Blockade of Central Angiotensin AT_1 Receptors Improves Neurological Outcome and Reduces Expression of AP-1 Transcription Factors After Focal Brain Ischemia in Rats

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Background and Purpose—Angiotensin-converting enzyme inhibitors have been shown to protect against stroke in hypertensive rats and to improve neurological outcome after cerebral ischemia in normotensive rats. The present study was designated to test the hypothesis that blockade of brain AT_1 receptors improves the recovery from focal cerebral ischemia and reduces expression of AP-1 transcription factors c-Fos and c-Jun, which have been associated with programmed cell death and neurodegeneration.

Methods—Experiments were carried out in normotensive male Wistar rats. Focal cerebral ischemia was induced by middle cerebral artery occlusion lasting for 90 minutes and followed by reperfusion. The selective AT_1 receptor antagonist irbesartan was infused intracerebroventricularly over a 5-day period before the induction of ischemia at a dose that inhibited brain but not vascular AT_1 receptors. Twenty-four hours after ischemia, neurological outcome was evaluated and expression of c-Fos and c-Jun proteins in the brain was studied immunocytochemically.

Results—Focal brain ischemia resulted in a strong induction of c-Fos and c-Jun proteins in the cortex, which positively correlated with the degree of neurological deficits. Treatment of rats with irbesartan significantly improved neurological outcome of focal cerebral ischemia when compared with the vehicle-treated group and markedly reduced the expression of c-Fos and c-Jun proteins in the cortex on the ligated side of the brain. Irbesartan pretreatment completely abolished the ischemia-induced c-Fos expression in the hippocampus.

Conclusions—The present study shows a relationship between c-Fos and c-Jun expression and neurological outcome after focal brain ischemia. Our data indicate that long-term blockade of central AT_1 receptors improves the recovery from brain ischemia and reduces the expression of c-Fos and c-Jun proteins in the brain. Pretreatment with an AT_1 receptor antagonist has beneficial effects after cerebral ischemia. (Stroke. 1999;30:2391-2399.)

Key Words: cerebral ischemia, focal receptors, angiotensin transcription factors rats

Focal cerebral ischemia leading to a loss of neuronal tissue and subsequent gliosis is a common, life-threatening disease in humans. The severity of clinical manifestations after cerebral ischemia depends mainly on the location and size of the infarcted area in the brain. Interruption of blood flow in the brain results in a structural damage of neuronal tissue. The events following brain ischemia comprise activation of membrane receptors and their signaling pathways, loss of energy stores, disintegration of membranes and macromolecular breakdown, activation of proteolytic enzymes, formation of free radicals, and fragmentation of DNA. Both neuronal necrosis and apoptosis can be observed after brain ischemia. Cerebral hypoxia also leads to alterations in protein synthesis and gene expression. The latter is, among others, under the control of inducible transcription factors (ITFs). Activation of ITFs after hypoxia-ischemia represents one of the links between the extracellular signals and the initiation of intracellular genomic and metabolic events that are associated with regeneration and survival or lead to a selective delayed neuronal death. Focal cerebral ischemia has been shown to induce the expression of gene products of the c-fos and c-jun families in the nervous system. c-Jun in particular is believed to initiate degeneration via de novo protein synthesis of apoptotic effectors.

There is increasing, though controversial, data showing that angiotensin II (Ang II), the effector peptide of the renin-angiotensin system (RAS) and natural agonist for angiotensin AT_1 and AT_2 receptors, may be involved in the initiation and regulation of processes occurring in brain ischemia. Long-term treatment with angiotensin-converting enzyme (ACE) inhibitors or AT_1 receptor antagonists has been reported to prevent the occurrence of stroke in stroke-prone spontaneously hypertensive rats and salt-loaded Dahl salt-sensitive rats. It is generally accepted that ACE...
inhibitors and AT1 receptor antagonists prevent against brain ischemia in hypertensive rats primarily by reducing blood pressure. In addition, ACE inhibitors have been demonstrated to exert beneficial effects on the metabolic and circulatory derangement in the ischemic brain in spontaneously hypertensive rats.\textsuperscript{13} However, treatment with ACE inhibitors also improved neurological recovery from cerebral ischemia in normotensive rats.\textsuperscript{13}

The mechanisms of the protective effects of ACE inhibitors in cerebral ischemia have not yet been elucidated. They may be related to the reduction of Ang II synthesis or to an accumulation of bradykinin or other peptides, such substance P or enkephalins, which are degraded by ACE. Because most of the known central actions of Ang II, including the stimulation of ITFs, are mediated through the AT1 receptor,\textsuperscript{14,15} we tested the hypothesis that a selective blockade of this receptor in the brain improves recovery from stroke and attenuates the ischemia-induced expression of ITFs in the brain. For this purpose, we investigated the effect of long-term inhibition of central AT1 receptors by the selective, nonpeptide AT1 receptor antagonist irbesartan on the neurological and c-Jun proteins induced by transient MCA occlusion focal cerebral ischemia and reduced the expression of c-Fos dipsogenic responses to ICV Ang II but did not modify the used dose of irbesartan effectively inhibited the pressor and cardiovascular responses to ICV Ang II during 5 consecutive days before MCA occlusion. Irbesartan is a potent, selective, high-affinity antagonist for AT1 receptors with no antagonist activity and affinity for AT2 receptors.\textsuperscript{16} To avoid peripheral actions of the AT1 receptor antagonist, irbesartan was infused intracerebroventricularly (ICV) during 5 consecutive days before MCA occlusion. The used dose of irbesartan effectively inhibited the pressor and dipsogenic responses to ICV Ang II but did not modify the pressor responses to intravenously injected Ang II. Our data demonstrate that long-term ICV treatment of rats with irbesartan significantly improved the neurological outcome of focal cerebral ischemia and reduced the expression of c-Fos and c-Jun proteins induced by transient MCA occlusion followed by reperfusion.

Materials and Methods

Male normotensive Wistar rats weighing approximately 250 g were obtained from Charles River (Sulzfeld, Germany). Animals were kept under controlled conditions with respect to temperature and humidity and were housed on a 12 h light/12 h dark cycle with free access to food and water.

Surgical Methods

Implantation of the ICV Cannula

For ICV injections, polyethylene cannulae (PP-20) were implanted into the left lateral brain ventricle using a stereotaxic apparatus (David Kopf Instruments) and fixed to the skull with dental cement. The stereotaxic coordinates for the ICV cannula were 0.6 mm caudal to bregma, 1.3 mm lateral to the midline, and 5.0 mm vertical from the skull surface.

Implantation of Osmotic Minipumps

Osmotic minipumps (ALZET Model No. 2002), which continuously deliver dissolved substances at a rate of 0.5 \(\mu\)L/h into the desired tissue area, were filled with vehicle or the AT1 receptor antagonist. The concentration of irbesartan in the solution was 4 nmol/L. The flow modulator of the pump was connected with a polyethylene catheter (PP-60) to a curved (right-angled) metal cannula (21-gauge) to allow for long-term ICV infusions of drugs. The pumps were placed in a sterile saline solution (0.9\%) for 4 hours at 37°C before implantation to initiate their operation at a constant pumping rate and to minimize the possibility of clot formation in the catheter or in the metal cannula. The head of the rat 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 rat. The osmotic pump was placed into the pocket, and the right-angled metal cannula was inserted through the skull into the brain (depth 5 mm) to reach the ventricle. The external part of the metal cannula was fixed to the skull with screws and dental cement, and the wound was sutured.

Implantation of the Femoral Artery and Vein Catheters

Three days after implantation of ICV cannulae, a polyethylene catheter (PP-50) was inserted through the femoral artery into the abdominal aorta. Another catheter was inserted into the femoral vein. Both catheters were filled with heparinized saline, passed through a subcutaneous tunnel, sealed, and secured at the back of the neck. The arterial catheter was used for blood pressure measurements, the venous catheter for intravenous Ang II administration.

Oclusion of the Middle Cerebral Artery

In this study, an intraluminal occlusion method with subsequent reperfusion was used.\textsuperscript{17} This method permits to induce reversible ischemia without craniectomy. Briefly, under general anesthesia the right MCA was occluded for 90 minutes with a 4–0 nylon monofilament inserted into the common carotid artery at the bifurcation of the common carotid artery and the external carotid artery and advanced via the internal carotid artery. Reperfusion was achieved by pulling out the monofilament. Sham-operated rats underwent the same surgical procedures except that the occluding monofilament was not inserted. Chloral hydrate (400 mg/kg body weight) injected intraperitoneally was used as anesthetic for all surgical procedures.

General Procedures

Measurement of Cardiovascular Responses

All experiments were carried out in conscious, freely moving rats. The femoral artery catheter was connected to the transducer. The experiments were started when the animals were resting and when basal mean arterial pressure (MAP) and heart rate were stable. Ang II was injected ICV in a volume of 1 \(\mu\)L and flushed with 4 \(\mu\)L of physiological saline, and the pressor responses to the peptide were recorded. In experiments carried out in rats in which osmotic minipumps had been implanted, Ang II (25 ng/kg body weight) was injected intravenously via the femoral vein catheter, and the pressor responses to the peptide were recorded.

Measurements of MAP were performed via the arterial catheter with use of a pressure transducer (DTX/Plus; Spectramed) connected to a pressure processor (Gould) coupled to a Gould Brush recorder. The analog output signal of MAP from the pressure processor was digitized and then processed with a computer program. The analysis of the MAP responses has been described in detail.\textsuperscript{18}

Determination of Drinking Response

Water intake was determined by weighing the water that the rat drank during a 20-minute period starting immediately after the ICV injection of Ang II.

Evaluation of Neurological Deficits

The evaluation of neurological deficits was carried out 24 hours after reperfusion using the neurological grading system developed by Bederson et al.\textsuperscript{19} This method includes the evaluation of the grade of the forelimb flexion contralateral to the injured hemisphere, resistance to lateral push, and observation for circling behavior. A grading scale of 0 to 3 was used to assess the effects of MCA occlusion on neurological deficits. Rats with no observable deficits were graded 0; rats displaying circling behavior...
Immunocytochemical Detection of c-Fos and c-Jun Proteins
Immediately after neurological examination, rats were deeply anesthetized and intracardially perfused with phosphate buffered saline followed by 4% paraformaldehyde solution for fixation of brain tissue. The brains were removed, postfixed overnight, and subsequently cryoprotected in 30% sucrose for 72 hours at 4°C. Cryostat-cut coronal sections of 50 μm were processed for cytochemistry as free-floating sections. Immunocytochemistry was performed by the conventional avidin-biotin complex (ABC) method. Brain sections were incubated with primary antibody for 48 hours at 4°C, then the reaction was visualized by using the ABC method (Vectastatin, Vector Laboratories Inc). Immunoreactivities were visualized by diaminobenzidine. The anti-c-Fos and anti-c-Jun antibodies were diluted 1:18,000 and 1:4000, respectively. The specificity of the polyclonal antibodies used has been described elsewhere.

Experimental Protocols

1. Effect of ICV Pretreatment With the AT1 Receptor Antagonist Irbesartan on Pressor and Drinking Responses Induced by ICV Ang II
These experiments were carried out to establish the lowest dose of irbesartan that effectively inhibits the pressor and drinking responses to ICV Ang II. Rats were pretreated ICV with vehicle (controls, n=8) or with irbesartan (0.5 nmol, n=8; 2 nmol, n=9; and 5 nmol, n=5) 10 minutes before ICV injection of Ang II (10 pmol). Pressor responses and drinking responses to the peptide were recorded.

2. Effect of Long-Term ICV Infusion of Irbesartan on Pressor Responses to Intravenous Ang II
This experiment was carried out to determine whether irbesartan after continuous ICV infusion over a 5-day period at the dose of 2 nmol/h was able to exert cardiovascular effects by interacting with vascular AT1 receptors.

Using osmotic minipumps, rats were continuously (5 days) treated ICV with vehicle (controls, n=9) or irbesartan at a dose of 2 nmol/h (n=6). On the third day after the implantation of the osmotic minipumps, rats were anesthetized and catheters were inserted into the femoral artery and vein. On day 6, the pressor responses to intravenously injected Ang II (25 ng/kg body weight) were recorded in conscious animals. The MAP values expressed as AUC (mm Hg× min) represent the sum of MAP changes over a period of 3 minutes.

3. Effect of ICV Infusion of Irbesartan on the Neurological Outcome After Focal Cerebral Ischemia
Animals were divided into 3 groups: sham-operated group (n=5), vehicle-treated group (n=19), and irbesartan-treated group (n=14). Using osmotic minipumps, sham-operated and vehicle-treated rats received ICV infusions of vehicle over the 5-day period. Irbesartan at a dose of 2 nmol/h was continuously infused ICV in the respective group. On day 6, the MCA was occluded for 90 minutes in all rats, then the reaction was visualized by using the ABC method. Statistical analyses were performed with the Student t test for unpaired samples. Comparisons of neurological deficits induced by MCA occlusion with reperfusion in vehicle- and irbesartan-treated rats were performed with the Student t test for unpaired samples. Some rats in the vehicle-treated group (n=4 of a total number of 19) did not display neurological deficits or signs of brain ischemia 24 hours after MCA occlusion. These animals were excluded from further analysis. Rats in the irbesartan-treated, MCA-occluded group without neurological deficits were corrected accordingly by applying the formula A=B−C×(D/E)+F×0.5, with A being the corrected number of rats without neurological deficits; B, the number of rats without neurological deficits before correction; C, the total number of rats used in irbesartan-treated group; D, the number of rats without neurological deficit in vehicle-treated group; E, the total number of rats used in vehicle-treated group; and F, the integer of a number. Statistical significance was accepted at P<0.05.

4. Effect of ICV Infusion of Irbesartan on Expression of c-Fos and c-Jun in the Brain After Focal Cerebral Ischemia
The animals that underwent the previous protocol were also used to investigate the expression of AP-1 transcription factors in the brain. Immediately after the neurological evaluation, rats were perfused intracardially with 4% paraformaldehyde under deep anesthesia, and the brains were removed and fixed for immunohistochemical detection of c-Fos and c-Jun.

These experimental protocols have been approved by the State Governmental Committee for Ethical Use of Animals.

Drugs
Ang II for intravenous injections was dissolved in physiological saline (25 ng Ang II/100 μL) and injected as a bolus at the dose of 25 ng/kg body weight. Ang II for ICV injections was also dissolved in physiological saline. Ten picomoles of the peptide was injected ICV in a total volume of 1 μL and flushed with 4 μL of physiological saline. The nonpeptide AT1 receptor antagonist irbesartan was a gift from Dr W.M. Petkun, Bristol-Myers Squibb, Princeton, NJ. Irbesartan for ICV infusions was dissolved in physiological saline by neutralization with stoichiometric equivalent of NaOH. The concentration of irbesartan in the solution was 4 nmol/L, and the final pH of the solution was 8.5 to 9.0. The pH of the vehicle solution was adjusted with NaOH to the same pH value.

Statistical Analyses
Results are expressed as mean±SEM. The effects of ICV treatment with various doses of irbesartan on pressor and drinking responses to ICV Ang II were analyzed by ANOVA followed by a post hoc Bonferroni test. Pressor responses to intravenous Ang II after long-term ICV treatment with either vehicle or irbesartan were analyzed by the Student t test for unpaired samples. Comparisons of neurological deficits induced by MCA occlusion with reperfusion in vehicle- and irbesartan-treated rats were performed with the Student t test for unpaired samples. Some rats in the vehicle treated group (n=4 of a total number of 19) did not display neurological deficits signs of brain ischemia 24 hours after MCA occlusion. These animals were excluded from further analysis. Rats in the irbesartan-treated, MCA-occluded group without neurological deficits were corrected accordingly by applying the formula A=B−C×(D/E)+F×0.5, with A being the corrected number of rats without neurological deficits; B, the number of rats without neurological deficits before correction; C, the total number of rats used in irbesartan-treated group; D, the number of rats without neurological deficit in vehicle-treated group; E, the total number of rats used in vehicle-treated group; and F, the integer of a number. Statistical significance was accepted at P<0.05.

Results

1. Effect of ICV Pretreatment With the AT1 Receptor Antagonist Irbesartan on Pressor and Drinking Responses Induced by ICV Ang II
Ang II injected ICV at the dose of 10 pmol induced an immediate increase in MAP of about 20 mm Hg followed by the drinking response. Irbesartan pretreatment had significantly, dose-dependent effects on the pressor responses to Ang II (F3,26=41.87; P<0.001). Although 2 and 5 nmol were more effective than 0.5 nmol to inhibit the Ang II–induced pressor responses, the dose of 5 nmol did not reduce the pressor response to Ang II more effectively than the dose of 2 nmol (Table 1). All doses of irbesartan completely abolished the drinking response to ICV Ang II (F3,26=13.56, P<0.001) (Table 1).

2. Effect of Long-Term ICV Infusion of Irbesartan on Pressor Responses to Intravenous Ang II
Basal MAP values on day 6 after implantation of the osmotic minipumps were not different between rats given long-term ICV infusion of irbesartan and rats infused with vehicle (Table 2). In both groups, injection of Ang II (25 ng/kg body weight IV) caused an immediate increase in MAP of approximately 40 mm Hg, which peaked within 20 seconds after angiotensin injection and rapidly returned to preinjection
TABLE 1. Effect of Irbesartan Administered ICV on MAP Increase and Drinking Response Induced by Ang II (10 pmol ICV)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Dose, nmol</th>
<th>n</th>
<th>MAP response, mm Hg</th>
<th>Water intake, mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0</td>
<td>8</td>
<td>20.8±2.4</td>
<td>4.0±1.0</td>
</tr>
<tr>
<td>Irbesartan</td>
<td>0.5</td>
<td>8</td>
<td>5.6±1.3*</td>
<td>0.2±0.0*</td>
</tr>
<tr>
<td>Irbesartan</td>
<td>2.0</td>
<td>9</td>
<td>0.5±0.6*†</td>
<td>0.1±0.0*</td>
</tr>
<tr>
<td>Irbesartan</td>
<td>5.0</td>
<td>5</td>
<td>0.1±0.4*†</td>
<td>0.1±0.0*</td>
</tr>
</tbody>
</table>

Values are the mean±SEM of n rats. Vehicle or irbesartan was infused ICV 10 minutes before Ang II (10 pmol ICV).

*P<0.001, statistical comparison to the vehicle-pretreated group; †P<0.05, statistical comparison to the group of rats of pretreated with 0.5 nmol irbesartan icv, calculated with 1-way ANOVA followed by a post hoc Bonferroni test.

values. The MAP responses to the peptide, expressed either as the maximal increase in MAP or as the sum of MAP changes integrated in time (AUC), did not differ significantly between the 2 groups (Table 2).

3. Effect of ICV Infusion of Irbesartan on the Neurological Outcome After Focal Cerebral Ischemia

Neurological evaluations were carried out 24 hours after MCA occlusion by a person who had no knowledge of the treatment that the rat had received. Twenty-four hours after focal cerebral ischemia, most of the rats infused ICV with vehicle (n=15) suffered from severe neurological deficits (grades 2 and 3). Four rats had a neurological grade of 1, 8 rats a grade of 2, and 3 rats a grade of 3. Rats treated with irbesartan and exposed to ischemia (n=11) had neurological grades of 0 (1 rat), 1 (6 rats), and 2 (4 rats). None of the irbesartan-treated rats showed neurological grade of 3. These rats showed a significantly lower neurological deficit grade than rats treated with vehicle (T24=2.48, P<0.02) (Figure 1).

4. Effect of ICV Infusion of Irbesartan on Expression of c-Fos and c-Jun in the Brain After Focal Cerebral Ischemia

Expression of c-Fos and c-Jun in the Brain of Sham-Operated Rats

The ICV cannulae did not affect c-Fos and c-Jun expression, apart from the neurons around the cannula, which showed a slightly increased level of c-Fos and c-Jun immunoreactivities. The basal c-Fos and c-Jun expression in other brain areas did not differ between the 2 brain hemispheres. c-Fos expression in sham-operated, vehicle-treated rats was very weak; only a few immunoreactive nuclei were scattered throughout all cortical layers (Figure 2). In contrast, c-Jun was expressed distinctly in the cerebral cortex, especially in the piriform, parietal, and insular cortices, with the highest density in the layer II (Figure 3). In the hippocampus, the expression of c-Fos was hardly visible, except that some neurons in the CA3 pyramidal cell layer showed c-Fos expression (Figure 4). Compared with c-Fos, c-Jun showed stronger expression in the hippocampal dentate gyrus, whereas a low level of expression was observed in the CA3 and CA1 neurons (data not shown). These distribution patterns of c-Fos and c-Jun expression in sham-operated rats were similar to those described in intact rats.

Expression of c-Fos and c-Jun in the Brain of Rats After Focal Cerebral Ischemia

The expression of c-Fos and c-Jun in the cortex and hippocampus on the nonligated brain side of stroke rats and sham-operated rats was similar. No c-Fos and c-Jun immunoreactivities were seen in the necrotic tissue. The majority of the animals in the vehicle-treated stroke group had severe neurological deficits (scores of 2 and 3) after focal ischemia. These rats showed dramatically increased c-Fos and c-Jun immunoreactivities in the cortex on the ligated side. c-Fos was more strongly induced than c-Jun (Figure 2 and 3). Rats in the irbesartan-treated stroke group, which had an average neurological score of 1, showed only a weak increase in c-Fos and c-Jun expression in the cortex on the ligated side of the brain (Figure 2 and 3). Focal ischemia produced a slight increase in c-Fos in the hippocampus. Irbesartan pretreatment completely abolished the ischemia-induced c-Fos expression in this brain area (Figure 4).

Discussion

Permanent or transient occlusion of the MCA constitutes a widely used animal model of focal cerebral ischemia. Unilateral occlusion of the MCA in rats induces necrosis mainly in the frontal and sensorimotor cortices and in the caudate-putamen region. The ischemia-induced changes and functional impairments closely resemble those observed after...
focal occlusion of the MCA in humans, the most common cause of ischemic stroke.1

The present study demonstrates for the first time that inhibition of central AT1 receptors can improve the neurological outcome of focal brain ischemia induced by temporary MCA occlusion in normotensive rats. Numerous studies have demonstrated that ACE inhibitors and AT1 receptor antagonists may have beneficial effects with respect to ischemic brain injury. These compounds, when administered peripherally in stroke-prone spontaneously hypertensive rats or salt-loaded Dahl-salt sensitive rats, have been reported to improve the neurological status and to reduce the infarct volume in animals exposed to brain ischemia.9,11,24

Interestingly, in some studies the protective effects were also achieved with doses that had little effect on blood pressure, which suggests that the beneficial effects of AT1 receptor antagonists and ACE inhibitors may result from the reduction of the specific actions of Ang II in ischemic brain

Figure 2. Frontal section through the parietal cortex stained for c-Fos protein in vehicle-treated, sham-operated rats (top). Focal ischemia in rats infused ICV with vehicle strongly increased c-Fos expression in the parietal cortex (middle). The ischemia-induced c-Fos expression is almost completely abolished in rats infused ICV with irbesartan (bottom).

Figure 3. Frontal section through the parietal cortex stained for c-Jun protein in vehicle-treated, sham-operated rats (top). c-Jun expression is increased 24 hours after focal ischemia in rats infused ICV with vehicle (middle). Long-term ICV infusion with irbesartan reduced the ischemia-induced c-Jun expression in the parietal cortex (bottom).
tissue rather than from the blood pressure–lowering effects.8–11 ACE inhibitors administered peripherally decreased ischemic brain injury in normotensive rats and attenuated metabolic derangement in the ischemic brain of spontaneously hypertensive rats.12,13 Because ACE inhibitors and AT1 receptor antagonists are vasoactive drugs and, in general, can cross the blood-brain barrier,21,25 their beneficial effects associated with cerebral ischemia may result from peripheral as well as central sites of action.

In the present study, we investigated the effect of AT1 receptor blockade in the brain on neurological outcome and expression of transcription factors following brain ischemia. The high-affinity, nonpeptide AT1 receptor antagonist irbesartan was used to inhibit central AT1 receptors. Irbesartan selectively inhibits AT1 receptors while it possesses no affinity for AT2 receptors.16 Irbesartan does not significantly interact with α1- or α2-adrenoceptors, serotonin, histamine, neureotensin, vasopressin, or imidazoline receptors or receptors for other neurotransmitters or neuromodulators thought to be involved in ischemic injury. Moreover, irbesartan has no effect on voltage-dependent calcium channels, does not interfere with Na+/Ca2+ and Na+/H+ antiports, and does not inhibit renin or ACE.16

To reach an efficient, steady-state inhibition of central AT1 receptors at the time point of the MCA occlusion, irbesartan was infused ICV over a 5-day time period at a dose that efficiently inhibited brain but not vascular AT1 receptors. Bunting and Widdop26 have convincingly demonstrated in spontaneously hypertensive rats, in which an overactive brain RAS is believed to contribute to genetic hypertension, that long-term central infusion of an AT1 receptor antagonist lowered blood pressure only when sufficiently high dose of the antagonist was infused ICV. However, this dose of the antagonist also affected pressor responses to peripherally injected Ang II, indicating that peripheral AT1 receptor blockade contributed to the hypotensive action of the AT1 receptor antagonist. In the present study, the observed effects of irbesartan were mediated by an interaction of the antagonist exclusively with central AT1 receptors because long-term ICV infusion of irbesartan did not affect basal MAP and did not reduce the pressor responses to a low dose of intravenous Ang II (Table 2).

Several mechanisms may contribute to the improved neurological outcome of cerebral ischemia after the blockade of central AT1 receptors (Figure 1). As the activation of AT1 receptors generally stimulates growth, proliferation and migration in several cell lines,27 blockade of AT1 receptors may affect the proliferation and migration of various cell types in ischemic brain tissue. On the other hand, the proliferation of cells was demonstrated to be inhibited by AT2 receptor stimulation.27 AT2 receptors are also involved in the initiation of cell differentiation and mediate neurotrophic actions of Ang II.28–30 When AT1 receptors are inhibited, Ang II can increasingly interact with AT2 receptors, because AT1 receptor antagonists do not affect the AT2 receptor but rather expose it to increased Ang II levels. The AT2 receptor mRNA level was shown to be increased in the cortex and hippocampus 3 hours after short-term global ischemia with reperfusion, whereas mRNA levels of the AT1 receptor were not affected.31 It is, therefore, conceivable to assume that activation of central AT2 receptors can considerably contribute to the observed beneficial effects of the AT1 antagonist on neurological outcome of cerebral ischemia. This hypothesis needs further investigation.

Figure 4. Frontal section through the hippocampus stained for c-Fos protein in vehicle-treated, sham-operated rats (top). Focal ischemia in rats infused ICV with vehicle slightly increased c-Fos levels (middle). Irbesartan infused ICV abolished the induction of c-Fos in the hippocampus after focal ischemia (bottom).
Effect of Irbesartan on c-Fos and c-Jun Expression in the Brain After Focal Cerebral Ischemia

Cerebral ischemia dramatically increased the expression of c-Fos and c-Jun proteins in the cortex ipsilateral to the injury, especially in the piriform, cingulate, and parietal cortices (Figure 2, 3). In general, c-Fos and c-Jun were stimulated in cortical regions that survived the ischemic insult. An et al. have demonstrated that focal cerebral ischemia increases c-Fos and c-jun mRNA levels due to an increase in the transcription rate of the corresponding genes. The mechanisms that lead to the rapid and transient transcription of c-fos and c-jun genes in neurons after focal cerebral ischemia have yet to be elucidated; however, calcium influx and spreading depression can play a role. Ang II itself is capable of increasing the expression of a number of ITFs, including c-Fos and c-Jun, via stimulation of AT

1 receptors in the brain. However, it remains to be determined how the inhibition of AT

1 receptors in the brain entails a reduction in ITFs expression induced by focal cerebral ischemia.

The present study shows that the overexpression of c-Fos and c-Jun in the cortex and hippocampus was suppressed in the majority of rats treated with the AT

1 receptor antagonist irbesartan (Figure 2, 3, 4). The reduction in the AP-1 transcription factor expression correlated with the improved neurological status. Rats with the lower neurological deficit grade showed a lower expression of c-Fos and c-Jun compared with rats suffering from severe neurological deficits.

In addition to necrosis, many brain neurons undergo apoptosis after focal ischemic insult. It seems likely that increased expression of c-Jun, and in some cases also c-Fos, represents part of the genetic program mediating apoptosis. c-Jun in particular has been implicated in the process of neuronal cell death. There is evidence that proteins of the bcl-2 gene family are involved in the control of apoptosis. Overexpression of Bcl-2 was shown to block hypoxia-induced apoptosis in cells. It has been hypothesised that increased transcription of c-Fos/c-Jun down-regulates bcl-2 expression and thus contributes to the induction of neuronal apoptosis in brain areas after focal ischemia. In the present study, pharmacological blockade of central AT

1 receptors clearly attenuated the expression of c-Fos and c-Jun, which in turn might alter the activation or repression of genes, such as the bcl-2 gene family, involved in the regulation of programmed cell death. Recent studies have demonstrated that Ang II is capable of inducing apoptosis in PC12W cells via AT

1 receptor stimulation and that the signal transduction pathway involves the generation of ceramides. Ang II has been shown to trigger apoptosis in stretched myocytes, and this effect was abolished by the AT

1 receptor antagonist losartan, which indicates that AT

1 receptors were involved. Thus, both AT

1 and AT

2 receptors have been reported to be involved in apoptotic processes. The question of whether Ang II can induce apoptosis via activation of AT

1 receptors in ischemic brain tissue remains to be answered.

Our finding of beneficial effects of the AT

1 receptor blockade on the neurological outcome of focal cerebral ischemia may provide a basis for new therapeutic strategies in the prevention and treatment of stroke. The clinical relevance of this finding becomes apparent with the increasing use of AT

1 receptor antagonists in the antihypertensive treatment and prevention of end-organ damage related to hypertension. In addition to the protective effects of this new class of drugs against stroke, attributed mostly to the antihypertensive actions, treatment with AT

1 receptor antagonists may also improve recovery from stroke by mechanisms independent of blood-pressure reduction.

References


1 subtype angiotensin II receptor antagonist. J Pharmacol Exp Ther. 1993;265:826–834.
Blood flow levels are thought to be an important driving force in determining the severity of consequences after an ischemic insult. The events following brain ischemia comprise activation of membrane receptors and their signaling pathways, loss of energy stores, disintegration of membranes, activation of proteolytic enzymes, formation of free radicals, and fragmentation of DNA. Concomitantly, cerebral ischemia also leads to alterations in protein synthesis and gene expression. Activation of inducible transcription factors after ischemia represents one of the links between the extracellular signals and the initiation of intracellular events associated with either regeneration and survival or leading to a delayed neuronal death. Focal cerebral ischemia has been shown to induce the expression of gene products of the c-fos and c-jun families, the latter products thought to be particularly important in neuronal degeneration.1,2 Interestingly, there has been some evidence, though controversial, that Ang II may be involved in the regulation of processes occurring in cerebral ischemia. For example, angiotensin-converting enzyme (ACE) inhibitors and antagonists of the AT1 receptor have been reported to prevent the occurrence of stroke in stroke-prone spontaneously hypertensive rats and to exert beneficial effects on the metabolic and circulatory derangement in the ischemic brain of spontaneously hypertensive rats.3,4 Such beneficial actions have generally been ascribed to the ability of such agents to reduce systemic arterial blood pressure. Perplexingly, however, treatment with ACE inhibitors has also improved the neurological recovery from cerebral ischemia in normotensive rats.5

The preceding study provides evidence that the beneficial effects of an AT1 receptor antagonist may result from the reduction of Ang II–mediated effects in ischemic brain tissue rather than from the blood pressure–lowering effects of such an agent. In this study, the high-affinity AT1 receptor antagonist irbesartan was used to investigate the effect of AT1 receptor blockade in the brain on neurological outcome and expression of transcription factors after cerebral ischemia. Irbesartan, importantly, has been shown to have no affinity for AT2 receptors as well as little to no interaction with α1- or α2-adrenoceptors or serotonin, histamine, neurotension, or vasopressin receptors or receptors for other neurotransmitters thought to be involved in ischemic injury. At least two aspects of the present study are noteworthy. First, ICV irbesartan did not affect mean arterial blood pressure nor did it reduce the pressor response to an intravenous dose of Ang II, indicating that this antagonist interacted exclusively with central AT1 receptors. Second, the overexpression of c-Fos and c-Jun in the cortex and hippocampus was suppressed in the majority of rats treated with irbesartan. The reduction in the expression of these factors correlated with an improved neurological recovery from cerebral ischemia in normotensive rats.6

Blood flow levels are thought to be an important driving force in determining the severity of consequences after an ischemic insult. The events following brain ischemia comprise activation of membrane receptors and their signaling pathways, loss of energy stores, disintegration of membranes, activation of proteolytic enzymes, formation of free radicals, and fragmentation of DNA. Concomitantly, cerebral ischemia also leads to alterations in protein synthesis and gene expression. Activation of inducible transcription factors after ischemia represents one of the links between the extracellular signals and the initiation of intracellular events associated with either regeneration and survival or leading to a delayed neuronal death. Focal cerebral ischemia has been shown to induce the expression of gene products of the c-fos and c-jun families, the latter products thought to be particularly important in neuronal degeneration.1,2 Interestingly, there has been some evidence, though controversial, that Ang II may be involved in the regulation of processes occurring in cerebral ischemia. For example, angiotensin-converting enzyme (ACE) inhibitors and antagonists of the AT1 receptor have been reported to prevent the occurrence of stroke in stroke-prone spontaneously hypertensive rats and to exert beneficial effects on the metabolic and circulatory derangement in the ischemic brain of spontaneously hypertensive rats.3,4 Such beneficial actions have generally been ascribed to the ability of such agents to reduce systemic arterial blood pressure. Perplexingly, however, treatment with ACE inhibitors has also improved the neurological recovery from cerebral ischemia in normotensive rats.5

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An important unanswered question in this study relates to the mechanism whereby inhibition of AT$_1$ receptors in the brain results in a reduction of c-Fos and c-Jun expression induced by focal cerebral ischemia. Additionally, the role of unopposed activation of AT$_2$ receptors by Ang II during AT$_1$ receptor blockade in the observed neurological improvement following cerebral ischemia also merits investigation.

Thus, although the precise mechanisms are unclear, findings such as those in the present study illustrate that treatment with AT$_1$ receptor antagonists may improve recovery from stroke by avenues independent of blood-pressure reduction.

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**References**


Blockade of Central Angiotensin AT₁ Receptors Improves Neurological Outcome and Reduces Expression of AP-1 Transcription Factors After Focal Brain Ischemia in Rats

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