Angiotensin II AT\textsubscript{1} Receptor Blockade Reverses Pathological Hypertrophy and Inflammation in Brain Microvessels of Spontaneously Hypertensive Rats

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Background and Purpose—The spontaneously hypertensive rat (SHR) is vulnerable to brain ischemia and stress and exhibits a chronically stimulated brain angiotensin II system, cerebrovascular hypertrophy, and inflammation. Pretreatment with angiotensin II type 1 (AT\textsubscript{1}) receptor antagonists protects from brain ischemia and from stress and prevents the development of stress-induced gastric ulcers in part by reducing inflammation in the gastric mucosa. We studied whether AT\textsubscript{1} receptor antagonists could exert antiinflammatory effects in the brain vasculature as a mechanism for their protective effects against ischemia.

Methods—Ten-week-old SHR and normotensive Wistar-Kyoto male rats received the AT\textsubscript{1} receptor antagonist candesartan (0.3 mg/kg per day) or vehicle for 28 days via osmotic minipumps. We studied AT\textsubscript{1} receptors, intercellular adhesion molecule-1 (ICAM-1), endothelial nitric oxide synthase (eNOS), and number of macrophages by immunohistochemistry and Western blots.

Results—We found increased endothelial AT\textsubscript{1} receptor expression of brain microvessels and middle cerebral artery of SHR. Brain AT\textsubscript{1} receptor inhibition reversed the pathological vascular hypertrophy, increased and normalized eNOS expression, and decreased ICAM-1 expression and the number of adherent and infiltrating macrophages in cerebral vessels of SHR.

Conclusions—The antiinflammatory effects of AT\textsubscript{1} receptor antagonists may be an important mechanism in protecting against ischemia. (Stroke. 2004;35:1726-1731.)

Key Words: cerebral arteries ■ inflammation ■ intercellular adhesion molecule-1 ■ middle cerebral artery ■ cerebrovascular disorders

Arterial hypertension, arteriosclerosis, and inflammation of blood vessels are leading causes of stroke.\textsuperscript{1} An initial step in the process of arteriosclerosis is endothelial dysfunction with endothelial macrophage adhesion, followed by their infiltration into the blood vessel wall.\textsuperscript{1,2} Endothelial dysfunction reduces nitric oxide (NO) production and leads to vasoconstriction.\textsuperscript{2} In the spontaneously hypertensive rat (SHR), stimulation of brain and cerebrovascular angiotensin II (Ang II) systems contributes to vasoconstriction, increased expression of proinflammatory factors such as intercellular adhesion molecule-1 (ICAM-1), and increased microvessel permeability.\textsuperscript{3} Long-term brain angiotensin type 1 (AT\textsubscript{1}) receptor blockade protects from brain ischemia\textsuperscript{4-6} and from the pathological effects of stress,\textsuperscript{8,9} in part through peripheral antiinflammatory effects.\textsuperscript{9} Cerebrovascular inflammation and vasoconstriction as a consequence of increased Ang II AT\textsubscript{1} receptor stimulation might have been related to the increased vulnerability to ischemia\textsuperscript{4-6} and stress\textsuperscript{10} in SHR.

To investigate whether Ang II AT\textsubscript{1} receptor antagonists exerted antiinflammatory effects in brain vessels, we studied endothelial nitric oxide synthase (eNOS) and ICAM-1 expression, perivascular macrophage infiltration, and endothelial macrophage adherence in brain microvessels (minimum transverse diameter ≤50 μm) and carotid artery of SHR.

Materials and Methods

Animals

Adult, 10-week-old male SHR (weight, 250 to 320 g) and age-matched male Wistar-Kyoto (WKY) rats, (weight, 300 to 380 g) were purchased from Taconic Farms (Germantown, NY) and kept at 22°C with a 12/12-hour light-dark cycle and food and tap water ad libitum. The National Institute of Mental Health Animal Care and Use Committee approved all procedures.

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Treatment With AT₁ Receptor Antagonist

Groups of 10 rats were anesthetized with pentobarbital (50 mg/kg IP) and implanted with osmotic minipumps (2004 Alzet; osmotic minipumps; mean pumping rate, 0.28 µL/h; mean fill volume, 236 µL; Durect Corporation) subcutaneously to deliver candesartan (0.3 mg/kg per day) or vehicle (0.1N Na₂CO₃) for 28 days. We measured systolic blood pressure by the tail-cuff method on treatment days 0 and 28. Before treatment, blood pressures were higher in SHR than in WKY rats (162 ± 6 and 114 ± 5 mm Hg, respectively). After 28 days of treatment, blood pressures in SHR (119 ± 6 and 114 ± 5 mm Hg) were not different from those in WKY rats.

Tissue Preparation

After 28 days of treatment, rats were anesthetized with pentobarbital (50 mg/kg IP) perfused with physiological saline through the heart, and brains and carotid arteries were removed immediately, frozen in isopentane over dry ice at −30°C, and stored at −80°C. Brain microvessels from additional groups of SHR and WKY rats treated with vehicle or candesartan were isolated as described.

Western Blotting

We homogenized brain microvessels in buffer containing protease inhibitor cocktail (Complete Mini, Roche Molecular Biochemicals) at 4°C, subjected the supernatants to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% gels, transferred the protein electrophoretically to polyvinyl difluoride sheets, and immunoblotted the sheets with a rabbit anti-human Ang II AT₁ receptor polyclonal antibody (Santa Cruz Biotechnology) followed by peroxidase-conjugated goat anti-rabbit immunoglobulin G (Amer sham Life science Inc). We detected the protein using the ECL peroxidase-conjugated goat anti-rabbit immunoglobulin G (Amer sham Life science Inc). We detected the protein using the ECL immunoblotting detection system and quantified the amount of protein by the densitometric analysis software (Scion Corporation). Results were expressed as a relative percentage of vehicle-treated WKY rats.

Determination of Ratio of Lumen/Wall Area

Determination of the ratio of lumen/wall area in middle cerebral artery was performed as reported in horizontal 6-µm-thick sections. Measurements were performed in right and left hemispheres of 2 consecutive sections for each animal, for groups of 5 animals each, with the use of image analysis software (Zeiss LSM Image Browser, version 2.80). Brain microvessels (intraparenchymal arterioles 30 to 50 µm in minimum transverse diameter with a round circumference) were located in basal ganglia and cortex. Three to 5 vessels per section, 2 sections per animal, were randomly selected, and the ratio of the lumen/wall area of each vessel was averaged in each animal. Determinations were performed by investigators unaware of the treatment and strain of the animal studied.

Immunohistochemistry

Immunohistochemistry was performed in 6-µm-thick horizontal sections of brain to study microvessels of minimum transverse diameter of 50 µm and in transversal sections of carotid artery cut at −20°C, air-dried, fixed for 10 minutes in cold acetone, rinsed in PBS, and then incubated in 0.03% H₂O₂ for 20 minutes at room temperature. Specimens were rinsed again in PBS and incubated for 60 minutes in 10% goat serum in PBS.

AT₁ receptors were visualized with a mouse anti-human monoclonal antibody (Ang II AT₁ receptor antibody, 4H2, dilution 6:1000; University of Bern), cNOS with a mouse anti-rat monoclonal antibody (dilution 1:100, Transduction Laboratories), and ICAM-1 in microvessels with a mouse anti-rat monoclonal antibody (1A29, dilution 1:500; Seikagaku Corporation). The number of immunopositive microvessels was counted manually in randomly selected nonoverlapping 5 high-power fields (×200) in horizontal cortical sections of each animal to avoid contamination with other resident or infiltrating cells occasionally expressed ICAM-1. Groups of 5 animals each were measured individually, and the numbers obtained in each area were averaged for each animal.

We used a mouse anti-rat monoclonal antibody (ED1, dilution 1:100; Serotec) to visualize endothelium-adhering macrophages in 5 sections per animal, identified as oval immunopositive cells, and a mouse anti-rat monoclonal antibody specific for ED2-positive macrophages (BD PharMingen technical datasheet) to visualize perivascular macrophages, identified as flattened or oval immunopositive cells. Perivascular macrophages were counted bilaterally in 2 randomly selected areas (1×1 mm in size) of basal ganglia and cerebral cortex.

Antibodies were visualized with the DAKO Envision System (DAKO) with the use of the diaminobenzidine chromogen in a peroxidase reaction, counterstained with hematoxylin, dehydrated with graded ethanol, and cleaned with xylene. To examine the immunostaining specificity, the primary antibodies were replaced.
with nonimmune serum. Immunohistochemical studies were performed by investigators unaware of the treatment and condition of the particular animal from which the specimen was obtained in groups of 5 animals each and measured individually in 2 to 5 sections per animal, and the results were averaged for each animal.

**Statistical Analysis**

The data are expressed as mean±SEM. We used one-way ANOVA followed by post hoc analysis for significance with the Newman–Keuls multiple comparison test. *P*<0.05 was considered statistically significant.

**Results**

**Effect of AT$_1$ Receptor Antagonist on Pathological Hypertrophy**

Pathological hypertrophy in the middle cerebral artery and brain microvessels of SHR was revealed by markedly increased wall (intima plus media) area and decreased lumen area, resulting in a lower lumen/wall area ratio (Figure 1A and 1B), and increased thickening in the medial layer of carotid artery in SHR compared with WKY rats (Figures 3B, 4B, and 5C). Treatment with the AT$_1$ receptor antagonist normalized the cerebrovascular hypertrophy (Figure 1A and 1B). There was no change in the ratio of lumen/wall area in WKY rats after candesartan treatment (data not shown).

**Localization and Quantitative Analysis of AT$_1$ Receptors**

AT$_1$ receptors were localized mainly in endothelium of brain microvessels and middle cerebral artery in SHR and WKY rats (Figure 2A and 2B), and their expression was higher in SHR (Figure 2A and 2B). There was AT$_1$ receptor expression in the medial layer of the middle cerebral artery in SHR but not in WKY rats (Figure 2B).

Isolated brain microvessels were composed of capillaries, venules, and arterioles in both SHR and WKY rats, with no noticeable morphological differences between strains (Figure 2C). AT$_1$ receptor protein was quantitatively higher in SHR than in WKY rats (Figure 2C).

**Effect of AT$_1$ Receptor Antagonist on eNOS and ICAM-1 Expression**

Endothelial eNOS expression of brain microvessels and carotid artery was weaker in SHR than in WKY rats (Figure 3A and 3B) and was restored to the level of control WKY rats after candesartan treatment (Figure 3A and 3B). Earlier quantitative studies have revealed decreased eNOS expression in isolated brain microvessels and carotid artery from SHR and a significant increase after candesartan treatment (Figure 3C).

ICAM-1 expression was localized to brain microvessel and carotid artery endothelium, was remarkably increased in SHR compared with WKY rats (Figure 4A and 4B), and was decreased after AT$_1$ receptor blockade to a level similar to that of WKY rats (Figure 4A and 4B). The number of ICAM-1–positive microvessels was higher in untreated SHR than in WKY rats or treated SHR (Figure 4C).
Effect of AT1 Receptor Antagonist on Macrophage Infiltration

There were no ED1-positive macrophages attached to microvessel endothelium in WKY rats (data not shown) and only very few in microvessels of SHR (Figure 5A). ED2-positive cells, identified as normal perivascular resident macrophages, were found in vehicle-treated WKY rats (Figure 5A) and in untreated WKY rats (data not shown), visualized as flattened, elongated cells, closely attached to and never separated from the microvessel wall. The number of ED2-positive perivascular macrophages was increased in SHR (Figure 5A). In addition to perivascular resident macrophages, in SHR there were a number of rounded macrophages, detached from the vessel wall (Figure 5A).

In WKY rats, no ED1-positive macrophages were detected in the middle cerebral artery (data not shown), and only very few were found attached to the endothelium of the carotid artery (Figure 5C and 5D). In SHR, very few ED1-positive adhering macrophages were seen in the middle cerebral artery (Figure 5B), and some ED1-positive macrophages were clearly seen attached to the carotid artery endothelium (Figure 5C). There were no infiltrating macrophages in the walls of the middle cerebral or carotid arteries of WKY rats or SHR (Figure 5B and 5C).

In cerebral microvessels and carotid arteries, the number of ED1-positive, endothelium-adherent, or ED2-positive, perivascular-infiltrating macrophages was reduced in SHR after treatment with the AT1 receptor blocker (Figure 5D).

Discussion

In SHR, pathological cerebrovascular hypertrophy occurs not only in large cerebral blood vessels such as the middle cerebral and carotid arteries6,7 (and present results) but also in brain microvessels (present results). We confirm our observations of decreased eNOS expression in brain vessels from SHR,7 and we report that alterations in eNOS expression are localized to the cerebrovascular endothelium, correlating with increased endothelial Ang II AT1 receptor and ICAM-1 expression, higher numbers of endothelium-adhering macrophages in cerebral microvessels and carotid artery, and an increased number of perivascular infiltrating macrophages in microvessels of SHR.

The strong association of the Ang II and NO systems is well known.7,18 AT1 receptor stimulation decreases eNOS expression in brain microvessels7 and in the rat heart.11 Ang II increases expression of ICAM-1 in cultured endothelial
cells and in heart tissue and increases macrophage infiltration and adherence, signs of inflammation. NOS inhibition promotes ICAM-1 expression and macrophage infiltration.\(^{21,22}\) Increased AT\(_1\) receptor stimulation, by increasing infiltration of ED2-positive perivascular macrophages, increases production of interleukin-\(\beta\), a proinflammatory cytokine that upregulates ICAM-1 expression in endothelial cells or isolated brain microvessels, to a greater extent in SHR than in WKY rats.\(^ {27}\) Increased endothelial ICAM-1 expression increases endothelial macrophage adherence, and this may explain the increased number of adherent macrophages observed in our study.

Conversely, AT\(_1\) receptor blockade reverses the alterations in eNOS expression,\(^ {7}\) and upregulation of eNOS activity can decrease ICAM-1 expression,\(^ {13,29}\) preventing macrophage infiltration.\(^ {21,22}\) We studied the AT\(_1\) receptor blocker candesartan, a compound that when administered peripherally decreases experimental brain ischemia by protecting cerebrovascular flow.\(^ {5-7}\) Candesartan reverses the cerebrovascular morphological and biochemical alterations in SHR, indicating that cerebrovascular inflammation and hypertrophy are dependent on overstimulation of the cerebrovascular Ang II AT\(_1\) receptors.\(^ {7,11}\)

As a consequence of increased expression of adhesion molecules, there are increased numbers of ED1-positive endothelium-adhering macrophages and increased numbers and morphology of ED2-positive, infiltrating macrophages in microvessels of SHR. Similar alterations in macrophage adherence and infiltration were reported in microvessels during experimental autoimmune encephalomyelitis, after injection of ink particles into the perivascular spaces, and in hypertensive and aged rats.\(^ {33}\) AT\(_1\) receptor blockade reverses these alterations and reduces pathological hypertrophy not only in large vessels but also in cerebral microvessels. Our results agree with an electron microscopy study reporting a decrease in perivascular macrophages in SHR after AT\(_1\) receptor blockade and indicate that AT\(_1\) receptor blockade normalizes the brain microcirculation and, as a consequence, decreases the vulnerability to brain ischemia and stroke in chronically hypertensive rats.

The reversal of the cerebrovascular inflammation by AT\(_1\) receptor blockade was a major finding in our study, suggest-
ing AT1 receptor overstimulation as a molecular mechanism leading to brain inflammation. Our recent findings of a reversal of stress-induced inflammation in the gastric mucosa by AT1 receptor antagonists suggest common pathogenetic mechanisms for ischemic and stress-related disorders. The suppression of inflammation in brain vessels suggests important therapeutic advantages of AT1 receptor antagonists (Figure 6) not only in the prevention of brain ischemia but also in the treatment of inflammatory diseases of the brain.

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