood from the ischemic kidney-graft described above caused vasoconstriction while blood from the normally perfused kidney-graft did not. Again the same results were seen with samples from Starlings' heart-lung preparation. More importantly he found that after incubating extracted renin from the blood of the ischemic kidneys with blood serum a pressor substance was formed and when injected it caused similar effects on blood pressure as the blood from the ischemic kidneys. He also found that the time of incubation played a role since when incubated for a short time the effect on blood pressure levels were not as profound. Braun-Menéndez assumed that renin was an enzyme and the substrate

was a blood protein and the product caused elevated blood pressure. He called this protein hypertensin [22, 23].

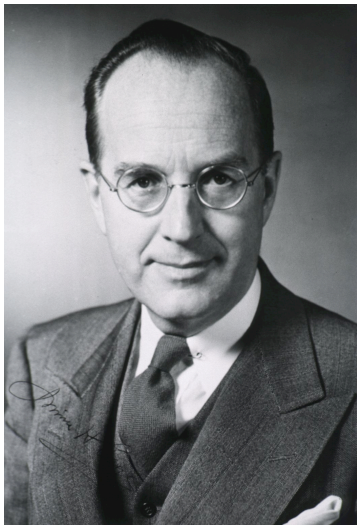

Figure 4. Irvine H Page. Image provided by the National Library of Medicine.

At the same time in Indianapolis, Page did research in both cats and dogs and he found that repeated injections of renin into the animals caused an increase in blood pressure but this effect diminished with repeated injections. Furthermore injecting blood from animals that had had repeated injections of renin did not result in vasoconstriction in the vessels of a rabbits ear. He concluded that the reason for a diminishing effect of the renin-injections was an anti-pressor state or loss of some substance necessary for the chemical reactions which culminate in the pressor action of renin in the blood and that the vasoconstricting effects caused by renin were activated by a substance he referred to as renin-activator [24]. Further research at the Lilly laboratories showed that it was actually renin that modified the renin-activator in blood serum to another vasoactive substance which he called angiotonin. Renin-activator thus was renamed renin-substrate to acknowledge the enzymatic properties of renin. He found that injecting this new substance into animals caused a sharp rise in blood pressure similar to the rise seen when injecting adrenaline but the effect was prolonged. The comparison of angiotonin and renin injections showed that the raised blood pressure in the animals injected with renin was not as sudden and not as marked but longer lasting than in the animals injected with angiotonin [25]. His group continued the extensive research and found that repeated injections of angiotonin also desensitized the animals to the elevating effects of angiotonin on the blood pressure although not as prominently as was seen with renin. He hypothesized that another substance was necessary to activate the vasopressor effects of angiotonin [26].

Research with hypertensin/angiotonin continued and two groups found that the product of renin action was a decapeptide which required further enzymatic breakdown into an octapeptide, the active substance [27, 28]. One of the groups called the resulting peptides hypertensin I and hypertensin II and the enzyme responsible for the process hypertensin converting enzyme.

The nomenclature was confusing, some groups used hypertensin and others used angiotonin. At an academic meeting in Michigan in 1956 Braun-Menéndez and Page met and agreed that a universal name for hypertensin and angiotonin was necessary [23, 29]. In a short article in 1958 they suggested the name angiotensin [30]. Over some years the name

caught on and these peptides, hormones and enzymes are now known as renin, angiotensinogen, angiotensin converting enzyme (ACE), angiotensin I and angiotensin II. Research in the RAS now continued at a faster pace and the physiologic properties of the components of the system were explored and still are to this day.

2. Cerebral Circulation

Figure 5. Charles S. Roy. Image provided by the National Library of Medicine.

The cells of the central nervous system (CNS) have a high metabolic demand and are dependent on an adequate steady flow of nutrients and therefore dependent on relatively constant CBF independent on the subject's physical activity or systemic blood pressure.

The cerebral vessels have an ability to keep CBF relatively constant over a wide range of systemic blood pressure. This ability is mediated via myogenic, neurogenic or metabolic mechanisms [31].

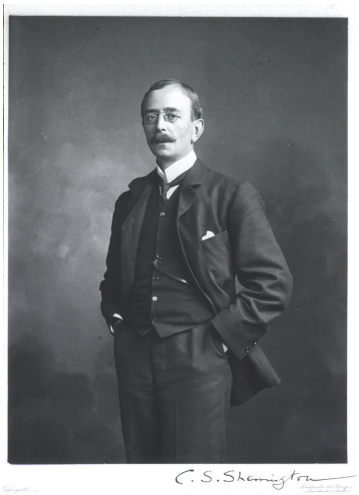

Figure 6. Charles S. Sherrington. Image provided by the National Library of Medicine.

Initial observation on the importance of the control of CBF were done by Roy and Sherrington in 1890 [32]. There was limited evidence of their observations but they suggested that local changes in cerebral activity and perfusion were coupled. In 1902 Bayliss published an article where he described the response of the arterial wall during changes in the internal pressure in a denervated hind limb of dogs [33].

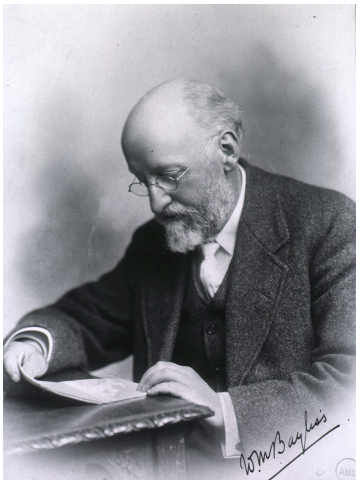

Figure 7. William M. Bayliss. Image provided by the National Library of Medicine.

One of the first observations of changes in the vasculature of the brain due to changes in systemic blood pressure was made by Mogens Fog in 1934. Fog saw that when blood pressure fell in a cat the pial arteries dilated and conversely the arteries contracted when blood pressure increased [34].

Estimating CBF indirectly from the arteriovenous oxygen difference of the brain was introduced by Lennox and Gibbs in 1932 [35].

Figure 8. Mogens Fog. Taken from www.document.no

Measurements of cerebral hemodynamics were crude or indirect until a new method was introduced by Kety and Schmidt in 1948 [36]. Before their work no methods were available that could measure CBF quantitatively. To measure CBF they used N_2O which is an inert gas. After about 10 - 15 minutes of inhaling a mix of N_2O and atmospheric air an equilibrium is achieved and the values of the differential rates in N_2O concentrations in the arterial blood and the blood in the cerebral venous blood can be used to estimate CBF. Kety and Schmidt found that the mean global CBF was 54 ± 12 ml * 100 g^{-1} * min^{-1}. This value has been reproduced several times in the 60 years since their discovery with more advanced equipment.

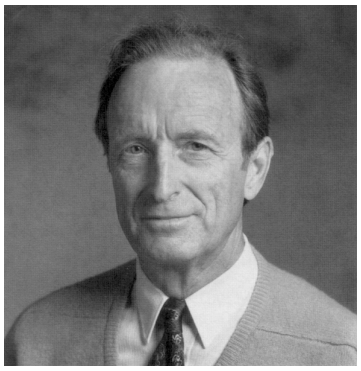

Figure 9. Niels Lassen. Taken from*Journal of Cerebral Blood Flow & Metabolism* (1997) **17,** 1005–1006.

An eminent description of the physiology and pathophysiology of the vasculature of the brain was supplied by Niels Lassen in 1959. Lassen took the term autoregulation from renal physiology and coined it to the control of blood flow to the brain under various blood pressure levels [37]. A curve depicting cerebral autoregulation was for the first time published in his paper, see figure 10. This concept of autoregulation was a leap forward in understanding the mechanisms of the cerebral circulation. We now know that the curve has some drawbacks. Primarily because it is comprised of measurements from several patients with different medical conditions. In Lassen's autoregulatory curve 11 points are shown and these represents means from 11 different patient groups, 2 points are from normal subjects with drug-induced severe hypotension, 2 points are from normal subjects with drug-induced moderate hypotension, one point was from pregnant females, one point from healthy males, one point from normal subjects with drug-induced hypertension, one point from females with pre-eclampsia and three points from patients with essential hypertension. Lassen's autoregulatory curve was compiled from data from 7 studies with 11 groups of patients and the figure was based upon 376 individual determinations.

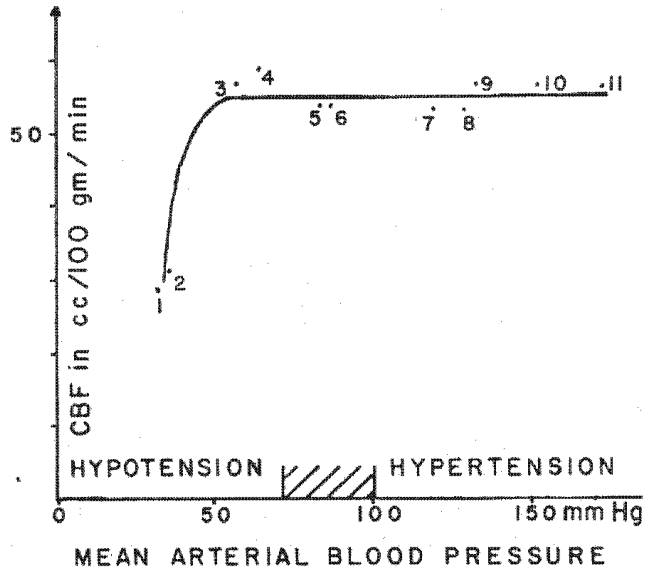

Figure 10. Niels Lassens autoregulatory curve published in 1959 [37].

Since the pioneering work of Fog, Kety, Schmidt and Lassen the techniques to measure CBF have advanced like every other field in medicine. ^{133}Xe-injection or inhalation to measure CBF is a modification of Kety and Schmidt's original method. These methods later developed into single-photon emission computed tomography (SPECT) scans to measure regional CBF. In 1981 the first reports on regional CBF in stroke patients employing SPECT was published [38]. Other new methods such as trans-cranial Doppler, laser Doppler flowmetry (LDF), near infrared spectroscopy (NIRS) has been used to study autoregulation of CBF. The laser Doppler method was used in the experimental work for this thesis. The method will be described later.

These newer methods have higher temporal resolution making it possible to analyze fast responses in CBF due to changes in MAP. This led to the introduction of the concept of dynamic autoregulation of CBF in 1989 by Aaslid and associates [39]. Dynamic autoregulation assesses the rate of regulation in cerebrovascular resistance (CVR) during sudden and steep changes in blood pressure. By using the trans-cranial Doppler method the flow velocity in the middle cerebral artery can be measured and this flow velocity is used to asses CBF. CBF and blood pressure can then be used to asses CVR and the rate of regulation, that is the time it takes to return to normal levels after sudden changes in blood pressure.

III. An Overview of the Renin-Angiotensin System

1. The Classical System

As described previously the RAS plays an important role in maintaining homeostasis of water, electrolytes and control of blood pressure.

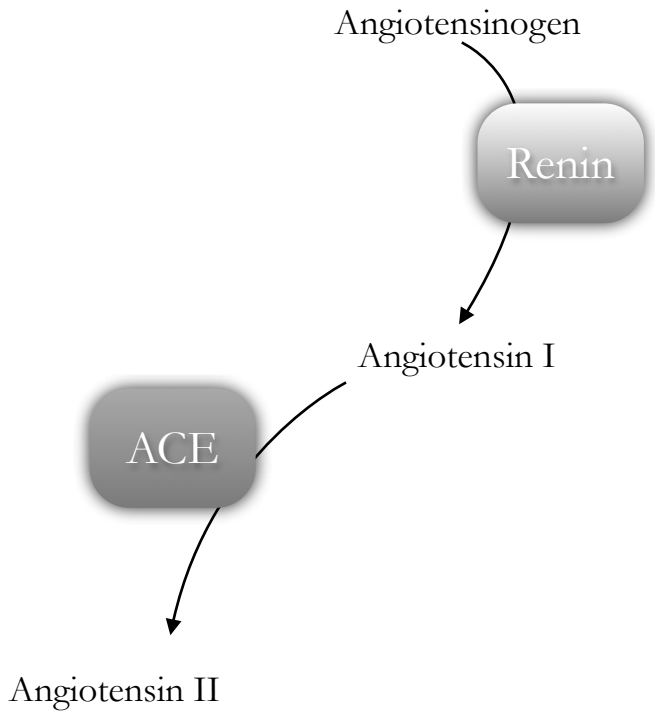

Figure 11. The classical renin angiotensin system pathway.

Renin is an enzyme secreted from the afferent arteriolar granules of the juxtaglomerular cells of the nephron. There are three major triggers for the release of renin from these cells; Stimulation of renal sympathetic nerves via β1-adrenoceptors, fall in renal perfusion pressure and a reduction in distal tubular salt delivery to the macula densa. Angiotensinogen is a protein made in the liver and is converted by renin to angiotensin I. Angiotensin I is then hydrolyzed to the active peptide angiotensin II by ACE, which is a membrane bound protein, primarily present in the endothelium of the lungs [40].

Angiotensin II is a potent vasoconstrictor, and a promoter of aldosterone secretion from the adrenal zona glomerulosa. Angiotensin II causes vasoconstriction increases in peripheral vascular resistance and elevates systemic blood pressure. Angiotensin II diminishes salt and water excretion in the kidneys and causes increased reabsorption of both salt and water from the tubular fluid [16].

2. Newer Aspects of the Renin-Angiotensin System

Research in the RAS is ongoing and insight into new components and interaction between both the new and the classic components of RAS are constantly being reported. The system is becoming more complex and an overview of it is shown in figure 12.

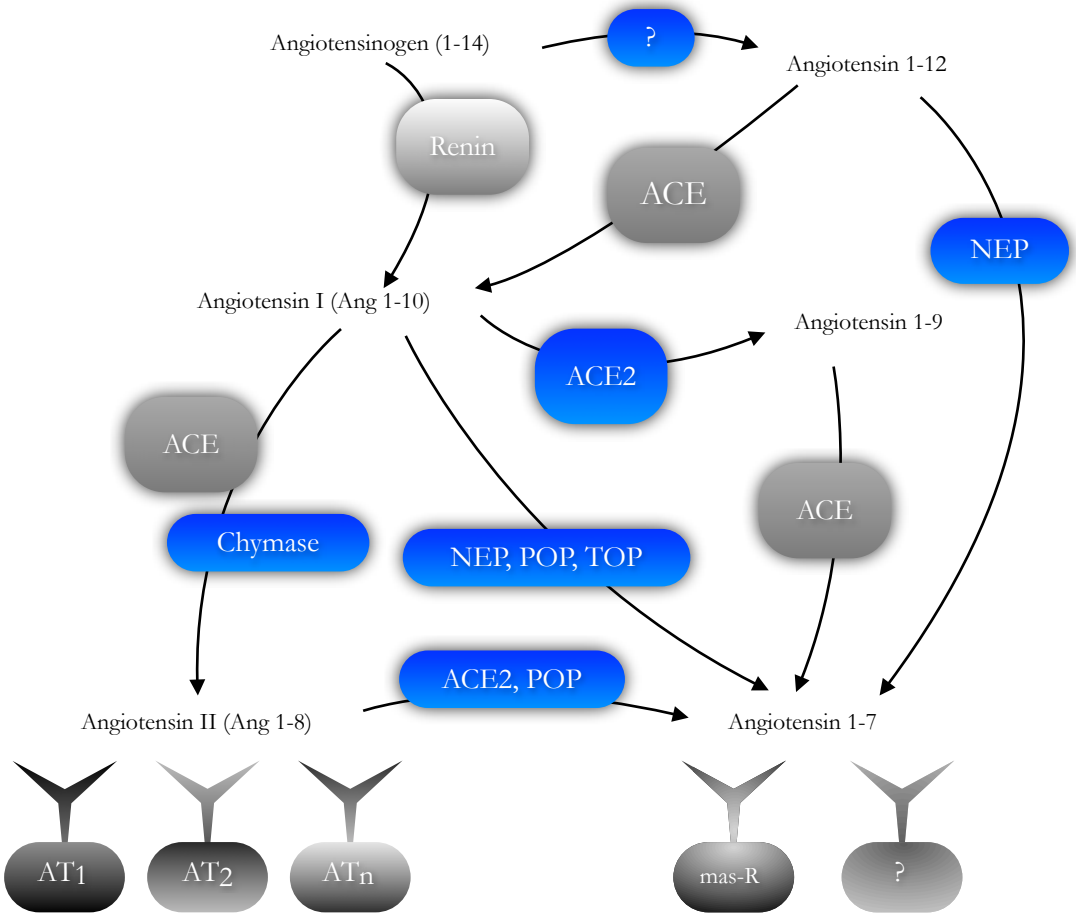

Figure 12. The novel renin angiotensin system pathway and some receptors. NEP: neutral endopeptidase; POP: prolyl oligopeptidase; TOP: thimet oligopeptidase. Ang 1-10 and Ang 1-8 are alternative names for angiotensin I and angiotensin II. 1-14 behind angiotensinogen represents the number of amino acids in angiotensinogen. Adapted from [23, 43].

Angiotensin converting enzyme 2 (ACE2) is a human homologue of ACE and shares 42% of the catalytic domains of ACE. ACE2 hydrolyzes angiotensin I to angiotensin 1-9 that again can be hydrolyzed to angiotensin 1-7 via ACE. ACE2 does not cleave bradykinin to inactive peptides and is not inhibited by ACEis [41-43]. Several endopeptidases can also participate in formation of angiotensin 1-7 [23]. Angiotensin 1-7 inhibits proliferation of smooth muscle cells in damaged carotid arteries in rats [44], and causes vasodilation of porcine coronary arteries through release of nitric oxide (NO) [45]. The Mas-receptor is

one of the known receptors for angiotensin 1-7 [42]. Chymase is also able to convert angiotensin I to angiotensin II [46]. The physiologic importance of these alternative pathways are and effects are not fully known in animals and to an even lesser extent in humans.

3. RAS and the Kallikrein-kinin System

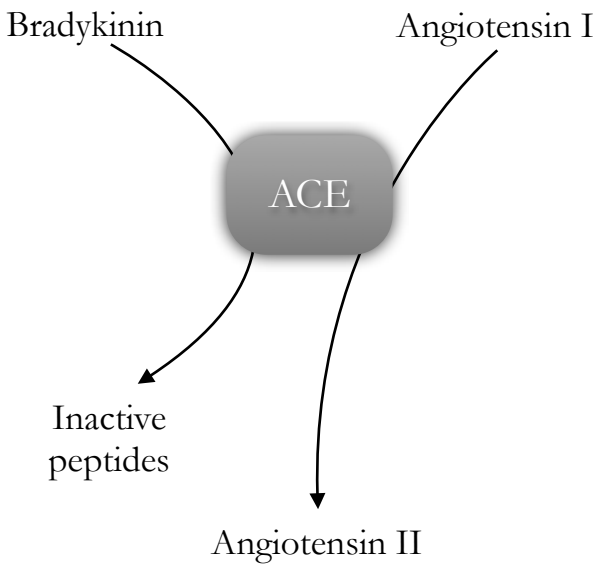

Figure 13. ACE both converts angiotensin I to angiotensin II and degrades bradykinin to inactive peptides..

Apart from converting angiotensin I to angiotensin II, ACE also degrades bradykinin to inactive compounds. RAS and the kallikrein-kinin system are thus intertwined. Both ACE and the bradykinin subtype 2 (B2) receptors can be found on the luminal side of the endothelium [47].

There is evidence that some of the positive effect of ACE-inhibition could be mediated not only by a fall in angiotensin II but also because of a local accumulation of bradykinin [48]. In one study in humans with hypertension it was found that ACE-inhibition caused a fall in systemic blood pressure, a fall in circulating angiotensin II and a rise in circulating kinins [49]. A report from 1994 suggested that ACEis potentiate the response in coronary blood flow to bradykinin in a way that is independent of ACE it self and is caused by an interaction between the ACEi and the B2 receptor [50]. This theory was substantiated by the observation that ACEis decreased endothelial bradykinin degradation which is accompanied by an increase in endothelium-dependent relaxation caused by bradykinin. In the same study ACEis also initiated endothelium-dependent relaxation in the coronary arteries that were stimulated with threshold concentrations of bradykinin. This relaxation could not be attributed to an inhibition of bradykinin [51].

The role of bradykinin via the B2 receptor in the blood pressure lowering effect of ARBs in hypertensive patients has been investigated by combining a B2 receptor antagonist to the ARB treatment. The results showed that the B2 antagonist did not block the blood

pressure lowering effect of the ARB valsartan [52]. The interplay between the B2 receptors and ACE inhibition or AT1 blockade is the subject of the first study in this thesis.

Bradykinin can via the B2 receptor cause NO synthesis via nitric oxide synthase (NOS). NO can relax vascular smooth muscle cells via guanylyl cyclase activation and a subsequent cGMP generation [53-55]. Bradykinin can also via its receptor lead to an increase in intracellular Ca^{2+} that causes activation of a phospholipase that liberate arachidonic acid from phospholipids in the membrane. The arachidonic acid is in turn changed to epoxyeicosatrienoic acid (EET) via a cytochrome P450 epoxygenase. EET can modulate the Ca^{2+} sensitivity of Ca^{2+}-dependent K^+ channels and via adenylyl cyclase can cause increased generation of cAMP. EETs and its metabolites can diffuse to smooth muscle cells in the vessel and activate large conductance K^+ channels that cause an efflux of K^+, hyperpolarization of the smooth muscle cell and subsequent relaxation [56].

4. RAS-blockers

Blocking elements of RAS is now common in antihypertensive therapy. The discovery of a substance that blocks ACE [57] led to the marketing of the first ACEi, captopril in 1981. The first ARB, losartan was marketed in the USA in 1995. Several ACEis and ARBs are now available to treat hypertension and the first renin-blocker, aliskiren, became clinically available in 2007. Renin-blockers will not be described further here.

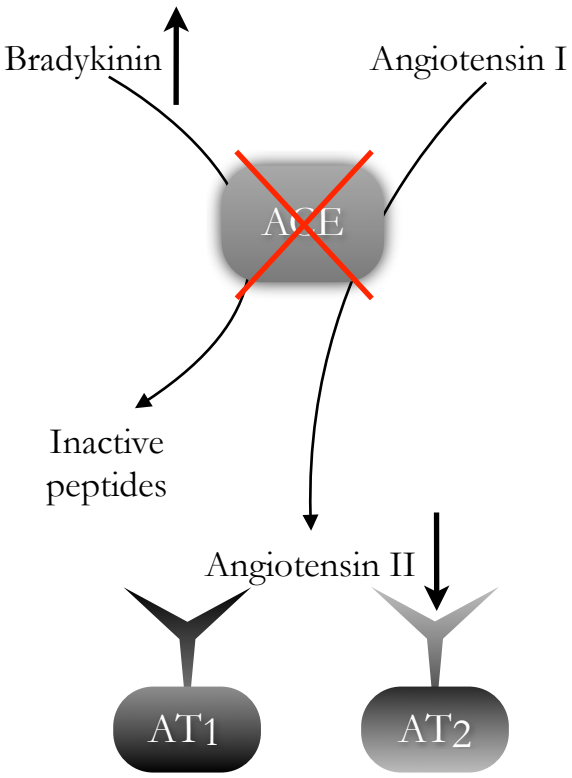

Figure 14. Effect of ACE-inhibition, bradykinin levels rise and angiotensin II levels fall.

ACEis are effective in treating hypertension. They cause as described above a fall in circulating angiotensin II and an increase in circulating bradykinin [49]. The ARBs like losartan and eprosartan cause an increase in circulating angiotensin II levels [58, 59]. Most ARBs do not cause an increase in

bradykinin apart from losartan that, in one study in hypertensive patients was shown to cause an increase in circulating bradykinin whereas the ARB eprosartan in the same study did not cause such an increase [59]. Some of the adverse effects of ACE-inhibition has been linked to an increase in bradykinin. Reports vary but an estimated 5 - 10% of patients on ACE-inhibitors will suffer from cough [60]. The explanation for the cough is most likely due to an increase in bradykinin due to the inhibition of ACE that is abundant in the pulmonary vessels. Fortunately this adverse effect disappears in all subjects within weeks after termination of the treatment [61].

The ARBs used in clinical practice to treat hypertension are selective AT1 receptor blockers [62, 63]. The increased amounts of circulating angiotensin II can thus bind to other angiotensin II receptor subtypes when ARBs are used. In this thesis the term ARB is used for angiotensin receptor blockers that block the AT1 receptor unless stated otherwise.

5. The Angiotensin II Receptors

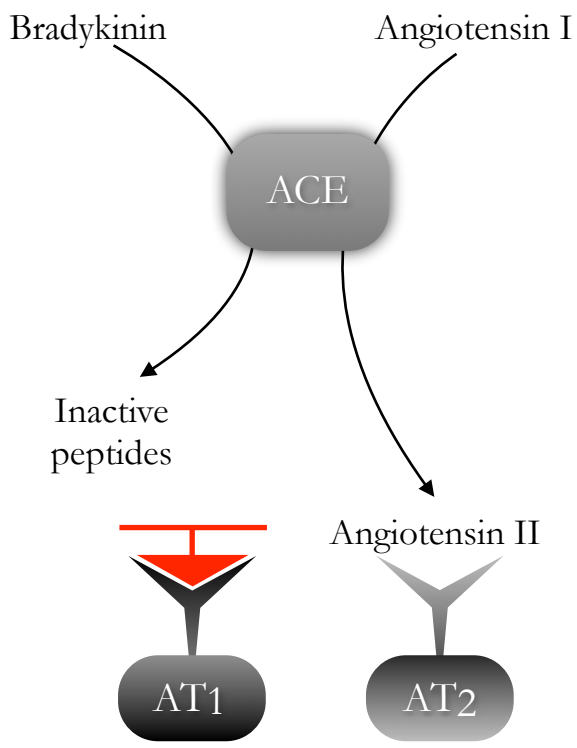

Figure 15. Effect of ARB-blockage, bradykinin and angiotensin II levels are not affected and angiotensin II binds to the AT2-receptor

Angiotensin II has a certain degree of duality depending on which subtype of angiotensin II receptor it binds to. Several angiotensin II receptor subtypes are known but the two main receptor subtypes that have been investigated are the AT1 and the angiotensin II subtype 2 (AT2) receptor. In rats the AT1 receptor has two isoforms, type A and type B, with 94% sequence identity [64]. Other angiotensin II receptors are known but their role has not been fully investigated and the focus will hereafter be on the AT1 and the AT2 receptors which are the best known angiotensin II receptors.

The effect of angiotensin II is determined by the receptor subtype it interacts with. When bound to the AT1 receptor, angiotensin II is a potent vasoconstrictor, has pro-inflammatory effects, and is also involved in electrolyte and water balance, thirst, hormone secretion and renal function [65-68]. When bound to the AT2 receptor angiotensin II probably opposes many of the effects of the AT1 receptor; by causing vasodilation, being anti-inflammatory and aiding in neuronal regeneration [69-71].

AT1 receptors are, not surprisingly, found in the vasculature and are most abundant in the smooth muscle cells and to a much lesser extent in the adventitia [72]. The AT1 receptors have been found in both the endothelium and smooth muscle cells of rat cerebral arteries [14]. In the kidney the AT1 receptor is most abundant in the interlobular arteries, followed by the tubulointerstitial fibrous regions surrounding those arteries and the glomeruli, and to a lesser extent in the glomeruli and the cortical tubules [73]. The AT1 receptor is found in the human endometrium and in the pregnant female can be found in the cytotrophoblast, syncytiotrophoblast, the extravillous trophoblasts and around the blood vessels in the placental villi [74, 75]. In the lung the AT1 receptor is found in the smooth muscle cells in the vasculature, in macrophages and in the stroma that lies under the airway epithelium [76]. In the brain AT1 receptors can be found in the areas that regulate autonomic and hormonal responses [77].

The AT2 receptor is not abundant in the adult human but it is expressed in several tissues in the fetus [78]. The receptor has been found in the brain, adrenal gland, heart, kidney, myometrium and the ovary. The interesting fact about the AT2 receptor is that under pathological condition it is upregulated. This has been shown in vascular injury, myocardial infarction (MI), congestive heart failure, kidney failure and brain ischemia [79-83]. It has also been shown that the AT2 receptors are important in stroke protection. The ARB irbesartan has been shown to elicit protection against stroke in ischemic stroke models. This effect is blunted during simultaneous AT2 receptor blockage. [84].

The exact mechanism of many of the effects mediated via the AT2 receptor are still not known. An overview of the effects of angiotensin II when bound to either AT1 or AT2 can be seen in table 1.

AT1 receptor Effect	AT2 receptor Effect
Vasoconstriction preferentially in coronary, renal and cerebral arteries	Antiproliferation
Sodium retention	Inhibition of cell growth
Water retention via vasopressin release	Cell differentiation
Renin-suppression via a negative feedback loop	Tissue repair
Hypertrophy in myocytes and smooth muscle cell	Apoptosis
Stimulates fibrosis of vasculature and myocardium	Vasodilatation
Inotropic, contractile in cardiomyocytes	Development of kidney and urinary tract
Chronotropic, arrythomgenic in cardioyocytes	
Stimulates plasminogen activating inhibitor-1 (PAI1)	
Activates sympathetic nervous system	
Increases endothelin secretion	

Table 1. Effect of angiotensin II mediated via AT1 and AT2 receptors. Modified from [63].

IV. Autoregulation of Cerebral Blood Flow

The intrinsic ability of an organ, or the vascular bed of an organ, to maintain adequate and in some cases constant blood flow despite variations in systemic blood pressure and perfusion pressure is called autoregulation. CBF autoregulation has been described in two terms depending on the recording method, steady state CBF autoregulation and dynamic CBF autoregulation, the latter to a great extent also representing the speed with which the autoregulation takes place. Steady state autoregulation will be described here and I will just use the term autoregulation.

CBF autoregulation has both upper and lower limits. When MAP is between the limits of autoregulation the organism is able to maintain a steady CBF. However when MAP exceeds either the lower or upper limit of autoregulation changes in CBF ensues. With increasing MAP above the upper limit the autoregulatory mechanisms of the vasculature of the brain cease to be able to maintain CBF at normal levels and an increase in CBF ensues. This may play a role in the pathogenesis of hypertensive encephalopathy [85-87]. On the opposite side, when MAP falls below the lower limit a fall in CBF is certain. The brain can to some extent compensate for this by increasing oxygen extraction from the blood but with a further fall in blood pressure there is risk of ischemic damage. CBF autoregulation is essential not only under physiologic conditions but also in several pathophysiological instances. An example is periods of hypotension where CBF is maintained even at severely low MAP where other organs might cease to function properly but cerebral ischemia is prevented due to the effective CBF autoregulation. Another example is in a sudden surge in MAP where damage to the fragile capillaries of the CNS vasculature might take place if the autoregulatory mechanisms would not raise CVR to maintain adequate CBF [31]. An autoregulatory curve can be seen in figure 16.

It is important to note that both the upper and lower limits and the plateau itself are not always fixed to a certain MAP but can be modulated in several ways. In healthy animals and individuals the reported lower limit is at 50 - 60 mmHg and the upper limit is at 150 - 160 mmHg [37, 88-90]. These limits can be shifted towards higher blood pressure levels in patients with chronic hypertension [91] and during sympathetic stimulation [92]. Medical intervention with compounds such as ACEis can shift the limits of CBF autoregulation to the left, toward lower blood pressure [12, 93]. Also, the level of CBF influences the limits of autoregulation. For instance, with CO_2 inhalation, CBF is increased and the

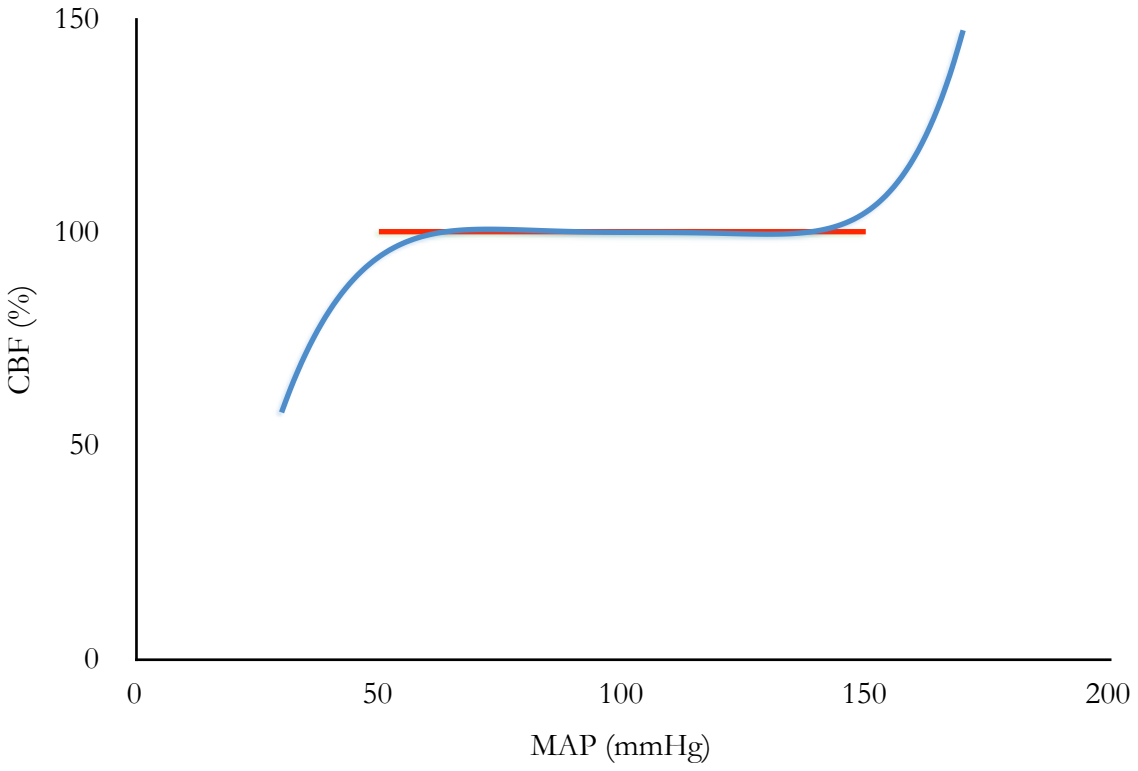

Figure 16. An example of an autoregulatory curve. CBF is expressed as percentage of normal CBF levels on the y-axis and MAP in mmHg on the x-axis. The autoregulatory curve is shown in blue while the plateau of CBF is shown in red. Blood pressure levels outside of the red line are either lower than the lower limit of CBF autoregulation if the values are to the left or higher than the upper limit if the values are on the right side of the plateau.

autoregulatory plateau is narrowed: the lower limit is raised, and the upper limit is lowered. Conversely, during hyperventilation with hypocapnia, CBF is lowered, and the plateau is widened, the lower limit lowered, and the upper limit raised [94]. CBF autoregulation can be impaired in patients with long lasting type I diabetes with clinical microangiopathy [95].

Several mechanisms have been proposed to explain autoregulation and it is known to be modulated by several other factors. The basic mechanisms proposed are myogenic, metabolic and neurogenic, and possibly endothelial, and modulators include perivascular nerves, the RAS and the actual level of CBF. This will be briefly reviewed below.

Mechanisms of control of the cerebral circulation are discussed in this chapter only in the context of their importance for autoregulation.

1. The Neurogenic Hypothesis

The theory for neurogenic regulation assumes that nerve stimulation triggers vasoconstriction or vasodilation depending on the changes in transmural tension [96].

The vasculature of the brain is surrounded by nerves both from the peripheral and the central nervous system. The extracerebral vessels are innervated by the peripheral nervous system. As these same vessels penetrate into the CNS and cross through the Virchow-Robin space the nerves from the peripheral nervous system, the "extrinsic innervation", cease to follow the vessel and as the vessel emerges from the Virchow-Robin space, it receives neural input from neurons that are located within the brain itself - the "intrinsic innervation" [97]. The extrinsic innervation has its origin in either the superior cervical ganglion where the sympathetic stimuli originates or in the sphenopalatine or the otic ganglia where parasympathetic activity originates. The intrinsic innervation stems from either subcortical pathways, originating from the locus coeruleus, raphe nucleus and the basal forebrain; or from local intercortical neurons [97-99]. Common with both the intrinsic and the extrinsic perivascular nerves is the fact that they lack the classical synaptic junction at the site of contact with the blood vessel [100]. Local CBF is affected and the microvascular tone is regulated via activity in the intrinsic nerves [101]. The extrinsic nerves are involved in regulation of the "inflow tract" and probably influence autoregulation only at very low and very high blood pressure [102]. This seems to be the clearest effect of perivascular nerves on CBF autoregulation.

2. The Myogenic Hypothesis

According to the concept of myogenic autoregulation the smooth muscle cells of the vessel wall react within seconds to any changes in transmural pressure. It assumes that the vessels of the brain constrict with increasing transmural pressure and dilate with decreasing transmural pressure. The transmural pressure is dependent on CPP, the higher the CPP the higher the transmural pressure is and inversely when CPP is low the transmural pressure is also low [103].

The close proximity of the endothelium and the smooth muscle cells in the vessels indicates that the myogenic mechanism may be linked to endothelial mechanisms and vice versa. There are however several studies which argue that the myogenic autoregulation is independent of the endothelium [104, 105].

3. The Metabolic Hypothesis

The brain is dependent on a steady and sufficient supply of energy to function properly [106]. A local increase in nerve cell activity causes increased metabolic demand and an

increase in local CBF follows [107]. Parenchymal cells release or take up local chemical factors that in turn change the composition of the interstitial fluid. These changes influence membrane potentials and conduction and are therefore able to cause contraction or dilation in the smooth muscle cells of the vessels and ultimately cause changes in CBF.

The metabolic regulation is dependent on a substance that is both vasoactive in cerebral vessels and the changes in the concentration of the substance are dependent on neuronal activity [108]. In simple terms the concept of metabolic autoregulation assumes that the vessels of the brain dilate or contract in response to lack of nutrients or accumulation of metabolites in the vessels.

Many possible mediators of chemical autoregulation have been put forward, including CO_2, O_2, H^+, and adenosine.

CO_2 is a potent vasodilator in cerebral vessels and is an end product of cerebral metabolism [109]. The response in CBF to changes in pCO_2 is not constant but dependent on pO_2, MAP and age [110]. Several ideas about the precise mechanisms of the effect of CO_2 on CBF exist however, consensus is lacking. Still there is an agreement that the vasodilation caused by CO_2 is mediated via H^+ [111] and that the effect of extracellular pH is probably more profound than intracellular pH changes [112].

The influence of changes in CO_2 on CBF can be influenced depending on the O_2 levels. During severe hypoxia, increases in CO_2 levels cause a decrease in CBF [113]. Low O_2 levels also causes vasodilation on its own via some local mechanism and might be the dominant force behind the vasodilation seen in hypotension as this effect was eliminated if the local tissue is hyperoxic [114].

Studies with erythrocytes have shown that they release adenosine triphosphate (ATP) in increasing amounts with decreasing oxyhemoglobin concentrations [115]. An interesting study supports the theory that increasing amounts of ATP in the collecting venule reflects a vasodilator response that is conducted upstream through the capillary network and the arteriole supplying the capillary bed in question [116]. In vitro studies showed that micro-application of ATP into cerebral arterioles caused a biphasic response of vasoconstriction followed by vasodilation and a conducted vasomotor response that traveled along the vessel and preceded the dilatory response [117].

The mechanisms described above can act individually, together or even have opposing effects on CBF depending on the physiologic circumstances. This means that under normal physiologic conditions where cerebral autoregulation is intact the cerebral vessels have vasomotor ability that alters their caliber to maintain constant CBF in spite of changes in CPP.

4. Autoregulation via Endothelium

The theory for endothelial autoregulation assumes that the endothelium releases vasoactive substances to influence the tone in the vascular wall. An intact endothelium not only plays a role in preventing thrombosis in the vessel but also produces vasoactive compounds that can either cause vasodilation or vasoconstriction which in turn causes changes in CBF [118-120].

Many vasoactive substances have been found in the endothelium, among the factors causing vasodilation are NO, endothelium-derived hyperpolarization factor, prostacyclin and prostaglandin E2 [121-123] while endothelin 1 (ET-1), prostaglandin F2α (PGF2α) and thromboxane A2 are among the vasoconstrictors [123-126].

An important note is that some of these substances have variable effect depending on the concentration of the substance and the place of action. ET-1 given in doses between 10 - 100 pmol injected in the carotid artery had vasodilatory effect, whereas it becomes a potent vasoconstrictor when given at amounts over 300 pmol [125]. In another more recent study the high doses (60, 150 and 300 pmol) of ET-1 caused a reduction in CBF when injected into the vicinity of the middle cerebral artery [127].

5. RAS and Autoregulation

The RAS has a role in regulation of CBF. Carbonic anhydrase inhibitor dilates the parenchymal resistance vessels of the brain to a maximum in rats. An increase in CBF was observed when an ACEi was added to the carbonic anhydrase inhibitor. This shows that RAS has a modulatory effect on CBF and that this effect takes place in the larger resistance vessels of the brain [128]. Interestingly, an interaction between RAS and sympathetic nerves is present in the modulation of the upper limit of the autoregulation [92]. Autoregulation of CBF and RAS is the main subject of this thesis and will be discussed further in the next chapter.

V. Cerebral Blood Flow and the Renin-Angiotensin System

1. Animal Studies, Autoregulation

Many studies on the influence of RAS on CBF and CBF autoregulation have been conducted in animals. The "end product" of the RAS, angiotensin II, causes constriction of the pial arteries when given into cerebrospinal fluid and causes constriction of the pial arteries and raises blood pressure when given intravenously (i.v.) [129]. Angiotensin II did not increase CBF when given i.v. in doses that caused a 15 - 20% increase in blood pressure [130].

The effect of several ACEis on CBF autoregulation has been investigated. The ACEi captopril shifts both the upper and lower limit of CBF autoregulation towards lower blood pressure [12]. An interesting finding in that study was that the effect was greater on the upper limit than on the lower limit and the autoregulatory plateau therefore was shortened. Another important finding from that study was that the effect of the ACEi did not influence the level of resting CBF but only the limits of autoregulation. In another paper the authors found that captopril did not cross the blood brain barrier and that the modulation of the limits of autoregulation only took place when the ACEi was given i.v. and not when it was injected into the ventricles of the brain [131]. The authors concluded that the effect of ACEi takes place on the endothelium of the vessels of the brain. The ACEi ceranapril significantly shifted the lower limit of CBF autoregulation towards lower blood pressure in both Wistar-Kyoto rats (WKY) and in spontaneously hypertensive rats (SHR) [11].

As mentioned earlier ACE degrades bradykinin to inactive peptides [48]. When SPRD rats were given the ACEi captopril intravenously it caused a shift in the lower limit of CBF autoregulation. When a selective B2 receptor antagonist Hoe 140 was injected prior to captopril injection the shift of the lower limit to the left was blunted. This could mean that some of the effect that the ACEi have on the autoregulation of CBF is in part mediated via bradykinin that accumulates during ACE inhibition [132].

ARBs have also shown ability to shift the lower limit of CBF autoregulation towards lower blood pressure levels. The ARB candesartan lowered both the upper and lower limit of CBF autoregulation towards lower blood pressure levels in both WKY and in SHR [13]. The ARB valsartan also causes a shift in the lower limit of CBF autoregulation towards lower blood pressure levels [14]. As mentioned previously, one study has shown that the

ARB losartan raised the limits of CBF autoregulation towards higher blood pressure levels [15].

The role of circulating renin and the influence it has on the modulating effect of the ACEi captopril has been investigated by our group. In WKY rats that were nephrectomized and dialyzed for 48 hours to eliminate any circulating renin, captopril still caused a shift in the lower limit of CBF autoregulation towards lower blood pressure levels in both the nephrectomized and sham-operated animals [133]. Hence, the effect of captopril was dependent on components of the RAS in the vessel wall.

ACEis have their primary effect on CBF in the larger cerebral arteries and that indicates that there is angiotensin II mediated tone in these vessels [128]. It is assumed that the ARBs exert their effect in the same arteries.

2. Animals Studies, Stroke

Several studies on the effect of ACEis on stroke have been performed. Captopril given i.v. 30 minutes prior to ischemia, caused by unilateral ligation of the carotid artery and hemorrhagic hypotension to 35 mmHg for 30 minutes, resulted in a better neurological outcome than controls in SPRD rats [134]. In a study in stroke-prone spontaneously hypertensive rats (SHSPR) where the animals were divided into three different groups, the first group got the ACEi cilazapril in the drinking water, the second group got hydralazine with hydrochlorthiazide in the drinking water and the third group was not medicated. A control group consisting of WKY rats was included and they did not get medication. After three months of treatment one middle cerebral artery was occluded. One day after occlusion the neurologic deficit was less in the rats that were treated with antihypertensive medication. On the third day however the neurologic deficit was only significantly decreased in the ACEi treated rats. The infarct size was reduced in both groups that received antihypertensive medications. The WKY rats had a smaller infarction and a better neurological outcome than any of the SHSPR. The authors concluded that antihypertensive therapy reduces infarct volume in SHSPRs [135].

Lowering blood pressure in WKY reduces infarct size. This was shown in a study with four groups of rats. In all groups the animals got an occlusion of the middle cerebral artery for 3 hours. At reperfusion the animals were either treated with enalaprilat in doses that did not influence the blood pressure, enalaprilat in blood pressure lowering doses,

hydralazine, or vehicle. The results showed that only in hydralazine and enalaprilat in blood pressure lowering doses had beneficial effect in form of a smaller infarction [136].

A direct head to head comparison of an ACEi and an ARB in experimental ischemic stroke has also been performed. Ramipril and candesartan were compared in WKY rats. The animals were treated for five days with either candesartan or ramipril in doses that did not lower blood pressure during occlusion of the middle cerebral artery, and a ramipril dose that lowered blood pressure during the occlusion of the artery or vehicle. Rats treated with candesartan had a smaller infarction and a better neurological outcome than any dose of ramipril or vehicle [137]. In one study the ACEi captopril and the ARB candesartan compared favorably to the Ca^{2+}-channel blocker nicardipine with regard to infarction volume in SPRD rats. These animals were treated for 28 days and the ARB group also had a significantly smaller infarction volume than the ACEi [138].

When the ARB irbesartan was given intracerebroventricularly for five days to simulate longterm ARB treatment in WKY the effect on neurological outcome was favorable when compared to controls [139].

A study has been performed on the importance of the time of administration of an ARB in ischemic stroke in SPRD rats. It was found that a single dose of candesartan before middle cerebral artery occlusion caused a smaller infarction when compared to controls but did not result in a better neurological outcome. A single dose of candesartan after occlusion of the middle cerebral artery resulted in a smaller infarction than controls and the animals that received a single dose of candesartan before occlusion. These treated rats furhtermore had better neurological outcome. [140]. Similar results were found in SPRD rats that were treated with the ARB olmesartan 7 days prior to an occlusion of the middle cerebral artery [141]. The effect of long-term blockade of the AT1 receptor with the ARB candesartan has also been studied. In WKY rats where candesartan was either given 4 hours before ischemic injury, twice daily for five days prior to injury or vehicle. The effect of 5 day treatment was significantly better than the other regimens at preventing neurologic injury [142].

The positive effects of ARBs in ischemic stroke in animal models have been reconfirmed in other studies [143, 144].

ARBs also seem to have positive effects in intracranial hemorrhage. A study in normotensive rats showed that rats treated with the ARB telmisartan 2 hours after intracerebral hemorrhage had a better neurological outcome. This observed effect was thought to be mediated by anti-inflammatory effects and antioxidant benefits [145].

The AT2 receptors might have a role in limiting ischemia. When the middle cerebral artery in AT2 receptor deficient mice was occluded the size of infarction and extent of

neurologic deficit was greater than in wild type mice [146]. When SPRD rats were treated for 24 hours with the ACEi lisinopril prior to an embolic stroke produced by injection of calibrated microspheres of 50 μm in diameter into the right internal carotid artery there was a statistically increased mortality and volume of infarction when compared to controls. In a group that was treated with the ARB candesartan in equihypotensive doses, and was subjected to the same cerebral insult, there was a better outcome than the ACEi group however they did not fare significantly better than a control group. A 5 day treatment with the ACEi or the ARB resulted in similar blood pressure lowering. Here the ACEi had detrimental effect when compared to the control. Both infarct volume and mortality were statistically increased while the candesartan group had lower mortality and a better neurological score when compared to the control. When the ACEi was added to a 5 day ARB treatment 24 hour before the cerebral insult the positive effect of the ARB was blunted. When an AT2 and/or an AT4 antagonist was added to the 5 day candesartan treatment the positive effect of the ARB was blunted [147]. A study in gerbils, that lack a complete circle of Willis, showed that the mortality was reduced when treated with either the ARB losartan or the ARB candesartan compared to treatment with either the ACEi enalapril or the ACEi lisinopril prior to ligation of one of the carotid arteries. When an ACEi was added to an ARB the positive effects were blunted. The authors' conclusion was that angiotensin II might have beneficial effect that are mediated via non-AT1 receptors [148].

Candesartan and enalaprilat treatment in mice showed that the expression of AT2 receptors increased in the mice treated with candesartan and that they also had a smaller infarction and a better neurological outcome than the mice treated with enalaprilat [149].

There are some controversies on the effect of both ACEis and ARBs on stroke in animal models, since some studies in animals point to a cerebroprotective effect of ARBs not readily seen with ACEis [150-152]. In summary the beneficial effect that is seen in the ACEi studies suggests that the positive effect is possibly mediated through blood pressure lowering effect where as the ARBs might have an effect that cannot be explained by blood pressure lowering alone. Changes in the lower limit of autoregulation of CBF might play a role. It is also possible is that the ACEis lower the metabolic rate in the ischemic brain as shown in SHR rats where the levels of lactate were lower and ATP levels higher in brains from rats treated with the ACEis captopril and SQ 29,852 (ceranopril) [153]. It is possible that the positive effects seen in ARBs are mediated via angiotensin II when it is bound to the AT2 receptor.

3. Human Studies

ACE-inhibitors and ARBs are widely used in antihypertensive therapy and it has been shown in controlled trials that these drugs may have a beneficial effect beyond blood pressure lowering, in particular protecting against stroke [9, 10, 154, 155]. Several studies on the relationship between RAS and CBF have been conducted in humans.

One of the earliest was conducted in patients with severe heart failure where the ACEi captopril taken for at least 48 hours caused a fall in MAP and an increase in CBF [156]. In another study where the acute effect of the ACEi captopril was investigated in patients with severe heart failure. These patients were treated with a single dose of captopril 6,25 mg and healthy individuals were treated with 25 mg. In the heart failure group that had a marked fall in blood pressure there was a slight increase in CBF. However when a correction for a change in arterial CO_2 levels was applied the slight rise in CBF disappeared. The control group did not have a fall in blood pressure and the CBF although showing a decline it was not significant [157]. The acute effect of captopril has been investigated in several studies with the same results i.e. CBF remains stable in spite of a fall in MAP [158]. In another study captopril was used for three weeks in hypertensive patients without a history of stroke. The findings were that the blood pressure levels were lowered during captopril treatment and that CBF remained unchanged [159]. In a study where captopril was used for a total of three months in hypertensive patients, it was found, after one month that CBF increased statistically and after another three months the CBF had returned to baseline levels. Measurements of muscle blood flow were undertaken at the same time as the CBF measurements and these measurements did not change during the study period [160].

One study in humans has been performed to assess the lower limit of CBF autoregulation. In healthy volunteers the lower limit of CBF autoregulation was shifted towards lower blood pressure limit, the difference was however not significant. In a group of hypertensive patients the lower limit fell in five of the subjects with a median value of 22 mmHg whereas the lower limit rose with 3 and 13 mmHg respectively in two of the patients [161]. Another study from the same laboratory investigated the effect of a single captopril dose 5 days after a stroke. In this study there was no effect on MAP or changes in CBF [162]. The ACEi perindopril has also been investigated in a study in stroke patients. In that study 24 patients with a recent stroke were randomized to either perindopril or placebo in a double blind study. The patients treated with perindopril had lower blood pressure after 2 weeks of treatment and no changes in CBF when compared to controls. There was no difference in neurologic outcome [163].

In patients with normal-pressure hydrocephalus the effect of captopril on blood pressure, intracranial pressure (ICP), CBF and CBF autoregulation was investigated. The results showed that after one hour blood pressure was lowered while CBF and ICP remained unchanged. The lower limit of CBF autoregulation was also assessed and it was found that the lower limit was shifted towards lower blood pressure levels [164].

The ACEi enalapril did in humans with moderate hypertension only lower blood pressure without affecting CBF [165]. Enalapril has also been shown to increase CBF in patients with heart failure after four weeks of treatment [166].

The ACEi fosinopril also lowers blood pressure while CBF remains stable after long term treatment in patients with moderate hypertension [167].

Very few studies of the effect of ARBs on CBF have been published. In one study where the ARB candesartan was used in patients with diabetes and hypertension the flow in the middle cerebral arteries increased after 3 - 4 months of treatment and this effect was independent of the changes in blood pressure [168].

There are several studies that assess other aspects of CBF with more hard endpoints such as stroke or mortality due to stroke. In the LIFE study the effect of the ARB losartan was compared to the β-blocker atenolol. The study enrolled over 9.000 patients that were followed for at least 4 years. With regards to fatal or non-fatal stroke the losartan group fared significantly better than the atenolol group.

Some evidence points to a positive effect of antihypertensive medication that increases angiotensin II levels. It has been suggested that ARBs, by way of increasing angiotensin II and influencing neuroprotective AT2-receptors in the brain, affords better stroke protection than ACE inhibitors [169]. However a recent meta-analysis of controlled clinical trials, found no difference between ACEis and ARBs with respect to stroke prevention, both groups of drugs being equally superior to placebo [170].

4. Conclusion

From animal models it is apparent that both ACEis and ARBs exert modulation of CBF autoregulation towards lower blood pressure levels. The same has been shown in humans regarding ACEis and for the ARB candesartan. When the lower limits of CBF autoregulation are shifted towards lower blood pressure levels it is to be expected that a patients tolerance against a cerebral insult would be raised. A fall in CPP due to hypotension or other causes, demands an adequate fall in CVR to maintain satisfactory CBF. The lower the lower limit of CBF autoregulation is the more efficient the response in

CVR is with falling CPP.

From large clinical trials it appears that the most important factor in preventing stroke is to lower blood pressure. There is however some data that suggest that the ARBs have a more positive effect than can be explained from the blood pressure lowering effect alone. Some evidence point to a positive effect of the medications that not only lower blood pressure but also cause an increase in angiotensin II [169, 171]. It is possible that a shift in the lower limit of CBF autoregulation towards lower blood pressure levels is part of this positive effect. The role of the AT2 receptor is interesting and a neuroprotective effect via stimulation of this receptor is certainly a possibility. Such a mechanism may have been stimulated by losartan in the LIFE-study rather than an effect on autoregulation, sicne losartan contrary to other ARBs may shift the lower limit of CBF autoregulation towards higher blood pressure [15].

In conclusion, several human studies show that chronic treatment with ACEi and ARB lowers the blood pressure with little effect on CBF. Such treatment however, shifts the lower limit of CBF autoregulation towards lower pressure.

VI. The Laser-Doppler Method and Calculation of the Lower Limit

1. Laser-Doppler Measurements

Several methods are available to measure CBF. Some of the techniques are suited for laboratory animals while others can be used in clinical practice. They all have their advantages and disadvantages.

The method used in our studies was the laser Doppler flowmetry (LDF). As the name implies it uses the Doppler effect to measure flow velocity [172]. LDF is an optical technique, similar to NIRS [173], and it is independent on the angle at which fluids are flowing and thus differs from the conventional acoustic Doppler that has found widespread use in clinical medicine. In vivo experimental animal CBF-studies frequently use LDF where the effect of medication on CBF is investigated [14, 132], in studies of the influence of infection such as meningitis [174], in stroke models [127], in models of liver failure [175] or in studies in traumatic brain injury [176]. The method has been validated in CNS in several studies [177-179].

To use LDF for studies of CBF it is important to use a cranial window or to thin the bone of the cranium. In our studies a cranial window was used and care was taken not to damage the dura mater. The laser is a near-infrared light with a wavelength of 780 nm (others wavelengths are available) and the light is pointed towards the brain. The photons from the laser are scattered according to the Doppler effect by moving particles, the erythrocytes, in the brain tissue [172]. The probe is made up of a transmitter and a receiver and the output of the measurements from the probe is computed to ensure the linear relationship between actual blood flow and the perfusion signal [180]. The shift in frequency due to random scattering when the photons hit the moving erythrocytes enables calculation of their velocity. The photons that hit nonmoving structures do not shift in frequency and the ratio between the frequency shifted and non-frequency shifted gives a measurements for the moving particles.

The resulting equation is:

$$\text{Flow} = \text{NBC} * \text{MV}$$

where:

NBC is the number of blood cells moving

MV is the mean velocity of moving blood cells

The measurement obtained this way is an arbitrary number, usually called perfusion units and the units normally applied with CBF measurements (ml * 100 g^{-1} * min^{-1}) cannot be used in this setting. The measurements are converted to percentages where the plateau of CBF is given the value 100%.

Several factors influence the tissue depth that the LDF can penetrate and measure: the properties of the tissue measured, the probe, the separation of the fibers and the wavelength of the laser. The longer the wavelength the deeper into the tissue the laser penetrates. In experimental models it measures the blood flow up to 3 mm into the cortex. Due to the arbitrary nature of the perfusion units it is imperative that the same probes and wavelengths are used throughout the studies. The ease of use and the high temporal resolution as shown in figure 17 are the main advantages of LDF.

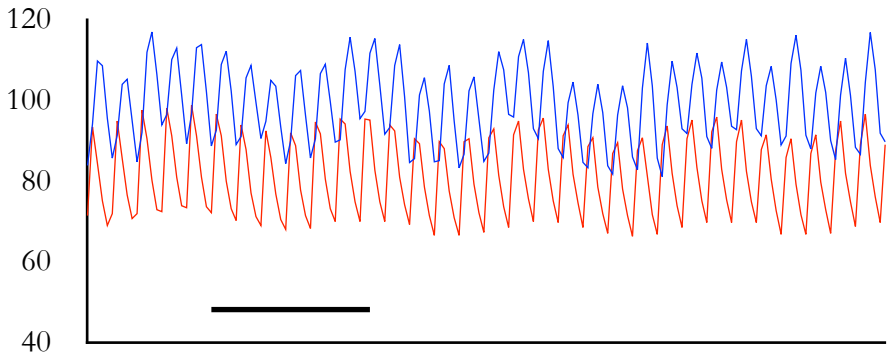

Figure 17. LDF measurements from one of the experiments. The Y-axis represents both CBF in percentage of the plateau in blue and blood pressure in mmHg in red. The black bar represents one second.

2. Assessing Autoregulation of Cerebral Blood Flow

There are two different type of analysis for autoregulation of CBF:

1. Steady state CBF autoregulation
2. Dynamic CBF autoregulation

Studies of steady state CBF autoregulation examines the relationship between CBF and MAP without taking the time domain into account. Studies on dynamic CBF autoregulation on the other hand are aimed at investigating the transient changes that take place in CBF in response to sudden changes in MAP [39].

The studies in this thesis are based on analysis of steady state CBF autoregulation. Therefore only this method will be described. This method has been used in our laboratory in many settings studying a wide variety of different mechanisms underlying modifications on CBF autoregulation [13, 133, 174, 179, 181]. The LDF method was used to collect CBF data in the last three studies.

In the classical autoregulatory curves drawn by Lassen (figure 10) the blood pressure interval with an intact autoregulatory response showed CBF values depicted as a flat horizontal plateau [37]. The concept that the plateau was conceived as being perfectly flat in many ways dominated research in CBF autoregulation. This view has been put into question and mathematical models suggest that perfect autoregulation would require some feedback gains that are greater than those found in most biological systems [182-185]. The possibility to use thousands of measurements during each autoregulatory experiment instead of tens of measurements has further elucidated the nature of the plateau.

Before advanced computing made its inroad into science the limits of CBF autoregulation was assessed visually [37, 91]. The use of computer programs has made this assessment easier and more reliable. Both the visual assessment and the calculated assessment of the lower limit require paired observations of MAP and CBF. The steps involved in the assessment of the lower limit of CBF autoregulation are shown in figures 18 to 20.

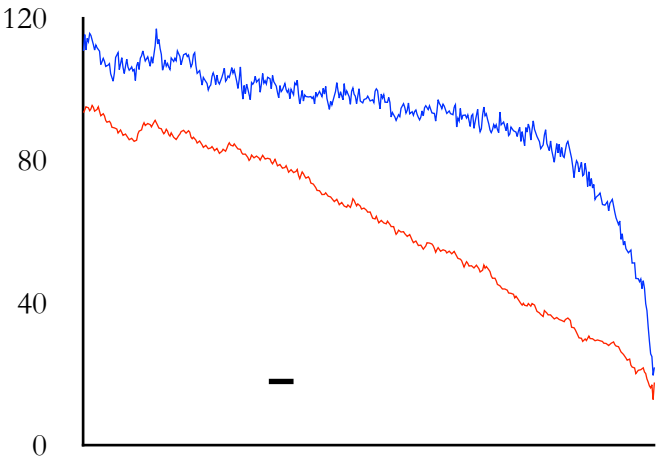

Figure 18. A graph showing CBF (in blue) and MAP (in red) during controlled bleeding. The black bar represents one minute.

Figure 18 shows data from one of the experiments. Here CBF was measured with LDF through a window in the cranium and blood pressure was measured via arterial catheter in a femoral artery. In the example shown, CBF falls slightly during the controlled bleeding that lowers systemic blood pressure. At about 35 mmHg the autoregulatory mechanisms are exhausted and we see a steep fall in CBF. It is very difficult to determine an exact lower limit using figure 18 alone. An easier method is using the autoregulatory

curve. Figure 19 shows an autoregulatory curve from the data used in figure 18. The paired CBF and MAP values are plotted on an XY-plot. CBF values are shown on the Y-axis and the values are given in percentage of CBF at plateau level. On the X-axis MAP values are given in mmHg. The plateau does have a slight slope but autoregulatory function is judged to be intact.

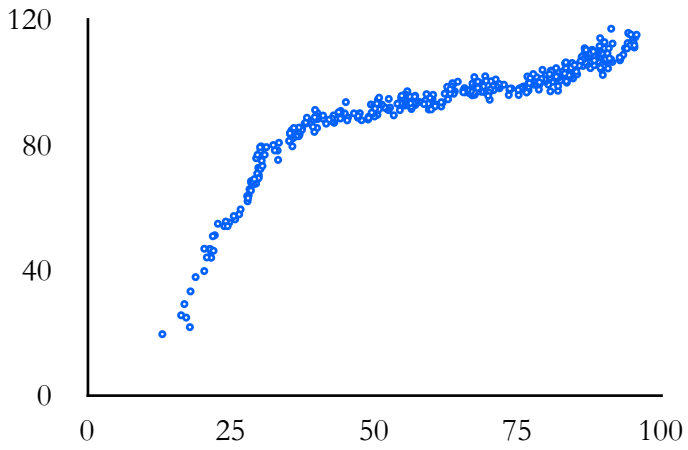

Figure 19. A XY-plot showing the relationship between CBF and MAP visualizing an autoregulatory curve. Same data as in figure 18. CBF is shown on Y-axis and MAP is shown on the X-axis.

As blood pressure falls a "knee" can be seen in the autoregulatory curve as the blood pressure levels reach 30 - 40 mmHg. As blood pressure falls below this value, CBF falls dramatically and autoregulation has certainly ceased to function. It is possible to assess the lower limit visually by drawing a line from the "knee" towards the X-axis to find the blood pressure levels that define the lower limit of CBF autoregulation. This method has its drawbacks. In some cases the "knee" is not well defined and the assessment becomes more subjective than objective.

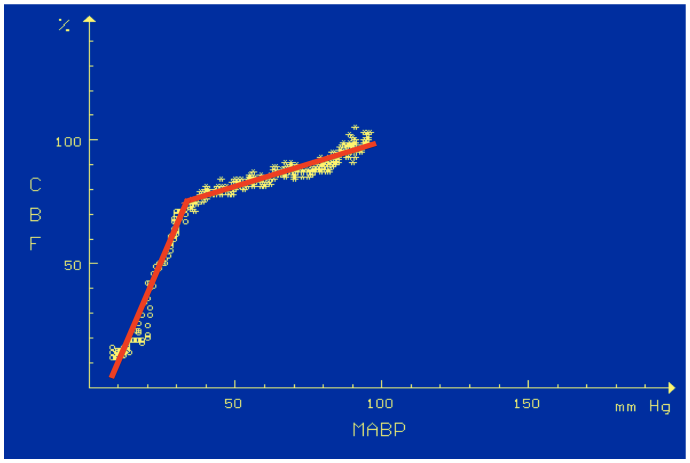

Figure 20. Computer analysis from the same data as in figure 18, showing the lines representing both the plateau and the slope, lower limit of CBF autoregulation was calculated to lie at 34 mmHg. For clarity the lines for both the slope and the plateau have been augmented.

Using a computer program to define both the slope and the plateau gives more reliable results and reduces the risk of human error while defining the lower limit. Figure 20 shows an image capture from a computer program designed to calculate the limits of CBF autoregulation. The computer program has been used in several settings at our laboratory [133, 174, 181] and the mathematical models the program uses have been described [133, 186]. In the previous studies the plateau has been defined as a horizontal line. In the present study we have taken the advantage of the many measured data sets obtained with the LDF method and allowed the plateau to have a slope.

The program as used in the present study draws 2 regression lines, one for the slope and one for the plateau. At least two data points are used for each regression line and then every other possible regression line combination is tested (slope i points, plateau n-i points, $i > 2$). The best fit is defined as the fit of the two regression lines with the sum of least squares [133]. Constrains are used, either for the slope or the plateau regression line forcing that regression line to cross the other in the blood pressure interval between the last point on the slope (i) and the first point on the plateau (i+1) [133, 186]. In the studies presented in this thesis the slope was constrained. Using constrains on the slope or the plateau regression line yielded essentially the same results because of the many data sets acquired by the LDF method.

Two methods have been put forward to give a better estimate of the lower limit. The first one was introduced in 1992 and it defines the regression lines for the slope first by introducing additional points (i) into the regression line until the sum of least squares for the two regression lines (slope and plateau) is found. The plateau is then constrained to lie between blood pressure point corresponding to i or i+1 [186]. A similar method can be used to constrain the slope. In this method the plateau defines its level first by introducing additional points (i) until the sum of least squares is found. The slope is then constrained to cross between points i and i +1. The slope is then set by the sum of least squares [133].

Some minimum requirements are necessary to ascertain that the program gives reliable results. A minimum blood pressure range should be no less than 40 mmHg, sum of squares for the two regression lines should be lower than the least sum of squares for a single linear regression line for the whole blood pressure range for that animal and that the blood pressure value of the lower limit must be physiologically acceptable where the lower limit is no less than 10 mmHg higher than the lowest blood pressure levels measured in the experiment. Furthermore the difference between the slope and the slope of the plateau (if any) should be at least threefold. When these criteria are satisfied autoregulation is judged to be intact.

VII. The Author's Studies

1. Study I

The aim was to investigate the effect of the ACEi enalaprilat and the ARB candesartan on the lower limit of autoregulation of CBF, and to see if the response could be modulated with the bradykinin B2 receptor antagonist Hoe 140.

a. Animals

The studies were carried out in male SPRD rats obtained from Charles River, Germany. The Danish Animal Experiments Inspectorate approved the present study and the protocol was approved by the veterinarians at The Department of Experimental Medicine at The Faculty of Health Sciences at Copenhagen University.

b. Surgical procedures and blood tests

All the animals were studied under general anesthesia with Isofluran (Baxter, USA) and N_2O. During induction the isofluran dose was at 5%, 2,5% during the surgical procedures and 1,7% during measurements. After induction of anesthesia and before surgical instrumentation blood samples were drawn from a tail vein for analysis for hemoglobin, creatinine, urea, K^+ and Na^+. A tube was surgically placed in the trachea for mechanical ventilation. Polyethylene catheters were inserted in both femoral arteries and both femoral veins. One arterial catheter was used for monitoring blood pressure and the other for arterial blood sampling to monitor pCO_2. One of the venous catheters was used to infuse study medication. After the insertion of the catheters the animal was placed in a stereotactic apparatus. With a small dental drill a craniotomy was done and care was taken not to damage the dura that was kept

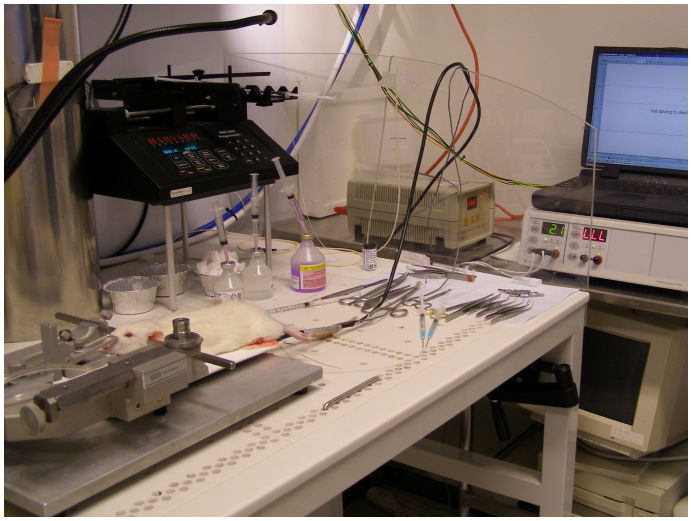

Figure 21. An SPRD rat in the stereotactic apparatus prior to drilling into the cranium. The equipment and PC used for data gathering are to right.

intact. A laser Doppler probe was placed onto the dura away from the larger vessels, to measure CBF via LDF. The setup, prior to drilling into the cranium can be seen in figure 21.

c. Groups of animals and medication

Six groups of animals were studied, a control group and five groups given drugs i.v.

1. control
2. enalaprilat, 2 mg/kg (Merck Frosst, Canada)
3. candesartan, 0,2 mg/kg (AstraZeneca, Sweden)
4. Hoe 140, 4 nmol/kg and 2 nmol/kg every 15 minutes hereafter (Zigma Aldrich, Germany)
5. Hoe 140 in the same doses as group d with the addition of enalaprilat in the same doses as group b, given 10 minutes after the first Hoe 140 injection
6. Hoe 140 in the same doses as group d with the addition of candesartan in the same doses as group c given 10 minutes after the first Hoe 140 injection.

The Hoe 140 doses given in our study were doses that in other experiments have averted bradykinin-induced hypotension [132]. To prevent an initial fall in blood pressure below the lower limit of the autoregulation of CBF, nor-epinephrine 2 - 8 μg/min was infused intravenously keeping MAP between 85 - 100 mm Hg. In a small additional study, the acute effect of candesartan and enalaprilat on blood pressure without nor-epinephrine infusion was investigated as described in appendix I.

d. Measurements and data analysis

After injection of study medication and a period of 20 minutes with stable blood pressure and CBF the experiment started by slowly reducing and finally discontinuing the nor-epinephrine infusion, followed by controlled stepwise bleeding to lower the blood pressure. Arterial blood samples were collected to measure pCO_2 at the start, during and at the end of the experiment. Arterial blood gases were analyzed immediately on an ABL 80 flex from Radiometer, Denmark. The respirator was adjusted as necessary to maintain stable pCO_2. Data were collected on a PC running Perisoft 2.5 from Perimed AB, Sweden.

The calculations to determine the lower limit were as previously described.

Statistical analysis were done running Prism 5.0b under OS X 10.5.8. Data were analyzed with one way analysis of variance followed by Dunnett's multiple comparison test where the 5 treatment groups were compared to the control group.

2. Study II

The aim in study II was to investigate the effect of suppression or stimulation of the RAS with high Na^+ diet and low Na^+ diet, respectively on the modulation by the ARB candesartan on the lower limit of CBF autoregulation compared to controls in SPRD rats.

a. Animals

The studies were carried out in male SPRD rats obtained from Charles River, Germany. The Danish Animal Experiments Inspectorate approved the present study and the protocol was approved by the veterinarians at The Department of Experimental Medicine at The Faculty of Health Sciences at Copenhagen University.

b. Surgical procedures and blood tests

Anesthesia and instrumentation were the same as in study I. After induction of anesthesia and before surgical instrumentation blood samples were drawn from a tail vein and for analysis for renin, hemoglobin, creatinine, urea, K^+ and Na^+.

c. Groups of animals and medication

The animals were initially divided into 2 groups.

1. a group on high sodium diet, 4% Na^+ (Brogården, Denmark) for 7 days.
2. a group on low sodium diet, 0,004% Na^+ (Zeigler, USA) for 7 days.

Each of these two groups was then again divided into two subgroups.

1.a a control group

1.b a group given candesartan 0,2 mg/kg intravenously

2.a a control group

2.b a group given candesartan 0,2 mg/kg intravenously

To prevent an initial fall in blood pressure below the lower limit of the autoregulation of CBF, nor-epinephrine 0,1 - 9,5 μg/min was infused intravenously keeping MAP between 85 - 100 mm Hg.

d. Measurements and data analysis

After injection of study medication and a period of 20 minutes with stable blood pressure and CBF the experiment started with slowly reducing and finally discontinuing the nor-epinephrine infusion, followed by controlled stepwise bleeding to lower the blood pressure. Arterial blood samples were collected to measure pO_2 and pCO_2 at the start and at the end of each experiment. Arterial blood gases were analyzed immediately on an ABL 80 flex from Radiometer, Denmark. The respirator was adjusted as necessary to maintain stable pCO_2. Data were collected on a PC running Perisoft 2.5 from Perimed AB, Sweden.

CBF and blood pressure were measured by the same methods as described for study I and calculations of the lower limit were the same as in study I. The statistical analysis differed from study I. Statistical analysis were done running Prism 5.0c under OS X 10.6.2. Data were analyzed with one way analysis of variance followed by Tukey's multiple comparison test where the 4 groups were compared to each other. To examine different effect of high and low sodium chow on Urea, Creatinine, Na^+, K^+, Renin, hemoglobin (hgb), and initial MAP levels t-test was used.

VIII. Results

1. Study I

Both enalaprilat and candesartan caused a shift in the lower limit of autoregulation towards lower blood pressure levels that was statistically significant when compared to the control group. The B2 receptor antagonist Hoe 140 by itself did not influence autoregulation. The addition of the B2 receptor antagonist to either enalaprilat or candesartan abolished the effect of both antihypertensive drugs on the lower limit of CBF autoregulation.

	Control (n=10)	Enalaprilat (n=10)	Candesartan (n=12)	Hoe 140 (n=11)	Enalaprilat & Hoe 140 (n=10)	Candesartan & Hoe 140 (n=10)
	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE
Lower limit of CBF autoregulation (mmHg)	54 ± 3	46 ± 2*	39± 2*	53 ± 2	52 ± 2	50 ± 2

Table 2. Lower limit of autoregulation. SE, standard error of means. * $p < 0,05$ compared to the control group using Dunnett's post analysis after anova.

The mean and standard error of means (SE) for the lower limit of CBF autoregulation in the six groups of animals are shown in table 2.

	Control (n=10)	Enalaprilat (n=10)	Candesartan (n=12)	Hoe 140 (n=11)	Enalaprilat & Hoe 140 (n=10)	Candesartan & Hoe 140 (n=10)
	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE
start pCO_2 (kPa)	5,7 ± 0,10	5,8 ± 0,14	5,3 ± 0,14	5,5 ± 0,10	5,6 ± 0,14	5,8 ± 0,09
end pCO_2 (kPa)	5,8 ± 0,11	5,8 ± 0,22	5,3 ± 0,12	5,8 ± 0,19	6,0 ± 0,21	5,4 ± 0,28

Table 3. There was no difference in pCO_2 levels. SE, standard error of means.

There was no difference in hgb, K^+, Na^+, urea or creatinine. There was no significant difference between the six groups in the animals' level of pCO_2 at the beginning and at the end of the study as shown in table 3.

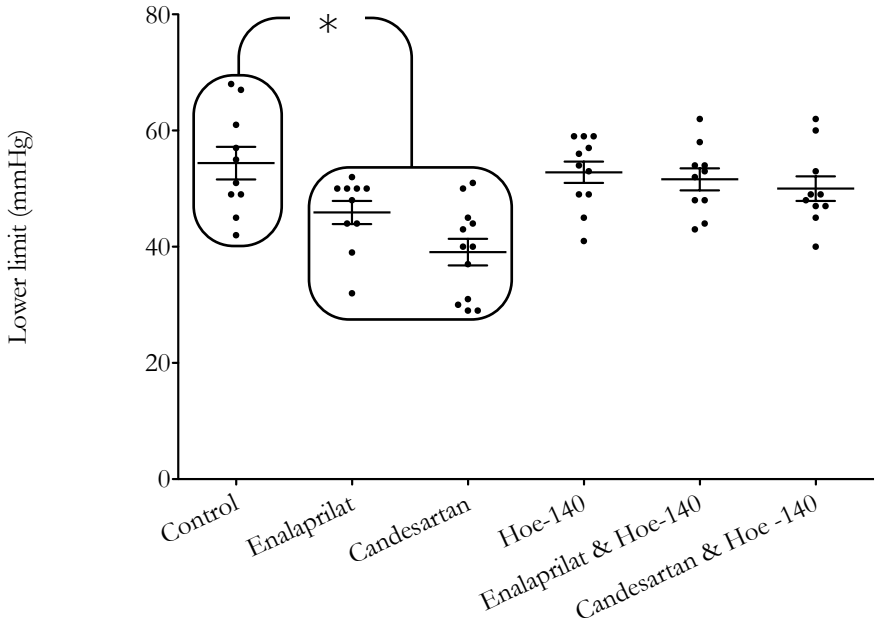

Figure 22. Lower limit of CBF autoregulation between the groups. Small bars represent standard error of means. * $p < 0,05$ Dunnett's post analysis after anova.

All lower limit values and their means and standard error of means are shown in figure 22.

2. Study II

There was no difference in the weight of the animals in the two groups. There was however a statistically significant difference in weight gain between the two groups. as can be seen in table 4.

	High sodium mean ± SE	Low sodium mean ± SE
Weight at start of special chow (g)	238 ± 4,9	241 ± 4,1
Weight at day of experiment (g)	258 ± 4,2	257 ± 4,0
Weight gain (%) *	9,3 ± 0,9	6,5 ± 0,7

Table 4. Weight in both groups at start of special chow and at start of experiment and the difference in weight gain. SE, standard error of means. * $p < 0,05$ t-test.

Initial blood pressure levels were significantly raised in the group eating low salt chow after induction of general anesthesia. All blood samples except for potassium were statistically different between the two groups. Data are shown in table 5.

	High sodium mean ± SE	Low sodium mean ± SE
MAP (mmHg) #	83 ± 3,5	102 ± 3,1
K^+ (mmol/l)	4,1 ± 0,15	4,1 ± 0,1
Na^+ (mmol/l) †	148 ± 0,5	144 ± 0,3
Hgb (mmol/l) †	7,5 ± 0,1	8,3 ± 0,1
Creatinine (mmol/l) ‡	12,1 ± 0,6	16,4 ± 1,1
Urea (mmol/l) †	5,1 ± 0,14	8,0 ± 0,7
Renin (mIU/L) †	10,0 ± 0,77	73,7 ± 7,3

Table 5. Initial MAP levels under general anesthesia. All blood samples were drawn after induction of general anesthesia. SE, standard error of means. # $p < 0,0005$, † $p < 0,0001$ and ‡ $p < 0,005$ in t-test.

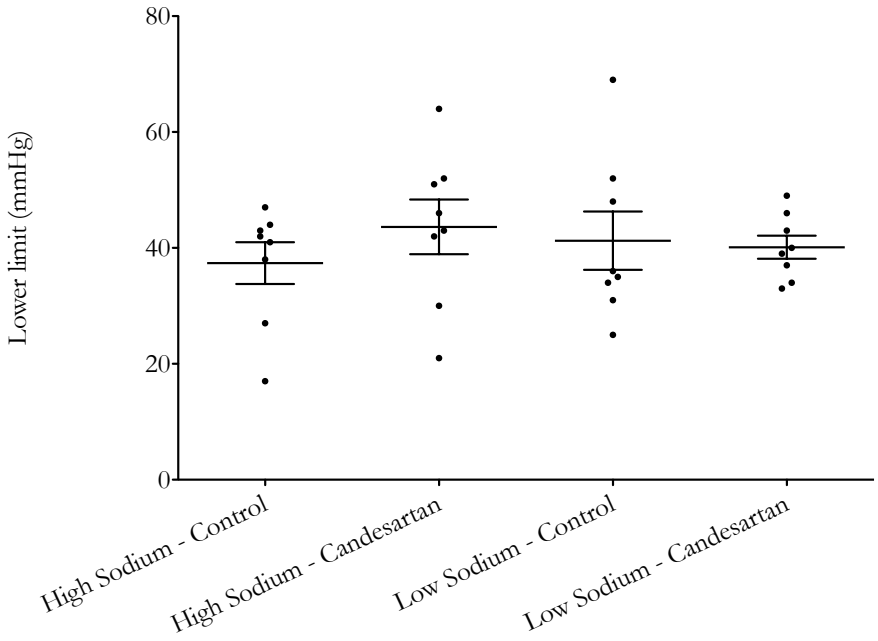

Figure 23. Lower limit of CBF autoregulation between the groups. Small bars represent standard error of means.

All lower limits are shown in figure 23.

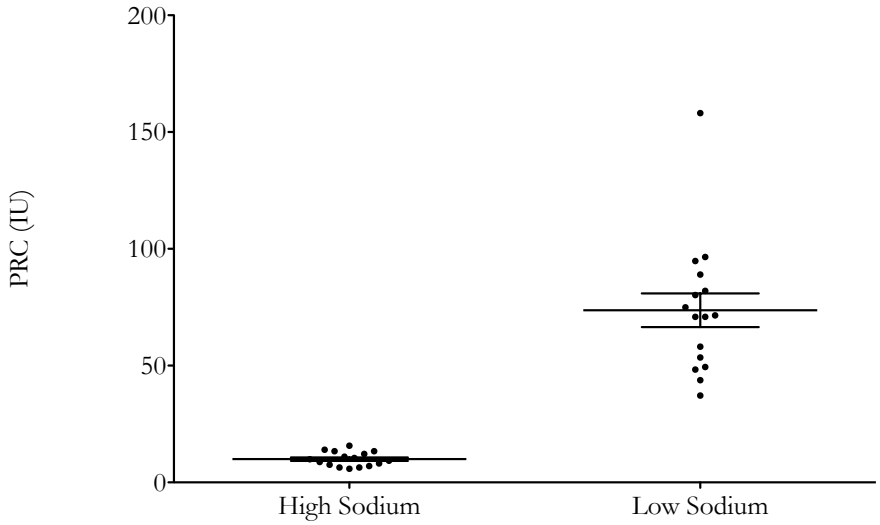

Figure 24. Renin-levels were statistically different between the groups. Small bars represent standard error of means. $p < 0{,}0001$ t-test.

Seven days of either high or low sodium diet did modulate the renin levels in the animals. Seven days of high sodium depressed renin whereas low sodium for seven days caused an increase in renin as can be seen in figure 24.

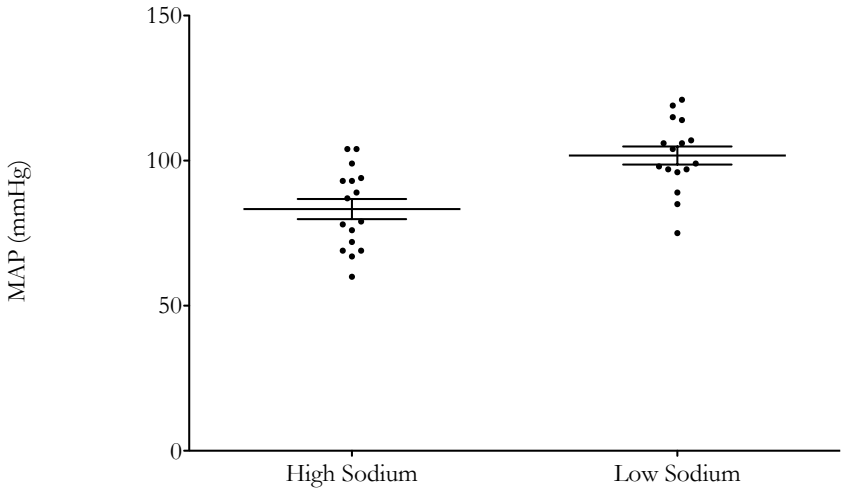

Figure 25. Initial MAP levels under general anesthesia. Small bars represent standard error of means. $p < 0{,}0001$ t-test.

After induction of general anesthesia and insertion of all catheters into the femoral vessels monitoring of MAP was started. Data for initial blood pressure levels, before opening the cranium, are shown in figure 25.

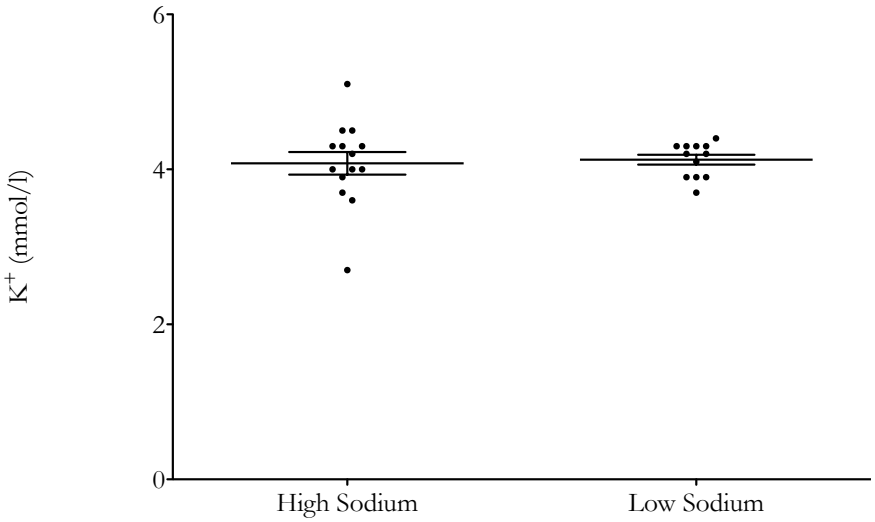

Figure 26. Potassium-levels were not different between the groups. Small bars represent standard error of means.

There was no difference between the high and low sodium groups regarding potassium levels. Data are shown in figure 26.

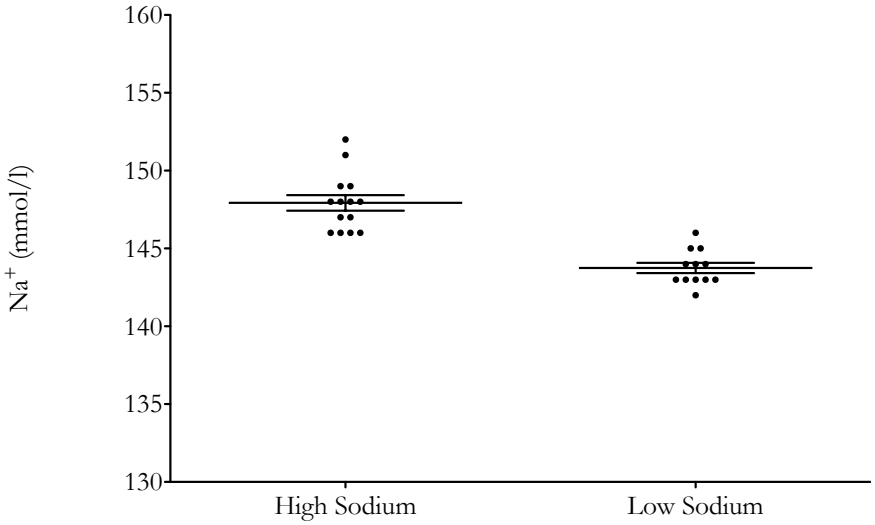

Figure 27. Sodium-levels were statistically different between the groups. Small bars represent standard error of means. $p < 0{,}0001$ t-test.

Sodium levels were raised in the animals on high sodium chow as can be seen in figure 27.

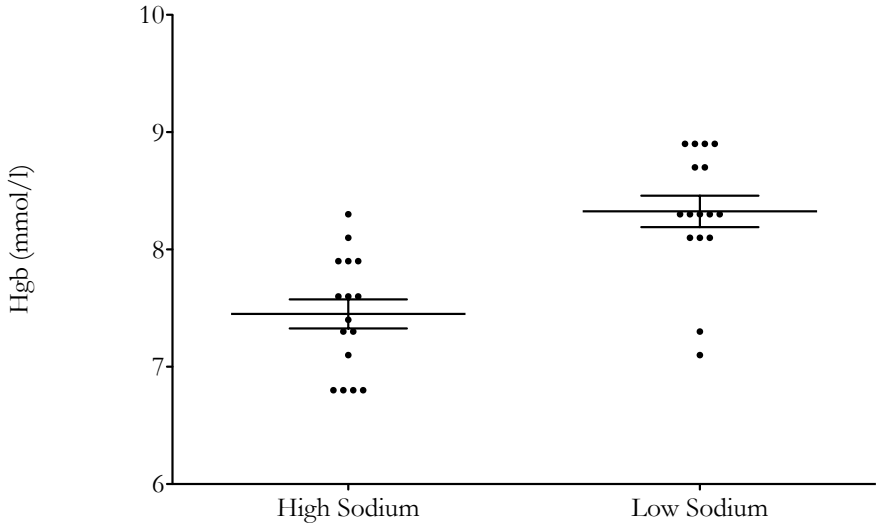

Figure 28. Hemoglobin-levels were statistically different between the groups. Small bars represent standard error of means. $p < 0{,}0001$ t-test.

Hemoglobin levels were also statistically different between the high and low sodium groups as shown in figure 28.

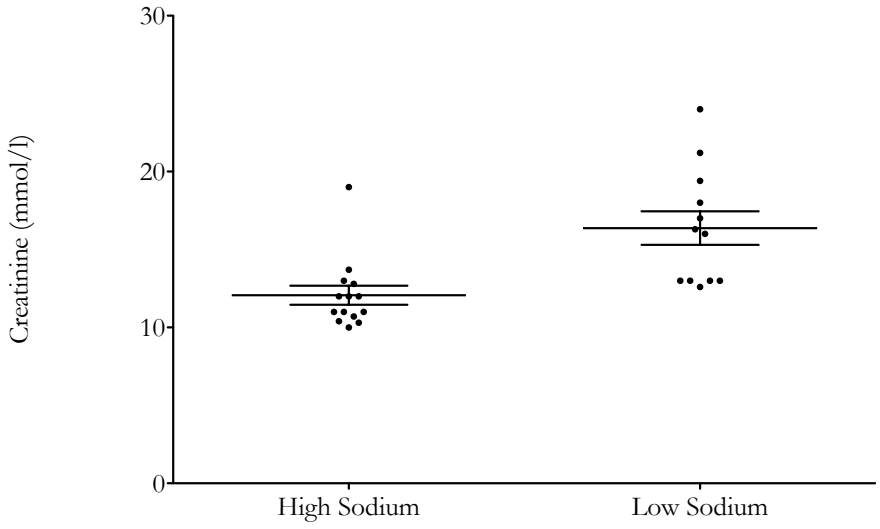

Figure 29. Creatinine-levels were statistically different between the groups. Small bars represent standard error of means. $p < 0{,}005$ t-test.

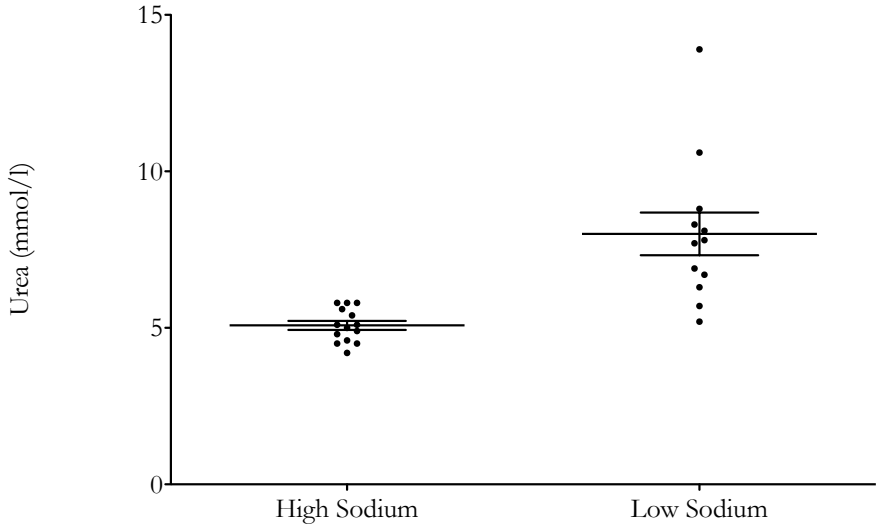

Figure 30. Urea-levels were statistically different between the groups. Small bars represent standard error of means. $p < 0{,}0001$ t-test.

Both creatinine and urea were significantly lower in the high sodium group compared to the low sodium group as can be seen in figures 29 and 30.

	High sodium		Low sodium	
	Control mean ± SE	Candesartan mean ± SE	Control mean ± SE	Candesartan mean ± SE
start pCO_2 (kPa)	4,7 ± 0,07	5,1 ± 0,07	4,9 ± 0,07	4,9 ± 0,16
end pCO_2 (kPa)	5,0 ± 0,08	5,1 ± 0,09	4,9 ± 0,08	5,1 ± 0,13
start pO_2 (kPa)	19,9 ± 1,8	19,2 ± 1,0	20,3 ± 1,0	18,3 ± 0,43
end pO_2 (kPa)	20,3 ± 2,1	21,1 ± 1,01	23,0 ± 0,95	21,3 ± 0,81

Table 6. There was neither difference in start pCO_2 and end pCO_2 nor in start pO_2 and end pO_2. SE, standard error of means.

pCO_2 and pO_2 levels remained similar over the experiment. Data shown in table 6.

	High sodium		Low sodium	
	Control mean ± SE	Candesartan mean ± SE	Control mean ± SE	Candesartan mean ± SE
Lower limit of CBF autoregulation (mmHg)	37 ± 4	44 ± 5	41 ± 5	40 ± 2

Table 7. There was no difference in lower limit of CBF autoregulation. SE, standard error of means.

There was no difference in the lower limit of CBF autoregulation between the four groups, data shown in table 7. When data from the lower limit studies were pooled to see whether there was any difference between high sodium or low sodium or between the control and the candesartan animals, the results remained non-significant.

IX. Discussion

1. Study I

In study I there were two new findings.

The first and novel observation in study I was that the effect of candesartan on the lower limit of CBF autoregulation was abolished by the B2 receptor antagonist Hoe 140, which by itself did not influence autoregulation.

The second observation was that enalaprilat caused a shift in the lower limit of autoregulation of CBF towards lower blood pressure levels as has been shown in other studies of CBF autoregulation with other ACEis [11, 12] and ARBs [13, 14] and that this effect was annulled by Hoe 140.

The ACE inhibitors exert their effect on the CBF by dilating the larger resistance vessels [128], thereby contributing to autoregulatory vasodilatation further downstream. The ARBs would be expected also to preferentially dilate the larger cerebral resistance vessels.

Bradykinin accumulates during ACE-inhibition [49] and some of the positive effects, on blood pressure, seen during ACE-inhibition could be credited to this accumulation [187]. It has been shown that bradykinin via the B2 receptor plays a role in the modulation that the ACEi captopril has on the lower limit of autoregulation of CBF. By blocking the B2 receptor with Hoe 140 the shift of the lower limit of CBF autoregulation to the left caused by the ACEi captopril alone is averted [132]. The definition of a lower limit in that paper was different than the definition used in our study. In their study the lower limit was defined as a 20% fall in CBF from the baseline level during controlled bleeding to induce hypotension whereas in our study a lower limit was defined as the blood pressure level where the plateau phase of CBF changes to a slope as described above.. This definition is in our opinion the more accurate one. Despite this difference in methodology we were able to reproduce the observations that an ACEi (in our study enalaprilat) causes a shift in the lower limit of CBF autoregulation towards lower blood pressure levels and that this effect is blunted by simultaneous blockage of the B2 receptor by Hoe 140.

It is conceptually plausible that bradykinin would play a role in the modulation of CBF autoregulation with the ACEis while it is less obvious how the effect of ARBs would be influenced by bradykinin. It was unexpected to find that blocking the B2 receptor with Hoe 140 would also blunt the effect of the ARB candesartan on the lower limit of CBF autoregulation. As stated previously ACEis cause an increase in circulating bradykinin and a fall in angiotensin II while the ARBs probably do not influence bradykinin levels while

angiotensin II levels have been shown to rise [58]. It was therefore not expected to see an effect of the B2 receptor blocker on the modulatory effect of the ARB as we saw. As noted it has been shown that at least one of the ARBs, losartan, causes an increase in bradykinin while in that same study the ARB eprosartan did not cause such an increase [59]. It can not be ruled out that this is the case for the ARB candesartan that was used in our study while it is also possible that candesartan exerts its effect on the kallikrein system locally.

There are several in vitro studies that have shown that there exists a local kallikrein system in the vessel wall [188-191] and that angiotensin II via the AT2 receptor causes activation of this kinin system [192]. Bradykinin via the B2 receptor has been shown to have a role in the the vasodilation caused by the AT2 receptor [193, 194].

In vivo studies on the interaction of angiotensin II and bradykinin have been carried out. In one of these it was found that an increase in cGMP due to angiotensin II-infusion could be blunted by either Hoe 140 or an NO synthase inhibitor thus suggesting that the rise in cGMP caused by the angiotensin II-infusion was mediated via bradykinin and NO [195]. A study in kininogen-deficient rats showed a significant decrease in AT2 dependent vasodilation compared to wild type and that the same vasodilatory response was significantly suppressed in SPRD rats that were treated with the B2 antagonist FR173657 [196]. The B1 receptor has also been shown to have some effect in ischemic stroke models in mice. Blocking the B1 receptor in this model caused a smaller infarction than blockade of the B2 receptor [197]. It is thus apparent that both the RAS and the kinin system have a role in the vasculature of the central nervous system.

We observed that Hoe 140 alone did not have any effect on the lower limit of CBF autoregulation on its own. That indicates that the ACEi enalaprilat either increases circulating bradykinin levels that in turn act locally in the vessel wall or increases bradykinin locally by inhibiting ACE in the vessels whereas the ARB candesartan activates a local kinin system in the vessel wall. When Hoe 140 is given prior to enalaprilat the circulating bradykinin is unable to bind to the B2 receptor in the vessel wall. The same applies to Hoe 140 given prior to candesartan except that the bradykinin may originate from a local kinin system within the vessel wall. Bradykinin causes vasodilation via the B2 receptor by increasing NO via NOS and via an increase in intracellular Ca^{2+} causes activation of a phospholipase that liberates arachidonic acid from phospholipids in the membrane. The arachidonic acid is in turn converted to EET, via a cytochrome P450 epoxygenase, that in turn can alone and with its metabolites diffuse to the smooth muscle cells in the vessel and activate K^+ channels that cause hyperpolarization and subsequent relaxation [56].

It has been proposed that the AT1 receptor and the B2 receptor can interact and make heterodimers that act in conjunction in the presence of both angiotensin II and bradykinin to enhance the effect of angiotensin II effect of the AT1 receptor [198]. The existence of these heterodimers has however raised debate and subsequent studies have not been able to confirm these findings [199]. Similarly a heterodimer consisting of the AT2 receptor and the B2 receptor has been described. Stimulation of this heterodimer with both the AT2 agonist CGP 42112A and B2 receptor agonist bradykinin caused an increase in both NO and cGMP from baseline when they were used together while the simultaneous use of the AT2 receptor agonists PD123319 and the B2 receptor antagonist Hoe 140 did not cause such an increase. Combining an AT2 receptor agonist with either a B2 receptor agonist or an antagonist caused an increase in NO and cGMP although that increase was not as profound as using agonists alone. A combination of the AT2 receptor antagonist with either the B2 receptor agonist or the B2 receptor antagonist did not cause an increase in NO or cGMP from baseline levels [200].

Experimental studies such as this one suggests that the positive effects of the RAS-blockers seen in clinical trials may be due to a shift in the lower limit of autoregulation of CBF and thereby improving the tolerance to lowering of blood pressure. Bradykinin may be involved in this via the B2 receptor.

2. Study II

Finding no difference in the lower limit of CBF autoregulation during blockade of the AT1 receptor was not predicted. It was anticipated that candesartan would cause a shift in the lower limit of CBF autoregulation to the left towards lower blood pressure levels. Our group has previously shown that the effect of the ACEi captopril on autoregulation of CBF does not depend on circulating renin. This was shown in rats that were kept alive with peritoneal dialysis for 48 hours after bilateral nephrectomy. At that point circulating renin was no longer present but the shift of the lower limit of CBF autoregulation to the left, towards lower blood pressure was preserved during ACE inhibition with captopril, most likely due to an effect on the RAS in the vessel wall [133]. A similar effect on the lower limit of CBF autoregulation was expected in animals that had very low PRC due to high sodium intake. Vessel wall renin, on the other hand, would also expectedly be suppressed in these animals, and the lack of effect of candesartan on the lower limit of CBF autoregulation in high salt animals may simply be due to the suppression of both the renal and the extra-renal RAS leaving no room for pharmacological blockade.

Low sodium diet increased PRC as expected, but contrary to expectation candesartan had no effect on the lower limit in these animals either. Low sodium intake increases sympathetic activity in man [201] and rat [202]. High sympathetic activity was most likely also present in the low-salt animals of the present study, given the surprising finding that blood pressure under anesthesia was on average 19 mmHg higher in low-salt than in high-salt animals despite signs of a reduced extracellular volume in the former. In these animals, a marked alpha-adrenergic sympathetic effect on the "inflow tract" arteries may have overridden the effect of the RAS and its blockade on the lower limit. Interestingly, stimulation of the sympathetic cervical ganglia has been shown to completely eliminate the effect of captopril on the upper limit of CBF autoregulation [92].

A type 2 failure is possible due to the limited number of animals in the present study. We do however find this unlikely as the results from our study do not show any trend at all towards significant difference between the two groups that were either fed high or low sodium chow or in the four subgroups that were treated with the ARB or were not treated at all.

There were several signs of expected changes in body fluid volume in the animals, in contrast to the paradoxical difference in blood pressure. In low-sodium animals hemoglobin, creatinine and urea were significantly elevated compared to the high-sodium animals, which by contrast had hypernatremia and a significant increase in weight during the one week of diet. They also had an increased water consumption compared to the low-sodium animals, although the exact fluid intake and urinary output was not measured. Hence, the low-sodium animals expectedly would have a lowered extracellular volume and high-sodium animals an increased extracellular volume. It is unlikely that these changes influenced CBF and its autoregulation.

X. Conclusion

1. Conclusion

In the present thesis we conclude that the ARB candesartan and the ACEi enalaprilat both shift the lower limit of CBF autoregulation towards lower blood pressure in animals on a diet containing normal amounts of sodium and this effect is dependent on bradykinin. The bradykinin antagonist Hoe 140 does not influence autoregulation on its own, suggesting that an increase in bradykinin concentration is necessary for the effect of the RAS blockers to take place. Such an increase is seen in the systemic circulation during ACE inhibition, and may take place locally in the vessels during ARB treatment by way of an angiotensin II effect on the AT2-receptors. This suggests the importance of a local RAS in the vessels of the brain with compounds either taken up from the blood or synthesized locally. It seems possible that an interplay of local RAS and local kallikrein system in the larger arteries of the brain play an important role in the modulation of autoregulation of CBF.

Both high and low sodium chow abolishes the effect of the ARB candesartan on the lower limit of CBF autoregulation. The precise mechanisms for this are unclear. It is proposed that: In the low-sodium group, blood pressure was almost 20 mmHg higher under anesthesia than in the high-sodium group, most likely because of alpha-adrenergic sympathetic activation. Such activation might override the effect of the RAS and its blockade, explaining the lack of effect of candesartan. In the high-sodium group, the RAS may have been so suppressed that it was not able to react to pharmacological blockade, explaining the lack of candesartan in this group.

2. Future Perspectives

To advance our understanding on RAS's role on CBF autoregulation it would interesting to investigate the role of the AT2 receptor. An AT2 receptor agonist has been developed, compound 21, and this compound has already been shown to mediate positive results in a rat MI model [203, 204]. A study with this compound alone and in combination with the B2 receptor antagonist Hoe 140 could answer the question whether the effect seen in study I is caused by increased stimulation of the AT2 receptor and whether the effect is in part mediated via the B2 receptor. With the advent of a specific renin-blocker, aliskiren, the possibility to block the RAS at "its origin" has arisen without the need to manipulate the

chow of animals with varying sodium concentrations and subsequent changes in fluid volume. This kind of study would have to be taken in transgenic rats with the human renin if the effect of aliskiren were to be assessed. A further study with a ganglionic blocker to isolate the contribution from the sympathetic nervous system in study II might further explain whether the effect on CBF autoregulation during blockade with the ARB candesartan was dependent on a contribution from the sympathetic nervous system.

XI. Appendix

1. Aim

In order to make certain that the doses of the ACEi enalaprilat and the ARB candesartan were equipotent in their ability to lower blood pressure levels and to see whether these two drugs would cause a fall in MAP below the lower limit of CBF autoregulation a short pilot study was performed in SPRD rats.

2. Material and methods

The study was carried out in male SPRD rats obtained from Charles River, Germany. The Danish Animal Experiments Inspectorate approved the present study and the protocol was approved by the veterinarians at The Department of Experimental Medicine at The Faculty of Health Sciences at Copenhagen University.

Two groups of SPRD rats were used with 6 animals in each group. All the animals were studied under general anesthesia with isofluran (Baxter, USA) and N_2O. During induction the isofluran dose was at 5%, 2,5% during the surgical procedures and 1,7% during measurements. A tube was surgically placed in the trachea for mechanical ventilation. Polyethylene catheters were inserted in a femoral artery and a femoral vein. The arterial catheter was used for monitoring blood pressure and the venous catheter was used to inject study medication.

3. Results

The blood pressure lowering effect was similar between the two groups. MAP fell between 31% and 54% in the enalaprilat group and 40% and 54% in the candesartan group. The blood pressure lowering effect measured in mmHg was between 27 and 40 mmHg in the enalaprilat group and between 31 and 51 mmHg in the candesartan group. Figure 31 shows the mean MAP values and standard error of means in each group of animals during the experiments.

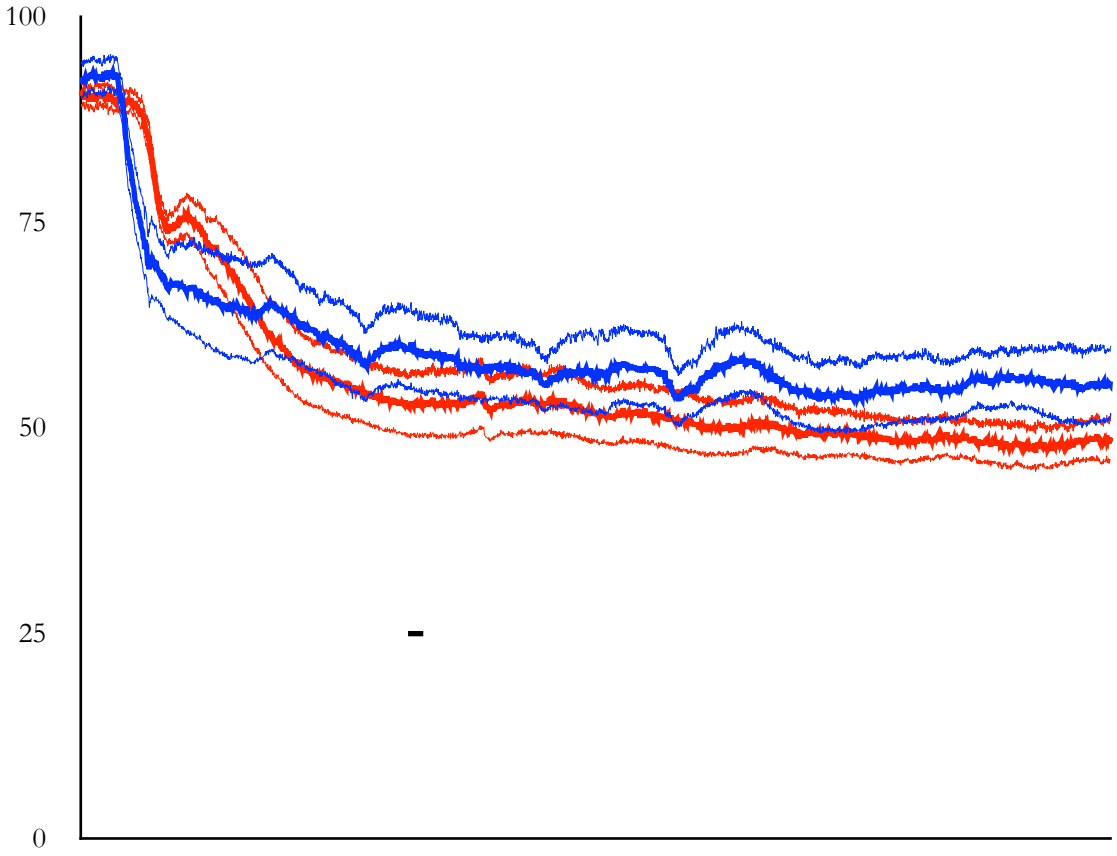

Figure 31. A graph showing mean values of MAP in the enalaprilat-group in blue and the candesartan group in red. The thin lines show standard error of means. The black line represents one minute. MAP in mmHg on the Y-axis.

There was no statistically significant difference between the two drug regiments and the level of blood pressure lowering can be seen in percentage of original measurement in figures 32 and in changes in MAP in mmHg in figure 33 and in table 8.

	Enalaprilat mean ± SE	Candesartan mean ± SE
MAP Δ %	40 ± 3,5	45 ± 2,9
MAP Δ mmHg	37 ± 3,4	40 ± 2,8

Table 8. There was no statistically significant difference between the two treatments.

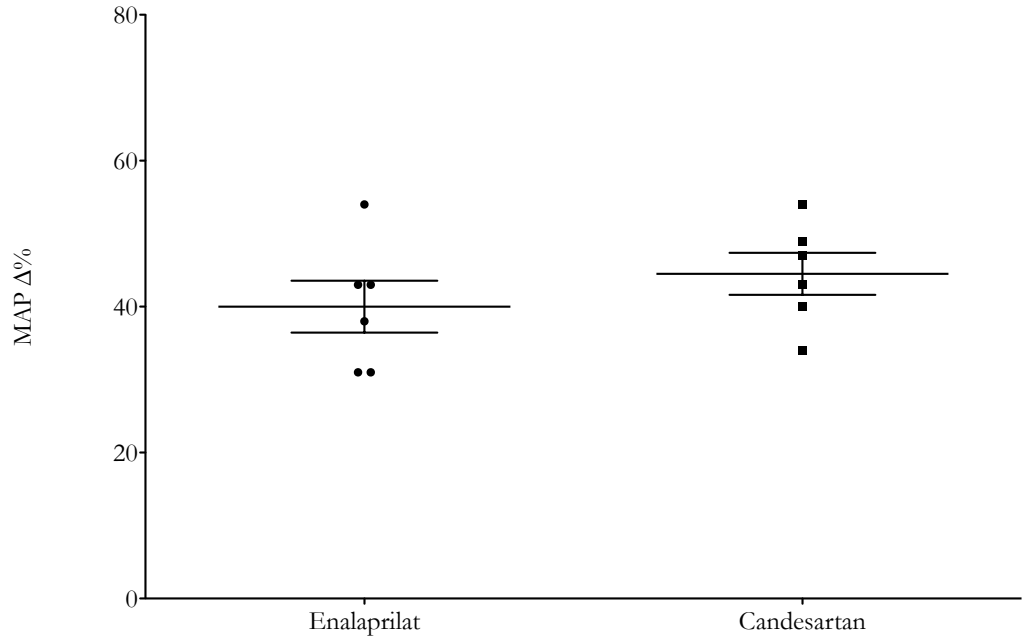

Figure 32. Changes in MAP in percentage from baseline. The bars show standard error of means. There was no statistically significant difference between the two groups.

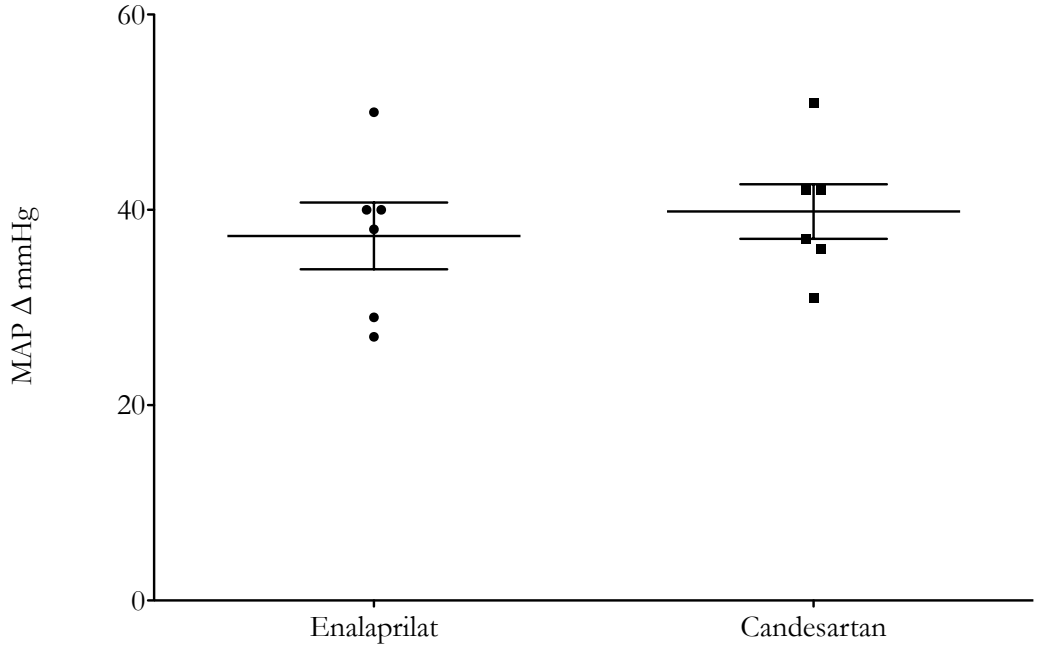

Figure 33. Changes in MAP in mmHg. The bars show standard error of means. There was no statistically significant difference between the two groups.

4. Comment

Candesartan and enalaprilat caused a similar acute, marked fall in blood pressure, and hence the doses chosen for the two main studies were equipotent. In most rats, blood pressure fell well below the lower limit of CBF autoregulation. Such profound hypotension might cause damage to autoregulation and other mechanisms regulating CBF. These observations demonstrate the necessity of using transient infusion of norepinephrine in the autoregulation studies, to be able to delineate the autoregulatory plateau and the lower limit.

XII. Paper I

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The bradykinin-antagonist Hoe 140 abolishes the effect of both candesartan and enalaprilat on the lower limit of autoregulation of cerebral blood flow

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Abstract

Background and purpose: The lower limit of autoregulation of cerebral blood flow (CBF) can be modulated with both angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB). The bradykinin 2 (B2) receptor antagonist (Hoe 140) abolishes the effect of ACE-inhibition on autoregulation of CBF. The influence of bradykinin antagonism on ARB-induced changes was the subject of this study.

Methods: CBF was measured in rats (SPRD) with laser Doppler technique. The blood pressure was lowered by controlled bleeding. Six groups of rats were studied: a control group and five groups given drugs intravenously: an ACE-inhibitor (enalaprilat), an ARB (candesartan), Hoe 140, a combination of enalaprilat and Hoe 140, and a combination of candesartan and Hoe 140.

Results: In the control group the lower limit of CBF autoregulation was 54 ± 9 mmHg (mean ± SD), in the enalaprilat group 46 ± 6, with candesartan it was 39 ± 8, with Hoe 140 53 ± 6, with enalaprilat/Hoe 140 52 ± 6, and with candesartan/Hoe 140 50 ± 7. There was a statistically significant difference between both the enalaprilat group and the candesartan group vs. control. Bradykinin-inhibition with Hoe 140 abolished the effect of enalaprilat and candesartan on autoregulation of CBF.

Conclusion: The bradykinin antagonist abolished not only the effect of the ACE inhibitor but also the effect of the ARB on the lower limit of CBF autoregulation. Thus, bradykinin seems to have an integral role in how the RAS modulates the cerebral circulation.

Introduction

The renin-angiotensin system (RAS) exerts a tone in the resistance vessels of the brain. Inhibition of this system with either an angiotensin converting enzyme-inhibitor (ACEI) or an angiotensin receptor blocker (ARB) shifts the lower and upper limits of autoregulation of cerebral blood flow (CBF) towards lower blood pressure levels. This has been shown with the ACE-inhibitors captopril, ceranopril and fosinopril[1, 2] and the ARBs candesartan and valsartan[3, 4] that block the angiotensin II subtype 1 (AT1) receptor. As a surprising exception to this the ARB losartan has in one study been shown to have the opposite effect[5]. The angiotensin II subtype 2 (AT2) receptor blocker PD123319 does not influence the lower limit of autoregulation of CBF[6]. ACE-inhibitors also inhibit kininase II which inactivates bradykinin and other kinins[7]. The ACE-inhibitors thus cause a rise in circulating bradykinin and a fall in angiotensin II levels while ARBs have no effect on circulating bradykinin and cause an increase in angiotensin II levels[8, 9]. Bradykinin is a potent vasodilator and acts by releasing prostacyclin, NO and endothelial-derived hyperpolarizing factor[10].

Takada et. al have shown that Hoe 140 which blocks the subtype 2 bradykinin (B2) receptor abolishes the effect of captopril on the lower limit of autoregulation of CBF[11]. It is thus possible that the effect of captopril on autoregulation of CBF is caused by accumulation of bradykinin. Bradykinin and the B2-receptor also seem to play a role when ARBs, blocking the AT1 receptor, are used as shown in rats with L-NAME induced hypertension. Here the effects of ARBs on endothelial vasodilator function were found to be mediated by bradykinin and the B2-receptor[12]. Angiotensin II can via the AT2-receptor induce vasodilatation and that effect is dependent on bradykinin and the B2-receptor[13]. Whether bradykinin antagonism modulates the

effect of ARBs on the lower limit of CBF autoregulation was the subject of the present study.

Methods

Studies of CBF autoregulation were carried out in male Sprague-Dawley (SPRD) rats obtained from Charles River, Germany. The Danish Animal Experiments Inspectorate approved the present study and the protocol was also approved by the veterinarians at The Department of Experimental Medicine at The Faculty of Health Sciences at Copenhagen University.

Surgical procedures

All the animals were studied under general anesthesia with Isofluran (Baxter, USA) and N_2O. The Isofluran dose was 5% during induction, 2,5% during the surgical procedures and 1,7% during measurements. A tube was surgically placed in the trachea for mechanical ventilation. Polyethylene catheters were inserted in both femoral arteries and both femoral veins. One arterial catheter was used for monitoring blood pressure and the other for arterial blood sampling to monitor pCO_2. One of the venous catheters was used to infuse study medication. After the insertion of the catheters the animal was placed in a stereotactic apparatus. With a small dental drill a craniotomy was done and care was taken not to damage the dura that was kept intact. A laser Doppler probe was placed onto the dura away from the larger vessels, to measure CBF.

Groups of animals and medication

Six groups of animals were studied. 1) a control group, whereas the other groups were given drugs intravenously as follows: 2) the ACE inhibitor enalaprilat, 2 mg/kg (Merck Frosst, Canada), 3) the ARB candesartan, 0,2 mg/kg (AstraZeneca,

Sweden), 4) the bradykinin B2 receptor antagonist Hoe 140, 4 nmol/kg and 2 nmol/kg every 15 minutes hereafter (Zigma Aldrich, Germany), 5) a combination of Hoe 140 in the same doses as group 4 with the addition of enalaprilat in the same doses as group 2, given 10 minutes after the first Hoe 140 injection and 6) a combination of Hoe 140 in the same doses as group 4 with the addition of candesartan in the same doses as group 3 given 10 minutes after the first Hoe 140 injection. The Hoe 140 doses given in our study were doses that in other experiments have averted bradykinin-induced hypotension[11].

Measurements and data analysis

To prevent an initial fall in blood pressure below the lower limit of the autoregulation of CBF, nor-epinephrine 2 - 8 μg/min was infused intravenously keeping MAP between 85 - 100 mm Hg. After injection of study medication and a period of 20 minutes with stable blood pressure and CBF the experiment started with slowly reducing and finally discontinuing the nor-epinephrine infusion, followed by controlled stepwise bleeding to lower the blood pressure. Arterial blood samples were collected to measure pCO_2 at the start, midway and at the end of the experiment. The respirator was adjusted as necessary to maintain stable pCO_2. Data were collected on a PC running Perisoft 2.5 from Perimed AB, Sweden.

The laser Doppler flowmetry is a validated method to estimate the lower limit of CBF autoregulation[14]. The method can continuously monitor local cortical cerebral perfusion by multiple measurements which give relative values of CBF with baseline registered as 100%.

To calculate the lower limit of autoregulation of CBF a computer program was used. In brief this program determines the lower limit of autoregulation of CBF by

ranking the measurements by blood pressure values from 1 to N. The lower limit is then calculated as the breaking point between a slope regression line which represents the measured levels below the lower limit and a plateau regression line which in turn represents the measurements above the lower limit. The lines are found by including more and more measurements in the slope until a best fit is found, defined as the least sum of squares of the deviations from the different sets of lines. The method of calculation represents a modification of the method described in details in a previously published paper[15]. In this paper a horizontal line was used to fit the plateau, but here we allowed this line to have a slope. This is a DOS program that was run on a MacBook Pro running OS X 10.5.8, emulating DOS using DOSBox v.0.72. Statistical analysis was done on a MacBook Pro running OS X 10.5.8 using Prism 5.0b. Data were analyzed with one way analysis of variance followed by Dunnett's multiple comparison test where the 5 treatment groups were compared to the control group.

Results

There was no significant difference between the six groups with regard to the weight of the animals and the level of pCO_2 at the beginning and at the end of the study (table 1).

The mean and SD for the lower limit of CBF autoregulation in the six groups of animals are shown in table 2, and all lower limit values are shown in figure 1. Both enalaprilat and candesartan caused a shift in the lower limit of autoregulation towards lower blood pressure levels that was statistically significant when compared to the control group. The B2 receptor antagonist Hoe 140 by itself did not influence autoregulation. The addition of the B2 receptor antagonist to either enalaprilat or

candesartan abolished the effect of both antihypertensive drugs on the lower limit of CBF autoregulation. Examples of individual autoregulation curves are shown in figure 2.

Discussion

There are two new findings in the present study. The first and novel observation in the present study is that the effect of the ARB candesartan on the lower limit of CBF autoregulation was abolished by the B2-receptor antagonist Hoe 140, which by itself did not influence autoregulation. Secondly, we found that the ACE inhibitor enalaprilat caused a shift in the lower limit of autoregulation of CBF towards lower blood pressure levels similar to the effects of other studied ACE inhibitors[1, 2] and ARBs[3, 4]. The ACE inhibitors exert their effect on the CBF by dilating the larger resistance vessels[16], thereby contributing to autoregulatory vasodilatation further downstream. The ARBs would be expected also to preferentially dilate the larger cerebral resistance vessels.

Bradykinin blockade has been shown in a study by Takada et al. to abolish the effect of the ACE inhibitor captopril on the lower limit of autoregulation of CBF[11]. We found a similar influence of bradykinin blockade on the effect of the ACE inhibitor enalaprilat on the lower limit. The definition of the lower limit of autoregulation of CBF in our study differs from that of Takada et al. who estimated the lower limit of the autoregulation during controlled hypotension in rats as the blood pressure where CBF was 20 % below baseline. In the present study we defined the lower limit as the point where the plateau phase of CBF changes to a slope. This would seem to be the more accurate method, as shown by examples of autoregulation curves in figure 2. Despite this difference in methodology, we were able to reproduce the

observations of Takada et al on how Hoe 140 abolished the effect of the ACE inhibitor on the lower limit.

It was an unexpected finding that bradykinin blockade also abolished the effect of the ARB candesartan on the lower limit of CBF autoregulation. Angiotensin converting enzyme degrades bradykinin to inactive substances and inhibiting this enzyme with ACE inhibitors causes an increase in blood bradykinin levels while the ARBs do not cause such an increase. It is therefore conceptually plausible that bradykinin could play a role in the modulation of CBF autoregulation with ACE inhibitors while it is less clear how the effect of ARBs should be similarly influenced. Several in vitro studies have however shown that a local kallikrein system exists in the vessel wall[17-20], that angiotensin II via the AT2-receptor causes activation of this kinin system[21] and that the bradykinin B2 receptor plays a role in vasodilatation caused by the AT2-receptor[13, 22]. An in vivo study performed by Gohlke et. al showed that an increase in cGMP during infusion of angiotensin II was mediated via bradykinin and NO as this effect could be abolished with either Hoe-140 or an NO synthase inhibitor[23]. Another interesting study in kininogen-deficient rats showed a significant reduction in AT2 dependent vasodilatory response compared to wild type and that the same vasodilatory response was significantly suppressed in SPRD rats that were treated with the B2-antagonist FR173657[24]. Finally a new study by Austinat et. al showed that blocking the B1-receptor in an experimental ischemic stroke model in mice caused a smaller infarction whereas the blocking of the B2 receptor did not cause a smaller infarction[25]. It is thus apparent that both the RAS and the kinin system exert a role in the vasculature of the central nervous system, the ARBs may modulate CBF autoregulation through a local kinin system in the

vessel wall. When Hoe 140 is given prior to either enalaprilat or candesartan the endogenous bradykinin released from the vessel wall may be unable to bind to the B2 receptor and cause vasodilatation via prostacyclin, NO and endothelial derived hyperpolarizing factor. Our group has shown that the effect of captopril on autoregulation of CBF is independent of circulating renin, since this effect persists 48 hours after bilateral nephrectomy when renin is no longer present in the blood and the animal is kept alive by peritoneal dialysis[15]. This suggests the importance of a local RAS in the vessels of the brain with compounds either taken up from the blood or synthesized locally. It seems possible that an interplay of local RAS and local kallikrein system in the larger arteries of the brain play an important role in the modulation of autoregulation of CBF.

ACE-inhibitors and ARBs are widely used in antihypertensive therapy and it has been shown in controlled trials that these drugs may have a beneficial effect beyond blood pressure lowering, in particular protecting against stroke[26-29]. Interestingly, several studies in animals point to a cerebroprotective effect of ARBs not readily seen with ACEIs[30-32]. Fournier and coworkers have suggested that ARBs, by way of increasing angiotensin II and influencing neuroprotective AT2-receptors in the brain, affords better stroke protection than ACE inhibitors[9]. A recent meta-analysis of controlled clinical trials, though found no difference between ACEIs and ARBs with respect to stroke prevention, both groups of drugs being equally superior to placebo[33].

From experimental studies such as the present it may be inferred that part of the beneficial effect of the RAS-blockers seen in the clinical trials may be due to a shift in

the lower limit of autoregulation of CBF, improving the tolerance to lowering of blood pressure. Bradykinin may be involved in this via the B2-receptor.

Conclusion

In the present study, candesartan and enalaprilat both shifted the lower limit of autoregulation of CBF towards lower blood pressure and this effect was dependent on bradykinin. The bradykinin antagonist Hoe 140 did not influence autoregulation on its own, suggesting that an increase in bradykinin concentration is necessary for the effect of the RAS blockers. Such an increase is seen in the systemic circulation during ACE inhibition, and may take place locally in the vessels during ARB treatment by way of an angiotensin effect on the AT2-receptors.

Table 1. Physiologic variables

	Control (n=10)	Enalaprilat (n=10)	Candesartan (n=12)	Hoe 140 (n=11)	Enalaprilat & Hoe 140 (n=10)	Candesartan & Hoe 140 (n=10)
	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
Weight (g)	336,8 ± 34,7	322,9 ± 27,0	377,4 ± 48,6	368,6 ± 17,3	376,1 ± 51,1	379,8 ± 30,2
start pCO_2 (kPa)	5,7 ± 0,3	5,8 ± 0,5	5,3 ± 0,5	5,5 ± 0,3	5,6 ± 0,4	5,8 ± 0,3
end pCO_2 (kPa)	5,8 ± 0,4	5,8 ± 0,7	5,3 ± 0,4	5,8 ± 0,6	6,0 ± 0,7	5,4 ± 0,9

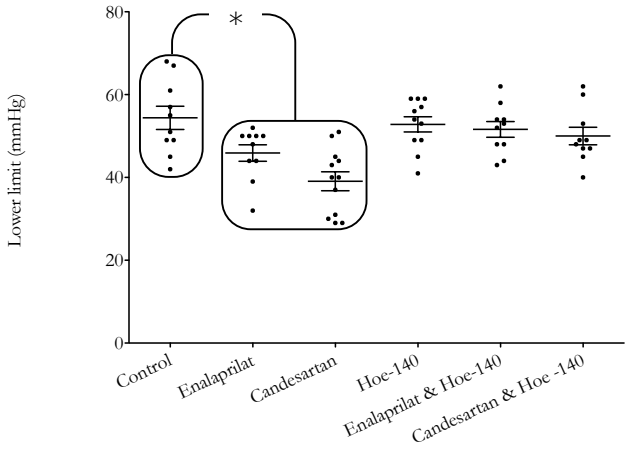
The groups are similar regarding basic physiological variables.

Table 2. Results

	Control (n=10)	Enalaprilat (n=10)	Candesartan (n=12)	Hoe 140 (n=11)	Enalaprilat & Hoe 140 (n=10)	Candesartan & Hoe 140 (n=10)
	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
Lower limit of CBF (mmHg)	54 ± 9	46 ± 6*	39 ± 8*	53 ± 6	52 ± 6	50 ± 7

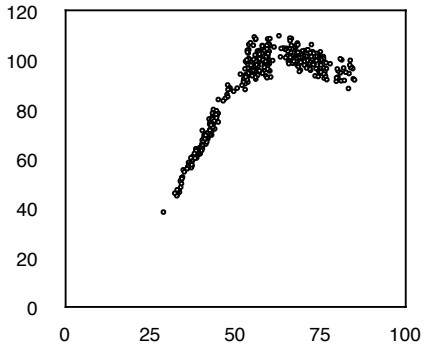
Lower limit of autoregulation. * $p < 0,05$ compared to the control group using Dunnett's post analysis after anova.

Figure 1. Results



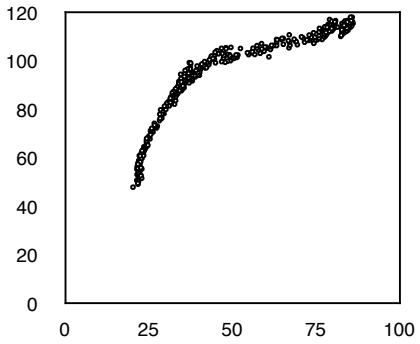
The figure shows a scatter plot of the results and the bars are standard error of means. There is a statistically significant difference between enalaprilat vs. control and candesartan vs. control (* p<0,05 anova with Dunnett's post analysis).

Figure 2.a. Autoregulation



An autoregulatory curve from a rat in the control group with blood pressure on the x-axis and CBF on the y-axis. The calculated lower limit of autoregulation of CBF is 55 mmHg.

Figure 2.b. Autoregulation



An autoregulatory curve from a rat in the candesartan group with blood pressure on the x-axis and CBF on the y-axis. The calculated lower limit of autoregulation of CBF is 37 mmHg.

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XIII. Paper II

Manuscript for study II.

Author information

Full title:

High and low sodium diet eliminate the modulatory effect of the ARB candesartan on the lower limit of cerebral blood flow autoregulation.

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Conflicts of interest

None.

Title Page

Full title:

High and low sodium diet eliminate the modulatory effect of the ARB candesartan on the lower limit of cerebral blood flow autoregulation.

Cover title:

High or low sodium intake, candesartan and CBF.

Tables:

Table 1: Basic physiologic properties between the groups

Table 2: Results

Key words:

Renin-Angiotensin System, Sodium-intake, Autoregulation, Cerebral Blood Flow

Abstract and keywords

Background: The lower limit of autoregulation of cerebral blood flow (CBF) can be modulated with both angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB). Sodium intake influences plasma renin concentration (PRC). The effect that either high or low sodium diet has on the modulatory effect of the ARB candesartan on the lower limit of CBF autoregulation has not been investigated and this was the aim of the present study.

Methods: The lower limit of CBF autoregulation was studied in four groups of Sprague Dawley rats. Two groups were fed high sodium diet (4,0% Na^+) and two groups were fed low sodium diet (0,004% Na^+). One group on high sodium and one group on low sodium was given a single dose of candesartan intravenously while the remaining two groups served as controls. CBF was measured with the laser Doppler technique. The blood pressure was lowered by controlled bleeding. Blood samples were analyzed for PRC, urea, creatinine, K^+, Na^+ and hemoglobin.

Results: There was no difference in lower limit of CBF autoregulation between the four groups. MAP was raised in anesthetized low sodium animals. The weight gain was larger in the high sodium animals and their hemoglobin, urea and creatinine levels were lower whereas sodium were elevated. PRC was raised in the animals on low sodium diet.

Conclusion: We conclude that both high and low sodium diet eliminates the modulatory effect of the ARB candesartan on the lower limit of CBF autoregulation.

Introduction

Cerebral blood flow (CBF) is kept constant despite fluctuations in systemic blood pressure [1]. The renin-angiotensin system (RAS) exerts a tone in the resistance vessels of the brain. Inhibition of this system with either an ACE-inhibitor (ACEi) or an angiotensin receptor blocker (ARB) shifts the lower and upper limits of autoregulation of CBF towards lower blood pressure levels. This has been shown with the ACEi's captopril, ceranopril, fosinopril and enalapril [2, 3]([Sigurdsson ST, Paulson OB, Nielsen AH and Strandgaard S], unpublished data, submitted to Stroke, STROKE/2010/582726) and the ARBs candesartan and valsartan ([Sigurdsson ST, Paulson OB, Nielsen AH and Strandgaard S], unpublished data, submitted to Stroke, STROKE/2010/582726)[4, 5] that block the angiotensin II subtype 1 (AT1) receptor. We found that a single dose of candesartan caused a significant shift of the lower limit of the order of 7 mmHg in SPRD rats fed standard chow with a sodium content of 0,25%. ([Sigurdsson ST, Paulson OB, Nielsen AH and Strandgaard S], unpublished data, submitted to Stroke, STROKE/2010/582726). A surprising exception to this consistent findings is one study where the ARB losartan has been shown to have the opposite effect i.e. Shifting the lower limit towards higher blood pressure [6].

Plasma renin activity (PRA) is influenced by changes in the sodium content in the diet, high sodium intake resulting in low PRA and low sodium intake in high PRA [7]. In a previous study our group has shown that the effect the ACEi captopril has on the lower limit of CBF autoregulation is not dependent on the presence of circulating renin [8]. The aim of the present study was to investigate the influence of manipulating

plasma renin concentration (PRC) by high sodium versus low sodium diet has on the effect of the ARB, candesartan on the lower limit of CBF autoregulation.

Methods

Studies of CBF autoregulation were carried out in male Sprague-Dawley (SPRD) rats obtained from Charles River, Germany. The Danish Animal Experiments Inspectorate approved the present study and the protocol was approved by the veterinarians at The Department of Experimental Medicine at The Faculty of Health Sciences at Copenhagen University.

Groups of animals, chow and medication

The animals were divided into two main groups. 1) a group fed high sodium diet (4,0% Na^+), supplied by Brogaarden, Denmark. 2) a group fed low sodium diet (0,004% Na^+), supplied by Zeigler, USA. All the animals had unlimited access to drinking water. Both main groups were further divided into a control group and a group that was given the ARB candesartan 0,2 mg/kg (AstraZeneca, Sweden). 32 animals were investigated, 8 in each group. To prevent clotting of the catheters all animals were given 200 IE of heparin. All medication was given intravenously.

Surgical procedures

All the animals were studied under general anesthesia with Isoflurane (Baxter, USA) and N_2O. During induction the dose of isoflurane was 5%, while it was set to 2,5% during surgery and 1,7% during CBF measurements. No muscle relaxant was given. A tube was surgically placed in the trachea for mechanical ventilation. Polyethylene catheters were inserted in both femoral arteries and both femoral veins. One arterial catheter was used for monitoring blood pressure and the other for arterial blood

sampling to monitor pO_2 and pCO_2. One of the venous catheters was used to infuse study medication. After the insertion of the catheters the animal was placed in a stereotactic apparatus. With a small dental drill a craniotomy was done and care was taken not to damage the dura that was kept intact. A laser Doppler probe was placed onto the dura away from the larger vessels, to measure CBF.

Measurements and data analysis

After induction of anesthesia and before surgical intervention venous blood was sampled from a tail vein and analyzed for K^+, Na^+, urea and creatinine by standard clinical chemistry methods at the department of clinical chemistry at our hospital. The method used to asses PRC was similar to a method described elsewhere [9, 10] with the exception that the plasma sample was incubated in the presence of plasma from nephrectomized sheep. The resulting PRC are reported as international units (IU) which is a WHO standardized unit.

To prevent initial fall in blood pressure below the lower limit of the autoregulation of CBF, nor-epinephrine 0,5 - 9 μg/min was infused intravenously keeping MAP stable at about 85 - 100 mm Hg. After intravenous injection of study medication and a period of 20 minutes with stable blood pressure and CBF, the experiment started with slowly reducing and finally discontinuing the nor-epinephrine infusion, followed by controlled stepwise bleeding to lower the blood pressure. Arterial blood samples were collected to measure pO_2 and pCO_2 at the start and at the end of the experiment. Initial hemoglobin levels were measured in the first arterial blood sample. The respirator was adjusted as necessary to maintain stable pCO_2. Data

were collected on a PC running MS Windows 98 SE running Perisoft 2.5 via Periflux 5000 and a PF 472 A/D Converter Box from Perimed AB, Sweden.

The laser Doppler flowmetry has been validated at our laboratory for estimating the lower limit of CBF autoregulation [11] and has been used in several disease models [12-14]. The method can continuously monitor local cortical cerebral perfusion by multiple measurements. From the laser Doppler signal a relative value for CBF is calculated, baseline being 100%. The method does not give absolute CBF values but arbitrary units.

The lower limit is defined as the breaking point between a slope regression line which represents the measured levels below the lower limit and a plateau regression line which in turn represents the measurements above the lower limit. The lines are found by including additional measurements in the slope until a best fit is found, defined as the least sum of squares of the deviations from the different sets of lines. The method of calculation represents a modification of the method described in details in a previously published paper [8]. In that paper a horizontal line was used to fit the plateau, but here we allowed this line to have a slope. Constrains on the breaking point was used on the slope as described in [8] but their significance is minor in the present study with so many measuring points obtained with the laser Doppler method and thereby better defined slopes. The program used to calculate the lower limit is a DOS program that was run on a MacBook Pro running OS X 10.6.2 and emulating DOS using DOSBox v.0.73. Statistical analysis was done on a MacBook Pro running OS X 10.6.2 using Prism 5.0c. Data were analyzed with one way analysis of variance followed by Tukey's multiple comparison test to compare the

four groups to each other. T-test was used to compare variables between the high sodium and low sodium groups.

Results

Both high sodium and low sodium diet eliminated the modulatory effect of the ARB candesartan on the lower limit of CBF autoregulation previously reported by our group in normotensive rats on diet containing 0,25% sodium ([Sigurdsson ST, Paulson OB, Nielsen AH and Strandgaard S], unpublished data, submitted to Stroke, STROKE/2010/582726)[4] and others in spontaneously hypertensive (SHR) rats [15]. There was no difference in pCO_2 and pO_2 levels during CBF measurements. Data for arterial blood gases and lower limits of CBF autoregulation are shown in table 1 and 2. Animals on low sodium diet had raised PRC compared to the animals on high sodium diet. In the low sodium animals Na^+ levels were lowered compared to the animals on high sodium diet whereas urea, creatinine and hemoglobin (HGB) levels were raised. K^+ levels were not statistically different. Data for biochemical variables are shown in table 3. The animals in the high sodium group gained more weight and MAP was lower in the high sodium group after induction of anesthesia compared to the animals on low sodium diet. The high sodium animals consumed water in much greater amount than the animals in the low sodium group and urinary output was also greater than in the low sodium group (not measured). Data for weight and blood pressure are shown in table 4.

Discussion

The finding that there was no difference in the lower limit of CBF autoregulation during blockade of the AT1 receptor in any of the four animal groups was not

predicted, as it was anticipated that candesartan would cause a shift in the lower limit of CBF autoregulation to the left towards lower blood pressure levels. Our group has previously shown that the effect of the ACEi captopril on autoregulation of CBF is independent on circulating renin. This was shown in rats that were kept alive with peritoneal dialysis for 48 hours after bilateral nephrectomy. At that point circulating renin was no longer present but the shift of the lower limit of CBF autoregulation to the left, towards lower blood pressure was preserved during ACE inhibition with captopril, most likely due to an effect on the RAS in the vessel wall [8]. A similar effect on the lower limit of CBF autoregulation was expected in animals that had very low PRC due to high sodium intake. Vessel wall renin, on the other hand, would also expectedly be supressed in these animals, and the lack of effect of candesartan on the lower limit of CBF autoregulation in high salt animals may simply be due to the suppression of the RAS leaving no room for pharmacological blockade.

Low sodium diet increased PRC as expected, but contrary to expectation candesartan had no effect on the lower limit in these animals either. Low sodium intake increases sympathetic activity in man [16] and rat [17]. High sympathetic activity was most likely also present in the low-salt animals of the present study, given the surprising finding that blood pressure under anesthesia was on average 19 mmHg higher in low-salt than in high-salt animals despite signs of a reduced extracellular volume in the former. In these animals, a marked alpha-adrenergic sympathetic effect on the “inflow tract” arteries may have overridden the effect of the RAS and its blockade on the lower limit. Interestingly, stimulation of the sympathetic cervical ganglia has been shown to completely eliminate the effect of captopril on the upper limit of CBF autoregulation [18].

A type 2 failure is possible due to the limited number of animals in the present study. We do however find this unlikely as the results from our study do not show any trend at all towards significant difference between the two groups that were either fed high or low sodium chow or in the four subgroups that were treated with the ARB or were not treated at all.

There were several signs of expected changes in body fluid volume in the animals, in contrast to the paradoxical difference in blood pressure. In low-sodium animals hemoglobin, creatinine and urea were significantly elevated compared to the high-sodium animals, which by contrast had hypernatriemia and a significant increase in weight during the one week of diet. They also had an increased water consumption compared to the low-sodium animals, although the exact fluid intake and urinary output was not measured. Hence, the low-sodium animals expectedly would have a lowered extracellular volume and high-sodium animals an increased extracellular volume. It is unlikely that these changes influenced CBF and its autoregulation.

Conclusion

Both high and low sodium diet eliminated the effect of the ARB candesartan on the lower limit of CBF autoregulation. The precise mechanisms for this are unclear but we propose the following explanations: In the low-sodium group, blood pressure was almost 20 mmHg higher under anesthesia than in the high-sodium group, most likely because of alpha-adrenergic sympathetic activation. Such activation might override the effect of the RAS and its blockade, explaining the lack of effect of candesartan. In the high-sodium group, the RAS may have been so suppressed that it was not able to react to pharmacological blockade, explaining the lack of candesartan in this

group. Clearly, further studies are necessary to confirm and explain the observations of the present work.

Table 1. Arterial blood gasses

	High sodium		Low sodium	
	Control mean ± SE	Candesartan mean ± SE	Control mean ± SE	Candesartan mean ± SE
start pCO_2 (kPa)	4,7 ± 0,07	5,1 ± 0,07	4,9 ± 0,07	4,9 ± 0,16
end pCO_2 (kPa)	5,0 ± 0,08	5,1 ± 0,09	4,9 ± 0,08	5,1 ± 0,13
start pO_2 (kPa)	19,9 ± 1,8	19,2 ± 1,0	20,3 ± 1,0	18,3 ± 0,43
end pO_2 (kPa)	20,3 ± 2,10	21,1 ± 1,01	23,0 ± 0,95	21,3 ± 0,81

Table 2. Lower limit of CBF autoregulation

	High sodium		Low sodium	
	Control mean ± SE	Candesartan mean ± SE	Control mean ± SE	Candesartan mean ± SE
Lower limit of CBF autoregulation (mmHg)	37 ± 4	44 ± 5	41 ± 5	40 ± 2

Table 3. Biochemical variables

	High sodium mean ± SE	Low sodium mean ± SE
K^+ (mmol/l)	4,1 ± 0,15	4,1 ± 0,1
Na^+ (mmol/l) *	148 ± 0,5	144 ± 0,3
Hgb (mmol/l) *	7,5 ± 0,1	8,3 ± 0,1
Creatinine (mmol/l) Ü	12,1 ± 0,6	16,4 ± 1,1
Urea (mmol/l) *	5,1 ± 0,14	8,0 ± 0,7
Renin (mIU/L) *	10,0 ± 0,77	73,7 ± 7,3

* $p < 0,0001$, Ü $p < 0,005$ in t-test.

Table 4. Weight and blood pressure

	High sodium	Low sodium
	mean ± SE	mean ± SE
Weight at start of special chow (g)	238 ± 4,9	241 ± 4,1
Weight at day of experiment (g)	258 ± 4,2	257 ± 4,0
Weight gain (%) *	9,3 ± 0,9	6,5 ± 0,7
MAP (mmHg) *	83 ± 3,5	102 ± 3,1

* $p < 0,05$ t-test

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