ANGIOTENSIN AT_1 RECEPTOR BLOCKADE PROTECTS THE BRAIN FROM ISCHEMIC DAMAGE

A thesis submitted in partial fulfillment of the requirements of the degree of Master of Science

By

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BVSc & A.H, College of Veterinary Science, Tirupati, 2004

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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Madhuri Penchikala ENTITLED Angiotensin AT1 Receptor Blockade Protects the Brain from Ischemic Damage BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Penchikala Madhuri. M.S., Department of Pharmacology & Toxicology, Wright State University, 2007. Angiotensin AT1 Receptor Blockade Protects the Brain from Ischemic Damage.1

1Angiotensin (Ang) AT1 receptors are considered to play an important role in ischemic stroke via degenerative processes leading to cell death. Recent clinical and basic studies show that systemic blockade of Ang AT1 receptors reduces brain lesion in ischemic stroke. In this study we evaluated whether blockade of central Ang AT1 receptors protects the brain from ischemia and inflammation during ischemic stroke. Adult male C57BL/6 mice were divided into two groups for chronic intracerebroventricular (ICV) infusion of a selective Ang AT1 receptor antagonist, losartan (Los, n=18, 2 ug/hr) or isotonic saline (Con, n=20) using osmotic minipump. Twelve days post infusion, focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO). The success of MCAO was verified by measurement of cerebral blood flow (CBF) using laser-Doppler flowmetry. Neurological deficits due to ischemic damage of neuronal cells were evaluated 24 hours after MCAO. Brains were removed 48 hrs after MCAO and the degree of damage due to ischemia was determined using triphenyltetrazolium chloride (TTC) staining. The expression of Ang AT1 receptors, matrix metalloproteinase-2 (MMP-2) and
myeloperoxidase (MPO) was carried out using western blot analysis.

Immunohistochemistry was performed to determine the inflammatory cell infiltration in control vs. losartan treated mice. Focal cerebral ischemia resulted in overexpression of Ang AT$_1$ receptor ischemic hemisphere compared to non-ischemic hemisphere (27%, $p<0.05$) suggesting a role of Ang AT$_1$ receptor in ischemia. We also found a significant increase in the expression of MPO and MMP-2 in ischemic vs. non-ischemic hemispheres. Pretreatment with losartan significantly improved neurological deficits and infarct volume compared to control mice ($p < 0.05$). Parelleling these effects on ischemia, losartan pretreatment also reduced (~50%) the reactive upregulation of MPO ($P < 0.05$) and inflammatory cells (neutrophils and macrophages, $P < 0.01$) in the ischemic area. These results support a role for Ang AT$_1$ receptors in cerebral ischemia and inflammation produced by stroke.

**Key words:** Stroke, angiotensin II, angiotensin type I (AT$_1$) receptor, middle cerebral artery occlusion, ICV infusion, losartan
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<td>RAS</td>
<td>Renin Angiotensin System</td>
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<td>AT₁</td>
<td>Angiotensin Type I receptor</td>
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<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
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<td>AT₂</td>
<td>Angiotensin Type II receptor</td>
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<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>ARBs</td>
<td>Angiotensin Receptor Blockers</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
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<td>Middle Cerebral Artery Occlusion</td>
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<td>PMN leukocytes</td>
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<tr>
<td>MMP-2</td>
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INTRODUCTION AND PURPOSE

Today, stroke is the second most frequent cause of death and the most frequent cause of invalidity. Stroke is defined as a new-onset neurological deficit of vascular origin lasting 24 hours or longer or until death. It can be classified into two major categories, ischemic & hemorrhagic; In about 80% of cases, stroke is caused by cerebral ischemia and in about 20% by intracerebral hemorrhage, subarachnoidal hemorrhage, venous thrombosis and other cerebrovascular diseases. Risk factors for stroke include, i) Risks that you cannot control: Increasing age, Male sex, Race, Family history of stroke and ii) Risks that can be controlled with Medical Treatment & Lifestyle Changes: High blood pressure, Tobacco use, High cholesterol, Obesity/overweight, Physical inactivity, Diabetes, Atrial fibrillation (irregular heartbeat), Unhealthy diet, Cardiac disease. Hypertension is a factor in nearly 70% of stroke cases (Bronner, Kanter, and Manson 1995).

PATHOPHYSIOLOGY OF ISCHEMIC STROKE

Ischemic stroke results from a transient or permanent reduction or interruption of cerebral blood flow (CBF). The normal CBF is approximately 50 to 60 ml/100gm/minute and varies in different parts of the brain. In response to ischemia, the cerebral autoregulatory mechanisms compensate for a reduction in CBF by local vasodilation, opening the collaterals and increasing the extraction of oxygen and glucose from the blood. However when the CBF is reduced to below 20 ml/100gm/minute, an electrical silence ensues and synaptic activity is greatly diminished in an attempt to preserve energy.
stores. CBF of less than 10 ml/100gm/minute results in irreversible neuronal injury (Astrup, Siesjo, and Symon 1981; Hakim 1998; Wise et al. 1983). Stroke results in a core area of cell death (infarct) due to interrupted circulation, with a larger surrounding area of compromised cell function resulting from reduced perfusion (penumbra).

At the molecular level, the development of hypoxic-ischemic neuronal injury is greatly influenced by “overreaction” of certain neurotransmitters, primarily glutamate and aspartate. This process called “excitotoxicity” is triggered by depletion of cellular energy stores. Glutamate, which is normally stored inside the synaptic terminals, is cleared from the extracellular space by an energy dependent process. The greatly increased concentration of glutamate (and aspartate) in the extracellular space in a depleted energy state results in the opening of calcium channels associated with N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxanole propionate (AMPA) receptors. Persistent membrane depolarization causes influx of calcium, sodium, and chloride ions and efflux of potassium ions (Siesjo 1981; Siesjo, Agardh, and Bengtsson 1989). Intracellular calcium is responsible for activation of a series of destructive enzymes such as proteases, lipases, and endonucleases that allow release of cytokines and other mediators, resulting in the loss of cellular integrity (Hademenos and Massoud 1997; Siesjo 1981).
Figure 1. Pathophysiology of Ischemic brain damage at molecular level.

The two processes by which injured neurons are known to die are coagulation necrosis and apoptosis. Necrosis evolves over 6-12 hrs and apoptosis develops with in days and weeks. Delayed edema and inflammation lead to secondary brain damage. Apoptosis is probably induced by ischemia and can cause secondary deterioration (Kajstura et al. 1997). The outcome of stroke mostly depends on the severity of cerebral blood flow reduction and on the duration of the ischemic insult (Dirnagl, Iadecola, and Moskowitz 1999).
BRAIN ANG II SYSTEM

The renin angiotensin system (RAS) is both a circulating and tissue based hormonal system that regulates homeostasis and tissue responses to injury. Angiotensin II (Ang II)) is the most important effector peptide of the RAS and has potent effects on vascular tone and sympathetic transmission and on the regulation of body fluid volume. It also influences endothelial function and stimulates inflammatory, proliferative, fibrotic and thrombotic processes (Schrader, Kulschewski, and Dendorfer 2007). Ang II, initially described as a peripheral circulating hormone regulating systemic blood pressure and fluid homeostasis, was later recognized as a brain neuromodulator inducing fluid and salt intake and blood pressure increase through stimulation of its physiological receptors, the AT₁ receptor type (Saavedra 2005).

All components of the classical RAS, such as angiotensinogen, renin, angiotensin-converting enzyme (ACE), and Ang II are present in the brain (Ganten et al. 1984; Phillips and Sumners 1998; Saavedra 1992). However, localization studies have revealed a puzzling picture, because there is not a single brain cell where all RAS components are normally expressed (Fig. 1). The postulate of brain Ang II formation through a classical RAS system requires multiple-cell interactions at long distances, enough to make the system unrealistically complex and inefficient. Conversely, the selective receptor localization clearly demonstrates that stimulation of receptors by Ang II is likely to result in specific, selective effects. Alternative hypotheses are necessary to explain how brain Ang II is produced and how the peptide reaches its receptors at physiologically active concentrations (Saavedra 2005).
There are two closely integrated central Ang II systems, one responding to Ang II generated in the brain and stimulating receptors inside the blood brain barrier, another with Ang II receptors in circumventricular organs, cerebrovascular endothelial and smooth muscle cells, responding to circulating Ang II of peripheral origin, and/or to locally generated Ang II (Saavedra 2005). AT₁ receptors located in the cerebrovascular endothelium and in specific brain areas participate in the regulation of cerebrovascular circulation. Many of the classical and hypothetical functions of brain Ang II are mediated by the stimulation of Ang AT₁ Receptors (Saavedra 2005).
Figure 2: RAS in the brain. Two possible ways of Ang II generation within the CNS are discussed: 1) volume transmission: extracellular generation of angiotensin peptides, acting as neurohormones; and 2) wiring transmission: uptake of angiotensinogen by neurons and formation of peptides within the neuron. Ang II acts as neurotransmitter or cotransmitter (Paul et al. 1993).
SIGNIFICANCE OF ANG II IN ISCHEMIC STROKE

The subtypes of angiotensin receptors, referred to as AT\textsubscript{1} and AT\textsubscript{2} receptors, are expressed in the brain. In the adult, the majority of brain areas express predominantly the AT\textsubscript{1} receptor that mediates most of the known central actions of Ang II not only on blood pressure and osmo control but also on neuroplasticity (Culman et al. 2001; de Gasparo et al. 2000). Beside its critical role in cardiovascular and fluid homeostasis, several lines of evidence implicate Ang II in ischemic neuronal injury. During the past decade, a number of studies have indicated that Ang II, the main effector peptide of renin angiotensin system, may be involved in the pathogenesis of cerebral ischemia (Culman et al. 2001). Maeda et al (Maeda et al. 1999), showed better collateral circulation and smaller lesion size in angiotensinogen knockout mice than in controls 1 hr after experimental stroke, indicating a beneficial effect of lower Ang II levels before the onset of ischemia. Walther et al. demonstrated a direct correlation between brain angiotensin II and the severity of ischemic injury in experimental stroke (Walther et al. 2002). In this study, they proved for the first time that activation of AT\textsubscript{1} receptors also initiates proapoptotic stimuli in neuronal cells.

There are several mechanisms by which Ang II, acting via the AT\textsubscript{1} receptor, could contribute to ischemic brain damage- for example, reduction of cerebral blood flow (CBF), induction of inflammation, production of reactive oxygen species (ROS) with consecutive cell destruction, or promotion of apoptosis (de Gasparo et al. 2000; Kaschina and Unger 2003; Stenman and Edvinsson 2004). Apoptosis may be directly due to Ang II; since, in vitro studies by (Kajstura et al. 1997) showed that programmed cell death in cells is due to Ang II. Inflammatory mechanisms are implicated, with the accumulation of neutrophils and the subsequent disruption of the blood–brain barrier (BBB). In addition,
the development of oedema, necrosis and scar formation brings about secondary brain damage (Tomita and Fukuuchi 1996). The CBF is also affected, with the induction of hypertrophy and hyperplasia of the vascular smooth muscle and an increase in vascular permeability (Unger 2001). It is assumed that cerebral ischemia induces a change (stimulates) in local vascular Ang II receptor function, which might influence the renin angiotensin system and a number of Ang II - mediated mechanisms of actions that may contribute to the worsening of ischemia and formation of the penumbral zone.

Figure 3: Proposed mechanisms of angiotensin II-induced worsening of brain ischemia (Unger 2001).
ANGIOTENSIN RECEPTOR BLOCKERS IN ISCHEMIC STROKE

Angiotensin receptor blockers (ARBs) have emerged lately as being very effective and perhaps superior to other antihypertensive drugs in the prevention of de novo or recurrent strokes. (Chrysant and Chrysant 2006). Walther et al showed that sustained blockade of AT$_1$ receptors with peripheral and centrally active AT$_1$ receptor antagonists reverse the cerebrovascular pathological growth and inflammation, increases cerebrovascular compliance, restores the eNOS/iNOS ratio and decreases cerebrovascular inflammation. These effects result in a reduction of the vulnerability to brain ischemia (Walther et al. 2002).

There is a good correlation between the area of blood flow below the required protective threshold and the area of neuronal injury and death, both significantly reduced by AT$_1$ receptor blockade (Ito et al. 2002). The underlying mechanisms of the ARB effect include a blockade of the vasoconstrictive and pro-growth effects of Ang II. Systemic treatment with AT$_1$ receptor antagonists has been reported to prevent the occurrence of stroke in spontaneously hypertensive rats and to reduce the degree of neuronal damage after ischemia in hypertensive and normotensive rats during ischemic stroke (Groth et al. 2003; Inada et al. 1997; Ito et al. 2002; Nishimura, Ito, and Saavedra 2000; Stier et al. 1993).

Nishimura et al (Nishimura, Ito, and Saavedra 2000) reported that blockade of AT$_1$ receptor by an ARB, candesartan, reduced the ischemic area after middle cerebral artery occlusion (MCAO) in genetically hypertensive rats. In the MCAO study done on rats, Emelie Stenman and Lars Edvinsson observed an increase in the relative amount of ACE mRNA in the occluded middle cerebral artery (MCA) and increased contractile AT$_1$
receptor-mediated responses in the ipsilateral MCA 48 hours after MCAO, suggesting a locally enhanced production of Ang II after cerebral ischemia (Stenman and Edvinsson 2004). These results support a role for AT\textsubscript{1} receptors in cerebral ischemia, and that AT\textsubscript{1} receptors might be future therapeutic target in ischemic stroke.

Losartan is an AT\textsubscript{1} receptor antagonist that is used clinically to lower blood pressure but has also been shown to decrease the incidence of stroke (Forder, Munzenmaier, and Greene 2005). Some studies have implied that blockade of the AT\textsubscript{1} receptor allows for a more active angiogenic and neuroprotective role of the AT\textsubscript{2} receptor in ischemic stroke (Achard et al. 2001; Shibata et al. 1998). Losartan Intervention For Endpoint reduction in hypertension (LIFE) study reported that a losartan-based treatment reduced stroke risk more than therapy based on atenolol in patients with cardiovascular risk (Kjeldsen et al. 2005). However, direct evidence of how ARBs act as cerebroprotective agents in stroke is currently lacking. Recent studies have shown that chronic, but not acute, pretreatment with an AT\textsubscript{1} receptor antagonist significantly improves neurological outcome and reduces the infarct size after temporary middle cerebral artery (MCA) occlusion followed by reperfusion in normotensive rats (Groth et al. 2003; Ito, Nishimura, and Saavedra 2001; Ito et al. 2002).

**BRAIN ANG II AND INFLAMMATION IN ISCHEMIC STROKE**

Inflammation is a critical process after stroke (Danton and Dietrich 2003). After the interruption of CBF, tissue injury begins with an inflammatory reaction, which is a common response of the cerebral parenchyma to various forms of insult. This requires the infiltration of leukocytes, both PMN leukocytes and monocytes/macrophages (but not lymphocytes), which are the cellular mediators of subsequent microvessel obstruction,
edema formation, cellular necrosis, and tissue infarction (Chou et al. 2004; Clark et al. 1993). Microglia/macrophages secrete a number of both toxic and protective effector molecules and thereby play a key role in inflammatory CNS processes (Schroeter et al. 2001).

Human data concerning the role of leukocytes in stroke includes an autopsy study demonstrating histologic PMN infiltration of the cerebral parenchyma 48 to 72 hours after stroke and evidence of radiolabeled leukocyte accumulation as early as 6 to 12 hours after stroke in partially perfused brain regions (Aspey et al. 1989). Taken further, leukocytes may be hypothesized to be the cells responsible for the progression from tissue ischemia to cerebral infarction (Dietrich et al. 1987; Zhang et al. 1994).

The recruitment of leukocytes in the ischemic regions requires a sequence of events beginning with leukocyte rolling on the activated endothelial blood vessel walls, neutrophil activation, adherence to endothelial cells, and transmigration into the cerebral parenchyma. Stimulation of brain and cerebrovascular Ang II systems may contribute not only to vasoconstriction but also to increased expression of inflammatory factors and increased microvessel permeability (Ito, Takemori, and Suzuki 2001).

Matrix metalloproteinases (MMPs) are a family of zinc dependent proteases responsible for the extracellular matrix turnover and degradation of bioactive proteins. MMPs play an important role in cell death and are upregulated after focal cerebral ischemia (Tsubokawa et al. 2006). The main forms of MMPs involved in the ECM alterations are MMP-2 and MMP-9. MMPs 2 and 9 have been shown to be elevated a few hours after ischemia (Gasche et al. 1999) and to maintain increased activity for days after the onset (Clark et al. 1997; Rosenberg et al. 1996). Animal studies revealed that early
increased expression of MMP-2/ MMP-9 in endothelial cells and infiltrating neutrophils is a significant response to cerebral focal ischemia (Romanic et al. 1998).
HYPOTHESIS

Intracerebroventricular (ICV) pretreatment with AT1 receptor antagonist (Losartan) protects the brain from ischemic brain damage in mice induced with focal cerebral ischemia.

SPECIFIC AIMS

1) To determine the effect of ischemic stroke on Ang AT1 receptor expression in mouse brain.

2) To determine the effects of brain Ang AT1 receptor blockade (Losartan) on ischemic brain damage.

3) To explore if AT1 receptor blockade has significant role in reducing the inflammatory response and activation of extracellular MMP-2 after stroke.
EXPERIMENTAL DESIGN

Materials and Methods.

Animals. Strain-related differences exist regarding vulnerability to cerebral ischemia. The most likely reason for such strain-related difference is the presence of variations in the cerebrovascular anatomy. The middle cerebral artery has a larger vascular supplying territory in C57BL/6 (Tamaki et al. 2006) mice compared to other strains. Adult male C57BL/6 mice (25 to 30 g; Harlan, Indianapolis, Inc) were used for all experiments in this study. Mice were fed standard chow (Harlan) and water ad libitum. All procedures were approved by the Wright State University Laboratory Animal Care and Use Committee (LACUC) and were in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health.

There are two survival surgeries in C57BL/6 mice, which are implantation of minipump for infusion of drugs into intracerebral ventricle (ICV) and placement of monofilament suture to induce focal ischemic stroke by occlusion of middle cerebral artery (MCAO) through carotid artery as described below.

Animal Surgeries: For anesthesia in Survival surgeries, ketamine:xylazine mixture was used (100:8 mg/kg). Anesthetic state was determined by visual inspection and lack of response to tail pinch. Surgical areas were shaved and all procedures were performed aseptically.
Implantation of ICV Minipump

Mice were instrumented with ICV minipumps for chronic (2 weeks) central infusion of AT₁ receptor specific antagonist, Losartan (2 µg/hr). Osmotic minipumps (ALZET Model No. 1002), which continuously deliver dissolved substances at a rate of 0.25 µL/hr into the desired tissue area, were filled with vehicle or the AT₁ receptor antagonist, Losartan. The flow moderator of the pump was connected with a polyethylene catheter to a curved (right-angled) metal cannula (30-gauge) to allow long-term ICV infusion of drugs. The pumps were placed in sterile saline solution (0.9%) overnight at 37°C before implantation to initiate their operation at a constant pumping rate and to minimize the possibility of occlusion or clot formation in the catheter. Head of the mouse was fixed in the stereotaxic apparatus, and the skull was exposed by a midline sagittal incision through the scalp. Then, a subcutaneous pocket was prepared at the back of the mouse. The osmotic pump was placed into the pocket, and the right-angled metal cannula was inserted through the skull into the brain (depth 3 mm) to reach the ventricle. The external part of the metal cannula was fixed to the skull with dental cement, and the wound was sutured. (Dai et al. 1999) After operation, mice were treated with buprenorphine (0.1 mg/kg, sc), 3 doses (36 hrs) with the first dose given immediately after accomplishment of the surgery.

Middle Cerebral Artery Occlusion

Middle cerebral artery occlusion (MCAO, Ito et al., 2002), is a common method used to induce ischemic stroke in rodents, a model that results in a profound decrease in blood flow predominantly localized to the ipsilateral cortical areas leading to relatively reproducible cortical infarct volumes. Focal cerebral ischemia was induced according to
the procedure described by Longa et al. (Longa et al. 1989). Briefly mouse was anesthetized using ketamine:xylazine mixture (100:8 mg/kg). Midline skin incision was made along the neck. One drop of bupivicaine (0.25% bupivicaine) was applied locally for anesthesia and hemostasis. Left common carotid artery was exposed by blunt dissection and the external carotid artery was ligated. A small incision was made on the dorsal side of the left common carotid artery to introduce a 7-0 nylon monofilament (Ethicon, Somerville, NJ, USA) treated with lysine and heat-blunted at the tip and the suture was advanced 10-13 mm distal to the carotid bifurcation. If left in place, this results in permanent MCAO and the skin incision was then sutured. The success of MCAO was verified by measuring the regional cerebral blow flow (CBF) using laser-doppler flowmetry. After operation, mice were treated with buprenophine (0.1 mg/kg, sc) for 3 doses (36 hrs) with the first dose applied immediately after accomplishment of the surgery.
Schematic explanation of embolization and the operation procedure

Figure 4: An assembled view (A) of brain and neck and an original image with skull (B) in normal C57BL/6J mice. ACA; anterior cerebral artery, BA; basilar artery, CCA; common carotid artery, ECA; external carotid artery, ICA; internal carotid artery, MCA; middle cerebral artery, PCA; posterior cerebral artery, PPA; pterygopalatine artery, SCA; superior cerebellar artery, VA; vertebral artery (Tamaki et al. 2006). Schematic representation of MCAO by passing a nylon monofilament through an incision on the common carotid artery (C).

Measurement of Cerebral Blood Flow

The regional cerebral blood flow (CBF) was measured by laser-doppler flowmetry (PF2B, Perimed, Stockholm, Sweden) and recorded on a data acquisition system (AD instruments, MA, USA). The mouse was anesthetized using ketamine:xylazine mixture. An incision was made along the site for fixing the probe on the surface of the brain. One drop of bupivacaine (0.25% bupivicaine) was locally applied for anesthesia and hemostasis. The fiberoptic probe tip (Perimed PF 319:2, diameter 0.5 mm) was fixed 2 mm posterior, 3 mm lateral to bregma on the ipsilateral
hemisphere. This site on the convex brain surface reflects the vascular territory supplied by distal segments of the middle cerebral artery. CBF values were recorded before and after MCAO to verify the success of occlusion.

Figure 5: Cerebral blood flow recorded using laser Doppler flowmetry immediately after middle cerebral artery occlusion (MCAO). The cerebral blood flow (CBF) dropped to less than 10 immediately after occlusion of the middle cerebral artery indicating the success of occlusion.

**Experimental protocol I (specific aims 1 & 3)**

To set up MCAO surgery with ICV minipump implantation, C57BL/6 mice were divided into 2 groups: Group 1) Vehicle + MCAO (ischemic stroke, n = 12), Group 2) AT₁ receptor blocker (losartan, n = 10) + MCAO. Mice were implanted with ICV minipump infusion kit (Alzet 3) for chronic infusion of vehicle or losartan (2 µg/hr). 12
days post infusion, MCAO surgery was done by occluding left middle cerebral artery to induce ischemic stroke.

**Table 1: Overview of Experimental protocol I**

<table>
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<tr>
<td>ICV minipump implantation</td>
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<td>ICV infusion of Losartan/ Vehicle</td>
<td>1-14</td>
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<tr>
<td>MCAO surgery</td>
<td>12</td>
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<td>Sacrifice mice</td>
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Mice were sacrificed on day 14 (48 hr after MCAO). Brains extracted were quickly frozen in dry ice (Western blotting) or fixed in 4% paraformaldehyde (Immunohistochemistry and Fluoro-jade staining) and stored at -80 °C for histological analysis of brain damage, inflammatory cell infiltration and protein expression.

**Experimental protocol II (specific aim 2)**

ICV minipump surgery was set up for chronic infusion of losartan (2 µg/hr)/vehicle followed by MCAO surgery. Mice were divided into 2 groups (8/group): Group 1) vehicle + MCAO stroke, Group 2) AT₁ receptor blocker (losartan) + MCAO stroke.

ICV minipump implantation surgery → ICV infusion of losartan / vehicle for 14 days → MCAO surgery on day 12 → Neurological evaluation on day 13 (24 hrs after MCAO) sacrificed mice on day 14 (48 hrs after MCAO). Brains extracted were sectioned immediately for the analysis of infarct volume.
Table 2: Overview of Experimental protocol II

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**METHODS/ GENERAL PROCEDURES**

**Evaluation of Neurological deficits**

The evaluation of neurological deficits was carried out 24 hours after MCAO according a 6-point scale: 0 = no neurological deficits, 1 = failure to extend right forepaw fully, 2 = circling to the right, 3 = falling/rolling to the right, 4 = no spontaneous walking with a depressed level of consciousness and 5 = death (Longa et al. 1989; Minematsu et al. 1992).

**Quantification of Infarct volume**

Infact volume was evaluated using 2,3,5- Tri phenyl Tetrazolium Chloride (TTC). TTC method is an established technique for the detection and quantification of cerebral infarction in experimental animals (Bederson et al. 1986). TTC is a colourless solution which is reduced by the enzymes of functioning mitochondria to yield a deep red formazan (Bederson et al. 1986). Infarcted brain regions cannot convert TTC and remain unstained appearing white/pale in colour. The animals that underwent the previous protocol were also used for quantification of infarct volume 48 hours after MCAO i.e one day after neurological evaluation, mice were sacrificed. Brains were removed and cut
into 5-6 sections of 1-mm thickness from frontal tip with a mouse brain matrix. The extent of ischemic infarction was revealed by reaction with a 2% solution of 2,3,5- TTC (Sigma Chemical, St.Louis, MO, USA) for 20-30 minutes at 37 °C in dark (Lundy et al. 1986). The sections were then washed with saline and fixed in 4% paraformaldehyde for 30 mins at room temperature. They were then stored at 4 °C in 10% formalin before analysis. 2,3,5-TTC is a marker for mitochondrial function and has been shown to be a reliable indicator of ischemic areas for up to 3 days after ischemia. Images of all stained slices were taken using a flat bed scanner and the border between infarct (unstained) and non-infarct (stained) tissue was outlined with an image analysis system(NIH, imageJ software). The area of infarction was measured by subtracting the area of non-lesioned ipsilateral hemisphere from the total area of the contralateral hemisphere (Lin et al. 1993). The volume of infarction was calculated by integration of lesion areas (area of infarct in square millimeters x thickness [1mm]).

**Fluoro-Jade Staining**

The animals were sacrificed 48 hrs after ischemic stroke and Fluoro-Jade labeling was performed on brains fixed in 4% paraformaldehyde overnight, cryo protected with 30% sucrose in PBS for 3 days. Cryostat sections were cut at 30 µm thickness and collected in buffer solution which were mounted on to gelatin coated slides. Fluoro-Jade staining was performed by placing 30 µm coronal sections in 0.001% Fluoro-Jade solution (Histochem, Jefferson, AR, USA) in 0.1% acetic acid as described previously (Schmued and Hopkins 2000). Briefly, 30-µm mounted sections were air dried and rehydrated in decreasing strengths of ethanol (100 to 75%) for 3 min then distilled deionized water for 1 min each, then the slides were incubated in 0.06% potassium
permanganate, washed in distilled water, and transferred to 0.001% Fluoro-Jade staining solution. After staining, the sections were rinsed in distilled water, dried, immersed in xylene, and coverslipped with DPX (Sigma-Aldrich, St. Louis, MO, USA) to observe under microscope for histological damage.

**Western Blotting**

To determine the expression of AT$_1$ receptor, MMP-2 and myeloperoxidase (MPO, marker for neutrophils) in brain tissue of mice after MCAO. The ischemic and non-ischemic hemispheres were separated, placed in cryotubes and flash frozen in dry ice. The tubes were stored in a -80 °C freezer until homogenization. Frozen tissue samples were homogenized in 400 µl cold Lysis buffer containing glycerol, HEPES (pH 7.4), EDTA, NaCl, MgCl$_2$ and protease inhibitor (Roche diagnostics, Indianapolis, IN, USA). The tissue was processed with a sonicator and centrifuged at 4oC for 10 minutes at 14000 g. The supernatants were separated, frozen and kept at -80 °C until use. The total protein concentration was determined by Bradford method. For gel loading, the volume equivalent to 30 µg of total protein was loaded into fresh made 10% or 12% polyacrylamide gels. Gels were electrophoresed at 100V, 150 mA, 75 W until the dye front neared the resolving gel and then increase the voltage to 150-170 volts until the dye reaches bottom of the gel. The proteins were transferred from the gel to 0.2 µm PVDF membranes for 2 hours at 70V, 30mA, 10W. The PVDF membranes were blocked with 10 ml of 10% NFDM (Carnation, Nestle) in washing buffer (0.1% Tween-20- tris-buffered saline, TTBS) overnight. After washing with wash buffer, the membranes were incubated overnight with either with rabbit anti mouse AT$_1$, rabbit anti-mouse MMP-2 or goat anti-mouse MPO polyclonal antibody (Santa Cruz biotechnology, CA, USA) and
monoclonal anti-β-actin (Sigma, Saint Louis, MO, USA) for actin (an internal standard). Membranes were washed again in TTBS, incubated with secondary antibody, goat anti-rabbit/donkey anti-goat IgG/donkey anti-rabbit IgG, horse radish peroxidase conjugated antibody (Jackson ImmunoResearch Laboratories, Inc, PA, USA) and anti-mouse actin (Sigma, Saint Louis, MO, USA) for 1 hour. After washing with washing buffer, the sample proteins were visualized via the chemiluminescence produced by the ECL substrate (Pierce) and captured by the Fuji LAS3000 imager. The relative amount of protein of interest (POI) is determined by normalizing to actin.

\[
\text{Normalized Value} = \frac{(\text{POI}_{\text{density}} - \text{background}_{\text{density}})}{(\text{actin}_{\text{density}} - \text{background}_{\text{density}})}
\]

**Immunohistochemistry**

To study the infiltration of blood borne cells (post-ischemic extravasation of macrophages and neutrophils which play a significant role in focal ischemia-induced brain damage (Hernandez et al. 1987; Zhang et al. 1994) and activation of microglia in order to assess the post ischemic effect of MCAO on inflammation. Brains were fixed in 4% paraformaldehyde overnight, cryo protected with 30% sucrose in PBS for 3 days and then stored at -80 °C until use. Temperature of frozen brains was resumed in a cryostat from -80 °C to -20 °C. 30 μm thick sections were rinsed in 0.01M PBS for 5 mins and incubated in blocking solution (5% normal horse serum, Vector laboratoties, in PBS containing 0.3% Triton X-100 1% BSA) for 1 hour at room temperature and then the specimens were incubated with either polyclonal goat anti-mouse MPO (Santa cruz biotechnology, CA, USA) or polyclonal rabbit anti-mouse CD-68 (marker for macrophages) antibody (Santa cruz biotechnology, CA, USA) for 2 days at 4 °C. The sections were washed with PBS and incubated in biotin–conjugated horse anti-goat IgG
or biotin conjugated donkey anti-rabbit IgG (Vector Laboratories, CA, USA) for 1 hour at room temperature. After washing, the secondary antibodies were visualized by treatment with horseradish-conjugated avidin/biotin complex (Vector Laboratories, CA, USA) for 30 mins-1 hr at room temperature. After washing with PBS, incubated in diaminobenzidine/H2O2 and sections were dehydrated, mounted with permount.

**Quantification of Immuno-positive cells (Neutrophils & Macrophages)**

The distribution of neutrophils (MPO-positive cells) and Macrophages (CD-68-positive cells) was studied at 6 levels (Figure 8 A) from 3 representative animals for each of control and losartan groups. The region of pathology from entire ipsilateral hemisphere of immunostained sections was photographed at four different fields using a Leica DMR microscope (Leica Wetzlar, Heidelberg, Germany, 20-fold objective) and an Optronix magnafure digital camera system. The number of MPO and MMP-2 positive cells was counted in these fields (512,000 µm², determined by NIH image J software) using NIH image J software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Statistical analysis**

All statistics are represented as mean ± SE. The changes in neurological scores were analyzed using the non-parametric Mann-whitney U-test. The data of Infarct volume, Western blotting and Immunohistochemistry was assessed in treated and untreated mice using unpaired student’s t-test. The data of western blotting in untreated mice was calculated using paired student’s t-test. For all procedures, p values <0.05 were considered statistically significant.
RESULTS

1. Effect of ischemic stroke on AT₁ receptor expression

The brain AT₁ receptor expression significantly increased ($p < 0.05$ with paired $t$-test, $n = 4$; Fig. 6) in the ischemic hemisphere at 48 hrs after Ischemic stroke compared to Non-ischemic hemisphere.

![Image of gel and densitometric analysis](image)

Figure 6: Comparision of AT₁ receptor expression in Non-stroke vs Stroke sides of brain tissue, 48 hrs after occlusion of middle cerebral artery. A) Representative gel showing an increased expression of AT₁ receptor in Ischemic hemisphere (Stroke) compared to Non-ischemic hemisphere (Non-stroke). B) Densiometric analysis for AT₁ receptor activity. ($n = 4$). *$p < 0.05$. 

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2. Effects of selective blockade of AT₁ receptor on neurological deficits

Neurological deficit evaluations were carried out 24 hours after MCAO. A scattergram of the neurological scores for vehicle versus losartan groups is presented in Fig. 7. Losartan was able to produce a significant reduction ($p < 0.05$; Mann Whitney U-test) in the neurological deficits seen in animals after ischemia when compared with vehicle-treated mice.

Figure 7: Scatter plots of neurological deficit scores in Vehicle versus Losartan treated groups evaluated 24 hrs after induction of focal cerebral ischemia. Statistical analysis was performed using the Mann-Whitney non parametric test. $n = 6$ per each group.
3. Effect of ICV infusion of losartan on infarct volume

Ischemia resulting from MCAO lead to brain injury of the ipsilateral hemisphere including cortical and subcortical areas, along with ipsilateral oedema. Mice pretreated with losartan showed a significant reduction in volume of injury, corrected for oedema compared to vehicle treated mice. The animals in vehicle-treated stroke group have severe neurological deficits (scores of 3, 4 and 5). These mice showed significant increase in lesion volume (Fig. 8A). Mice in losartan-treated stroke group, which had an average neurological score of 2, showed decreased lesion volume (Fig. 8B,C).

Figure 8: One millimeter thick, 2,3,5-triphenyltetrazolium chloride (TTC) stained coronal sections of brain numbered from frontal (section 1) to caudal (section 6) of vehicle treated (A) and losartan treated (B) mice 48 hrs after MCAO. Non-ischemic areas had converted TTC to a deep formazan compound and appear dark in figure. Ischemic areas failed to metabolize TTC and appear white in figure. Note the decrease in ischemic area of the mice pretreated with losartan.
Figure 8C: Morphometry of ischemic area determined with TTC staining and expressed as a percentage of total area in vehicle treated vs losartan treated groups. n = 4/group. *$p < 0.05$. Values are mean ± SEM.

4. Histological (qualitative) analysis of Ischemic brain damage due to MCAO

Fluoro-jade staining was performed to specifically identify the success of MCAO and to identify the areas in brain affected by ischemic brain damage. Fluoro-Jade is an anionic fluorochrome capable of selectively staining degenerating neurons in brain slices. The histochemical application of Fluoro-Jade results in a simple, sensitive and reliable staining of degenerating neurons and their processes resulting from exposure to a variety of neurotoxic insults (Schmued, Albertson, and Slikker 1997). Fluoro-jade positive cells were found throughout the ipsilateral cortex (Fig. 9A), caudate putamen (Fig. 9B, C), CA1 layer and Dentate gyrus of hippocampus (Fig 9D).
5. Analysis of MMP-2 and MPO expression in Vehicle versus Losartan treated groups.

The brain MPO expression significantly increased ($p < 0.05$ with paired $t$-test, $n = 4$; Fig. 10) in the ischemic hemisphere at 2 days after Ischemic stroke compared to non-ischemic hemisphere. There was also a significant increase in MMP-2 expression 48 hrs after
MCAO in ischemic hemisphere relative to non-ischemic hemisphere (n = 4, paired $t$-test, Fig. 11). Lastly there was a reduction in the levels of MPO and MMP-2 in losartan pretreated mice compared to untreated (vehicle) mice (n = 4, unpaired $t$-test, Fig. 12). Though the decrease in MMP-2 expression was not significant in treated group.

Figure 10: Comparision of MPO activity in Non-stroke vs Stroke sides 48 hrs after occlusion of middle cerebral artery. A) Representative gel showing an increased expression of MPO in Ischemic hemisphere (Stroke) compared to Non-ischemic hemisphere (Non-stroke). B) Densiometric analysis for MPO activity. (n = 4, *$p < 0.05$).
Figure 11: Comparison of MMP-2 activity in Non-stroke vs Stroke sides, 2 days after occlusion of middle cerebral artery. A) Representative gel showing an increase in MMP-2 activity in Ischemic hemisphere (Stroke) compared to Non-ischemic hemisphere (Non-stroke). B) Densiometric analysis for MMP-2 activity. (n = 4, *p < 0.05).
Figure 12: Chronic pretreatment with losartan reduced the ischemia-induced increase in MPO (80 KDa) and MMP-2 (63 KDa) expression. A) Representative gel showing the decreased expression of MPO and MMP-2 in mice pretreated with losartan compared to vehicle treated mice. B) Summary of densiometric analysis for MPO and MMP-2 activity (n = 4/group). *p < 0.05 (Vehicle [MPO] vs Losartan [MPO]).

6. Effect of Ang II/AT₁ receptor blockade on inflammatory cell infiltration

There was a significant increase in neutrophil and macrophage infiltration in the ipsilateral (ischemic) hemisphere (Fig. 13 A, 14 A) compared to contralateral hemisphere (almost none, Fig. 13 C, 14 C) at 2 days after focal cerebral ischemia. Losartan pretreatment significantly reduced the inflammatory cell infiltration relative to the vehicle treated mice (Fig. 13 B,E and Fig. 14 B,E).
Figure 13: Typical pictures of myeloperoxidase (MPO)-positive cells (neutrophils) in mice subjected to permanent occlusion of middle cerebral artery for 48 hours.

Pictomicrographs of ipsilateral hemispheres from untreated (A), treated (B) and contralateral hemisphere of untreated (C) and treated groups (D, arrows indicate neutrophils). Scale bar = 50µm. Insert shows magnified neutrophils (63x).
Figure 13E: Quantification of neutrophils in Vehicle vs Losartan treated groups (E) (n = 3/group), **p < 0.01 (Vehicle [Ipsilateral] vs Losartan [Ipsilateral]).
Figure 14: Typical pictures of CD-68-positive cells (macrophages) in mice subjected to permanent occlusion of middle cerebral artery for 48 hours. Pictomicrographs from ipsilateral hemispheres of untreated (A), treated (B) and contralateral hemisphere of untreated (C) treated groups (D, arrows indicate macrophages). Scale bar = 50 µm. Insert shows magnified macrophages (63x).
Figure 14E: Quantification of neutrophils in Vehicle versus Losartan treated groups (E) (n = 3/group, ***p < 0.001).
DISCUSSION

During the past decade number of studies have indicated that systemic treatment with AT₁ receptor antagonists can prevent the occurrence of stroke in spontaneously hypertensive rats and reduce the degree of neuronal damage and infarct volume in animals induced with ischemic stroke (Inada et al. 1997; Nishimura, Ito, and Saavedra 2000; Stier et al. 1991; von Lutterotti et al. 1992). However, it is not clear whether the benefit is from the peripheral or central effects of AT₁ receptor antagonist. For evaluating the role of brain AT₁ receptors in ischemic stroke, we blocked these receptors by direct infusion of losartan into brain. The present study demonstrates that blockade of central AT₁ receptors can protect the brain from focal cerebral ischemic damage by reducing the neurological outcome, extent of infarction and inflammation in normotensive mice.

In this study, we firstly determined the Ang AT₁ receptor expression after ischemic stroke. For the first time we showed that experimental stroke results in a significant upregulation of AT₁ receptors in the ischemic (ipsilateral) hemisphere compared to non-ischemic (contralateral) hemisphere (Fig. 7) of normotensive mice. Upregulation of AT₁ receptor expression during ischemic phase provides the evidence of activated Ang II/AT₁ signaling pathway in contributing to brain injury during ischemic stroke. Ang II AT₁ receptors mediate all of the known physiological effects of Ang II in the central nervous system and the periphery (Saavedra 1992; Timmermans et al. 1993). The AT₁ receptors are broadly distributed in the brain with high concentrations in the
forebrain, including hypothalamic nuclei and circumventricular organs (Hauser, Johren, and Saavedra 1998). Most of the brain Ang II receptors are localized in neurons, (Lind, Swanson, and Ganten 1985; Tsutsumi and Saavedra 1991) endothelial cells lining the cerebral vasculatures (Ando et al. 2004). The Ang II receptors in the circumventricular organs and cerebrovascular endothelial cells can respond to circulating Ang II; receptors located inside the blood barrier are activated by Ang II formed in the brain and/or transported to the brain from the circulation. In the current study, we determined the expression level of AT1 receptor only in the whole brain tissue using western blotting technique. Further investigation is required to see in which components (neuron, glia, cerebral blood vessel) of brain, the AT1 receptors are unregulated during ischemic stroke.

Following the finding that AT1 receptors are upregulated in stroke, we tested if the blockade of AT1 receptors can influence (reduce) the ischemia induced damage to brain. For specifically targeting the brain AT1 receptors, losartan was used to inhibit these receptors through ICV minipump infusion. Losartan is a high affinity, nonpeptide and a non-competitive receptor antagonist (Lucius et al. 1999). To reach an efficient, steady-state inhibition of central AT1 receptors at the time point of MCAO, losartan was infused over a 14-day period. Results showed that chronic blockade of brain AT1 receptors with losartan resulted in improvement of neurological deficits (Fig. 7) and reduced infarct volume (Fig. 8) compared with untreated animals that received the same surgical intervention. This constructive outcome after MCAO is supported by the reports of Maeda et al. (Maeda et al. 1999), who showed improved collateral circulation and smaller lesion size 1 hour after MCAO in angiotensinogen knockout mice than in controls, indicating a beneficial effect of lower Ang II levels before the onset of ischemia. Walther
et al. demonstrated a direct correlation between brain Ang II and the severity of ischemic injury in experimental stroke. This study revealed an enlarged infarct size in brain tissue 24 hours after MCAO in angiotensinogen-overexpressing mice compared to the wild type mice and a smaller lesion area in AT₁ knockout mice compared to wild type. They also demonstrated reduced cell damage in the neurons from AT₁ knock out mice. This data provides clear evidence that neuroprotection against ischemia/hypoxia can be achieved by a drug- as well as a transgene-induced suppression of AT₁ receptor function in vivo and in vitro (Walther et al. 2002). Comparable evidence of improved neurological outcome and infarct volume has been obtained by pharmacological blockade of Ang II synthesis or the AT₁ receptors in hypertensive or normotensive rats (Dai et al. 1999; Mark and Davis 2000; Nishimura, Ito, and Saavedra 2000; Werner et al. 1991; Yabuuchi et al. 1999). Losartan intervention for endpoint reduction in hypertension (LIFE) showed that a 25% stroke reduction in severely hypertensive patients with left ventricular hypertrophy treated with a losartan-based regimen compared to atenolol-based regimen. In a substudy of LIFE, patients with systolic hypertension showed 40% reduction in stroke risk with losartan. These results suggest AT₁ receptor blockers contributing to beneficial outcome of stroke by mechanisms other than blood pressure reduction (Chrysant 2004; Dahlof et al. 2002).

In this study, we used two different staining techniques to evaluate the brain damage after MCAO. TTC staining was used as a quantitative method to compare the severity of ischemic stroke between treated and untreated groups (Fig. 8) where as Fluoro-jade staining was used to determine the histochemical localization of neuronal degeneration (Fig. 9). TTC method is an established technique for the detection and
quantification of cerebral infarction in experimental animals (Bederson et al. 1986). TTC is a marker for mitochondrial function and has been shown to be a reliable indicator of ischemic areas for up to 3 days after ischemia. Fluoro-Jade stains the cell bodies, dendrites, axons and axon terminals of degenerating neurons but does not stain healthy neurons, myelin, vascular elements or neuropil (Schmued, Albertson, and Slikker 1997). Fluoro-jade staining revealed that unilateral occlusion of middle cerebral artery in mice induced neuronal necrosis in the frontal and sensorimotor cortices, caudate-putamen region and hippocampus (Fig. 9) that coincide with behavioral impairments. These ischemia induced changes and functional impairments closely resemble those observed after focal MCAO in humans, which is the most common cause of ischemic stroke (Dai et al. 1999).

Inflammation is thought to play an important role in the pathogenesis of cerebral ischemia and secondary damage (Danton and Dietrich 2003; Dirnagl, Iadecola, and Moskowitz 1999). As mentioned earlier, interruption of cerebral blood flow (CBF) leads to tissue injury followed by inflammatory reaction, which is a common response of the cerebral parenchyma to various forms of insult. This requires the infiltration of leukocytes, both PMN leukocytes and monocytes/macrophages (but not lymphocytes), which are the cellular mediators of subsequent microvessel obstruction, edema formation, cellular necrosis, and tissue infarction (Chou et al. 2004; Clark et al. 1993). Microglia /macrophages secrete a number of both toxic and protective effector molecules and thereby play a key role in inflammatory CNS processes (Schroeter et al. 2001). Ito et al. showed that stimulation of brain and cerebrovascular Ang II systems may contribute not
only to vasoconstriction but also to increased expression of inflammatory factors and increased microvessel permeability (Ito, Takemori, and Suzuki 2001)

To explore if losartan has beneficial effect on inflammatory response after MCAO, we measured the neutrophil and macrophage infiltration in treated vs untreated groups. We found that ischemic stroke induced a remarkable increase in neutrophil and macrophage infiltration as evidenced by the expression of MPO, marker for neutrophils (Fig. 10, Fig. 13 A) and CD-68, marker for macrophages (Fig. 14 A) in the ipsilateral hemisphere. Interestingly, the inflammatory cell infiltration (neutrophils and macrophages) was significantly reduced (Fig 12; 13 B,E; 14 B,E) in the losartan treated group. The data suggests that the reduction of inflammatory response to ischemic stroke can, at least partly, account for the beneficial effect of the central AT$_1$ receptor blockade during cerebral ischemia.

Earlier, Ang II has been reported to be a potent stimulator of the expression of matrix metalloproteinases (MMPs), which degrade the basal lamina constituents (Arenas et al. 2004). The potential of Ang II to increase cerebral edema can be mediated by the expression of MMPs. MMP-2 participates in the breakdown of collagen type IV, a major component of subendothelial basement membrane (Libby and Lee 2000; Sternlicht and Werb 2001). Moreover it has been reported (Fernandez-Patron, Radomski, and Davidge 1999; Fernandez-Patron et al. 2000) that MMP-2 may also lead to vasoconstriction and inflammation through the cleavage of endothelium derived peptides. Besides an increase in inflammatory cell infiltration, we also found a significant increase in MMP-2 expression during ischemic stroke. There was a significant increase in the expression of MMP-2 in the ischemic (ipsilateral) hemisphere compared to non-ischemic (contralateral)
hemisphere (Fig. 11) and losartan pretreatment reduced this ischemia induced over-expression of MMP-2 in the ipsilateral hemisphere. But the results were not significant (Fig. 12). This might be related to losartan treatment and needs to be further verified by infusing the animals with losartan for more than 48 hrs after inducing ischemic stroke (MCAO) or by treating the animals with losartan more than 14 days before inducing ischemic stroke. Inflammation and extracellular matrix damage (MMP-2), are thought to play an important role in the disruption of BBB, oedema, necrosis, scar formation leading to secondary brain damage and apoptosis.

AT$_1$ receptor stimulation also initiates a cascade of signaling events, resulting in the phosphorylation of constitutive transcription factors which regulate the transcription of inducible transcription factors (ITFs), such as c-Fos and c-Jun which act as transcription factors for other genes (Blume, Herdegen, and Unger 1999) leading to apoptosis. ITFs trigger the expression of a number of proinflammatory factors in injured neurons, glial cells, and adhesion molecules on the endothelial cell surface (Blume, Herdegen, and Unger 1999). Moreover Ang II via its AT$_1$ receptors was reported to enhance neutrophil accumulation via neutrophil chemoattractant factors released from vascular endothelial cells (Herdegen, Skene, and Bahr 1997). Therefore, a suppression of inflammatory reaction with reduction of ITFs and inflammatory infiltrates after the blockade of brain AT$_1$ receptors may also contribute to the improved neurological outcome in cerebral ischemia (Thone-Reineke et al. 2004).

BBB, which is formed by the endothelial cells that line cerebral microvessels, has an important role in maintaining a precisely regulated micro-environment for reliable neuronal signaling. At present, there is great interest in the association of brain
microvessels, astrocytes and neurons to form functional 'neurovascular units', and recent studies have highlighted the importance of brain endothelial cells in this modular organization (Abbott, Ronnback, and Hansson 2006). It is assumed that ischemia induces local AT₁ receptor activation in endothelial cells/neurons and leads to Ang II mediated multiple mechanisms which might contribute to generation of reactive oxygen species & membrane disruption, inflammation, increased expression of MMPs which lead to disruption of basal lamina and BBB. This might lead to leakage of various cellular and pathological constituents which can affect the associated astrocytes and neurons leading to secondary damage. An understanding of how these interactions are disturbed in pathological conditions and how these mechanisms are affected by inhibition of central AT₁ receptors could lead to the development of new protective and restorative therapies.

Long-term blockade of AT₁ receptors might result in Ang II accumulation which may lead to enhanced interface of Ang II with AT₂ receptors. Stimulation of AT₂ receptors was proposed to result in vasodilatation (NO-dependent or bradykinin-dependent) of existing vessels and inhibition of growth, balancing AT₁ receptor effects (Carey 2005). AT₂ receptors have been implicated in the process of wound healing and tissue repair and increased tissue AT₂ levels have been reported as early as 24 h after brain injury (Kimura, Sumners, and Phillips 1992; Nakajima et al. 1995; Nio et al. 1995; Lucius et al. 1999; Viswanathan et al. 1994). Hamai et al. showed that pretreatment with ARB, candesartan resulted in increased expression of AT₂ receptor in the ischemic area, 3 hours after MCAO in C57BL/6J mice (Hamai et al. 2006). So relative stimulation of AT₂ receptor signaling by ARB treatment could have a therapeutic advantage to prevent neurological disorders and tissue damage after stroke. However in accordance with
numerous studies on ischemic stroke, direct evidence is currently lacking and needs further investigation to prove this hypothesis.

Recent evidence comes from the study of Forder et al. (Forder, Munzenmaier, and Greene 2005) on normotensive rats which provides direct evidence of increased angiogenesis by increased microvascular measures of vessel density, increased surface vascularization, and maintained microvascular delivery resulting in increased collateralization in the cerebral vascular network ensuing little or no visible infarct after chronic administration of losartan followed by permanent focal ischemia on middle cerebral artery. Inhibition of oxidative stress may also play an important role in brain protection caused by ARBs in cerebral ischemia. It has been previously reported that brain ischemia results in increased oxidative stress due to generation of ROS (Dzau 2001; Laursen et al. 1997; Ushio-Fukai et al. 1996). Hamai et al. found an increase in superoxide production in the cerebral cortex including neurons and glial cells and in small arteries in the brain of C57BL/6J mice and this was found to be inhibited in the brain arterial wall as well as in cerebral cortex by ARB treatment (Hamai et al. 2006). The protection from ARBs against oxidative stress might contribute to neuroprotection and decreased vessel damage which protect against cerebral ischemia.

Our current data on AT₁ receptor expression, infarct volume, neurological deficits, inflammatory cell infiltration and MMP-2 expression combining with previous studies support the idea that Ang II/AT₁ receptor activation is involved in the worsening of ischemic brain damage and inhibition of central AT₁ receptors might have a potential role in reducing the brain damage by directly targeting the receptors at the site of original damage.
CONCLUSION

These results strongly suggest that inhibition of central angiotensin AT₁ receptors can protect the brain from ischemic damage and AT₁ receptor blockers could be used as a possible novel therapy of inflammatory conditions in ischemic stroke to complement the traditional antithrombotic drugs and surgical procedures.
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