AT_1 Receptor Blockade Regulates the Local Angiotensin II System in Cerebral Microvessels From Spontaneously Hypertensive Rats

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Background and Purpose—Blockade of angiotensin II AT_1 receptors in cerebral microvessels protects against brain ischemia and inflammation. In this study, we tried to clarify the presence and regulation of the local renin-angiotensin system (RAS) in brain microvessels in hypertension.

Methods—Spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) controls were treated with an AT_1 receptor antagonist (candesartan, 0.3 mg/kg per day) via subcutaneous osmotic minipumps for 4 weeks. The expression and localization of RAS components and the effect of AT_1 receptor blockade were assessed by Affymetrix microarray, qRT-PCR, Western blots, immunohistochemistry and immunofluorescence.

Results—We found transcripts of most of RAS components in our microarray database, and confirmed their expression by qRT-PCR. Angiotensinogen (Aogen), angiotensin-converting enzyme (ACE) and AT_1 receptors were localized to the endothelium. There was no evidence of AT_2 receptor localization in the microvascular endothelium. In SHR, (pro)renin receptor mRNA and AT_1 receptor mRNA and protein expression were higher, whereas Aogen, ACE mRNA and AT_2 receptor mRNA and protein expression were lower than in WKY rats. Candesartan treatment increased Aogen, ACE and AT_2 receptor in SHR, and increased ACE and decreased Aogen in WKY rats, without affecting the (pro)renin and AT_1 receptors.

Conclusions—Increased (pro)renin and AT_1 receptor expression in SHR substantiates the importance of the local RAS overdrive in the cerebrovascular pathophysiology in hypertension. AT_1 receptor blockade and increased AT_2 receptor stimulation after administration of candesartan may contribute to the protection against brain ischemia and inflammation. (Stroke. 2006;37:1271-1276.)

Key Words: circulation ■ endothelial cells ■ hypertension ■ renin-angiotensin system

In addition to the well-characterized role in the regulation of blood pressure and fluid homeostasis, the renin-angiotensin system (RAS) regulates cerebrovascular flow.1 Genetically (spontaneously) hypertensive rats (SHR) show a shift toward higher blood pressures at the upper and lower limits of cerebrovascular autoregulation indicating a decrease in vascular compliance, pathological remodeling, alterations in expression of nitric oxide synthase isoenzymes and inflammation of brain microvessels, leading to decreased tolerance to ischemia. These alterations are reversed by AT_1 receptor blockade.2–5 At least part of these effects are the consequence of antagonism of AT_1 receptors located in the endothelium of cerebral microvessels and the endothelium and smooth-muscle cells of small brain arterioles.5

Endothelial AT_1 receptors can be stimulated by circulating angiotensin II (Ang II) generated peripherally. The presence of angiotensin-converting enzyme (ACE) activity suggested the possibility of local synthesis of Ang II in cerebral microvessels.6 To explore the possible role of locally generated Ang II, we studied the expression of RAS-related genes in brain microvessels of SHR and their normotensive controls, Wistar Kyoto (WKY) rats, and the effect of AT_1 receptor antagonism.

Materials and Methods

Animals and Tissue Preparation
Twelve-week-old male SHR and WKY rats weighing 250 to 320 g were purchased from Taconic Farms, Germantown, NY, and housed under standard conditions at 25°C with a 12/12-hour light-dark cycle. The National Institute of Mental Health Animal Care and Use Committee approved all procedures.

SHR and WKY rats were randomly divided into 2 groups, treated with the AT_1 receptor antagonist candesartan, 0.3 mg/kg per day, or...
vehicle, delivered through Alzet 2004 osmotic minipumps (Durect Corporation) implanted subcutaneously for 28 days. Systolic blood pressure was monitored by the tail-cuff method at the 28th day of treatment. Measurements were the mean of 4 determinations in each individual animal. The blood pressures in SHR decreased significantly (203±4 versus 132±3 mm Hg, vehicle-treated versus candesartan-treated, respectively; P<0.001) and to a much larger extent than those in WKY rats (115±3 versus 103±1 mm Hg, vehicle-treated versus candesartan-treated, respectively; P<0.01). Isolation of brain microvessels was performed as described.3,7 Different groups of SHR and WKY rats, treated with candesartan or vehicle were used for microarray, qRT-PCR, Western blot and immunohistochemical studies (see below).

Microarray
We used the Affymetrix GeneChip Expression Analysis Technique (Affymetrix) after RNA extraction from brain microvessels and hybridization to the Affymetrix rat genome U34A array containing 8799 probe array sets. We performed at least 2 independent experiments, each sample consisting of RNA pooled from 5 rats. Array and sample quality was assessed according to Affymetrix guidelines from Data Analysis Fundamentals (https://www.affymetrix.com/support/downloads/manuals/data_analysis_fundamentals_manual.pdf). The results were analyzed with Affymetrix Microarray Suite 5.0 and Data Mining Tool 3.0 softwares. The statistical analysis logic and algorithms are extensively described in the Affymetrix website (http://www.affymetrix.com/support/technical/technote/statistical_reference_guide.pdf). Using the Affymetrix Microarray Suite 5.0 software, the detection call of "present" or "absent" was given if the transcript was reliably detected or not detected (set at P<0.05), respectively. To compare the change between 2 groups, the significant "Decrease" or "Increase" was considered only for transcripts showing the same change call in all pair-wise comparisons between 2 groups.

qRT-PCR and Western Blot Analysis
RNA extraction, qRT-PCR and Western blots were performed as described previously.3,5 The sequences of the primers used are as follows: 18s rRNA, 5’-ACCGGGTCTTCAATTGTGTT-3’ (sense) and 5’-CCCTCTTAAATCATGGCCTCA-3’ (antisense); ACE, 5’ATGTCAGAGCATCCAAGAAGG-3’ (sense) and 5’TGGCCTATACCAGGAGATCG-3’ (antisense); Aogen, 5’GATGATCGTGTGGGACCAAA-3’ (sense) and 5’-AAAGTTGTTCTGGGCGTCACT-3’ (antisense); (pro)renin receptor, 5’TGGGAA-3’ (antisense); ACE, aminopeptidases, endopeptidases and cathepsins. The gen gene-inducible enhancer-binding protein, renin-binding protein in microvessels from SHR when compared with WKY rats, and the endothelial cell marker von Willebrand factor (Factor VIII; 2143, dilution 1:50, Serotec), the smooth-muscle cell marker α-actin (0.N.5, dilution 1:400, Abcam) or the astrocyte marker GFAP (556329, dilution 1:100, BD Biosciences) at 4°C overnight to determine Aogen localization to specific cell types within microvessels. The slides were then incubated for 1 hour with fluorescein anti-rabbit IgG and Texas red anti-mouse IgG (both 1:100, Vector; Burlingame, CA).

For AT1 and AT2 immunofluorescence, isolated brain microvessels preparations were smeared on the slides and dried by vacuum overnight at 4°C. A polyclonal antibody against the glucose transporter-19 (AB1341, 1:100 dilution, Chemicon) combined with a monoclonal antibody against the AT1 receptor (TOMI-1, 1:100 dilution, Abcam) or against the AT2 receptor (10A11, 1:100 dilution, University of Bern, Bern, Switzerland), respectively, were applied overnight at 4°C to localize AT1 or AT2 receptors on brain microvessels. The slides were incubated for 30 minutes with biotinylated anti-mouse IgG, then with fluorescein streptavidin and Texas red anti-rabbit IgG (1:100 for each, Vector; Burlingame, CA) for another 30 minutes. Immunofluorescence slides were mounted with Vectashield (Vector) and observed under a Leica Confocal system (Leica). The specificity of the Aogen and AT2 receptor antibodies has been previously described in detail.10,11 To exclude any nonspecific staining, consecutive sections were incubated with PBS containing 2% normal serum and mouse/rabbit IgG (Vector, Burlingame, CA) omitting primary antibodies, which shows negative results.

Statistical Analysis
Data were expressed as means±SEM. Statistical analysis was performed by one-way ANOVA followed by post-hoc analysis using Newman-Keuls test for multiple comparisons. Differences were considered significant when P<0.05.

Results
Gene Expression of RAS Components
We found expression of many RAS transcripts in our microarray database (supplemental Table I, available online at http://stroke.ahajournals.org) including those for Aogen, Aogen gene-inducible enhancer-binding protein, renin-binding protein, ACE, aminopeptidases, endopeptidases and cathepsins. The renin transcript was not detectable. The AT1 receptor became clearly detectable only after treatment with candesartan. The AT1 receptor transcript was absent from the database, a finding inconsistent with reports based on immunohistochemical and qRT-PCR methodology.4,5 The (pro)renin receptor transcript was not included in the Affymetrix rat genome U34A array. We found selected differences in gene transcripts for Aogen and ACE. Both Aogen and ACE transcripts were decreased in SHR when compared with WKY rats, and administration of the AT1 receptor blocker increased Aogen mRNA only in SHR.

Immunochemistry and Immunofluorescence
After 28 days of treatment, rats were anesthetized with 50 mg/kg pentobarbital, administered intraperitoneally, perfused with physiological saline through the heart, the brains were immediately removed, frozen in isopentane over dry ice at −30°C, and stored at −80°C until used. We performed immunohistochemistry with the diaminobenzidine chromogen in a peroxidase reaction.4,5 A monoclonal antibody against ACE (CD143, dilution 1:100, Chemicon) and a polyclonal antibody against angiotensinogen (Aogen; dilution 6:1000, produced at the University of Bern, Bern, Switzerland) and a monoclonal antibody against was not included in the Affymetrix rat genome U34A array. We found selected differences in gene transcripts for Aogen and ACE. Both Aogen and ACE transcripts were decreased in SHR when compared with WKY rats, and administration of the AT1 receptor blocker increased Aogen mRNA only in SHR.

qRT-PCR and Western Blot Studies
We found lower Aogen, ACE mRNA and AT1 receptor mRNA and protein, higher (pro)renin receptor mRNA and AT1 receptor level in protein and mRNA, and no change in renin binding protein in microvessels from SHR when compared with WKY rats (Figures 1 and 2). Candesartan treatment increased Aogen and ACE mRNA and AT1 receptor mRNA and protein in SHR, decreased Aogen mRNA and increased ACE mRNA in WKY rats and did not affect (pro)renin receptor, renin-binding protein mRNA or AT1 receptor mRNA and protein in either strain (Figures 1 and 2).
Localisation of Aogen, ACE, AT1 and AT2 in Brain Microvessels

Figures 3 and 4 show the localisation of ACE and Aogen to endothelial cells of cerebral microvessels and arteries in brain sections. Aogen was also localized to astrocytes associated with microvessels, and not to smooth-muscle cells (supplemental Figure I, available online at http://stroke.ahajournals.org). We localized both AT1 and AT2 receptors in isolated microvessel preparations. Although AT2 receptors colocalized with the endothelial marker, AT2 receptors did not (Figure 5).

Discussion

Our results demonstrate expression of Aogen, (pro)renin receptor, ACE and AT1 and AT2 receptors in brain microvessels, revealing the presence of a local RAS.

The expression of Aogen mRNA indicates a tissue-specific Aogen transcription in our brain microvessel preparation, but the cellular localization of the transcript has not been determined. We have detected Aogen not only in microvessel-associated astrocytes, but also localized to endothelial cells. The expression of Aogen protein in endothelial cells of microvessels and cerebral arteries suggests local formation and uptake of Aogen from the circulation, and our data indicate that endothelial cells could be actively involved in local Ang II formation. Although the renin transcript was reported as "absent" in our microarrays, we detected the presence of renin-binding protein, which binds circulating renin and masks its protease activity. More importantly, we found expression of the (pro)renin receptor transcript in microvessels. The (pro)renin receptor specifically binds renin and prorenin from the circulation, increasing the catalytic efficiency of angiotensinogen conversion to Ang I. We have also localized ACE and AT1 receptors in the endothelium, confirming previous observations. Our results demonstrate that microvessels possess the necessary machinery to form Ang II locally, that both circulating (pro)renin and Ang I may contribute to the local formation of Ang II, and that Ang II effects in cerebral microvessels may be the consequence of AT1 stimulation by local as well as circulating peptide. The presence of other gene transcripts proposed to be involved in the formation and metabolism of Ang II, such as carboxypeptidases, endopeptidases, cathepsins and aminopeptidases, some of them previously localized by immunohistochemistry in brain microvessels, further indicates that Ang II can be actively metabolized in cerebrovascular endothelial cells.

The expression of both the (pro)renin and the AT1 receptors was higher in SHR, indicating a hyperactive RAS in cerebral microvessels from hypertensive rats, probably responding to circulating as well as locally active systems. A cerebrovascular RAS overdrive, increasing the vasoconstrictive, proinflammatory and growth effects of Ang II, explains the inflammatory and pathological remodeling characteristic of the cerebral vasculature in genetic hypertension, a model expressing normal circulating renin activity.

Our observations of lower Aogen and ACE in SHR are in agreement with previous findings of decreased ACE activity in microvessels from SHR and decreased aortic Aogen mRNA in this strain. We interpret these data as an attempt to compensate increased Ang II function by decreasing its synthesis or to other undetermined factors indicative of cerebrovascular dysfunction.

AT1 receptor antagonists block cerebrovascular AT1 receptors with no significant effect on receptor protein and mRNA expression and decrease aortic Ang II levels. Candesartan treatment normalized the RAS overdrive in SHR, in spite of increased circulating Ang II levels and lack of change in the high (pro)renin receptor expression (present results). The increase in Aogen and ACE mRNA after candesartan in SHR can be construed as a compensatory local increase in Ang II synthesis after AT1 receptor inhibition. In normotensive rats, candesartan
decreases Aogen and enhances ACE mRNA expression, indicating a strain-dependent effect of unclear significance.

Whereas the AT1 type is considered the physiological Ang II receptor, the role of the AT2 receptor type is controversial. We found that Ang II AT2 receptor mRNA and protein in microvessels was lower in SHR when compared with normotensive controls. The proposal has been advanced of opposite, beneficial effects of AT2 receptor stimulation (vasodilatation, antigrowth) in opposition to and in balance with AT1 receptor. Our data revealing higher AT1 and lower AT2 receptor expression can be interpreted as further evidence of a shift toward higher Ang II vasoconstrictor, progrowth and proinflammatory activity in hypertension.1–5

We found that blockade of AT1 receptors increased AT2 receptor mRNA and protein in SHR, a finding consistent with the observation that AT1 receptor antagonists upregulate AT2 expression in endothelial cells in culture, in isolated mesenteric resistance arteries and in aorta. The increase in AT2 receptor transcription and protein by AT1 receptor antagonism, by shifting the balance toward a predominance of AT2 receptor stimulation, enhances its vasodilatation, antigrowth and antiinflammatory effects. Such a shift toward predominance in AT2 receptor stimulation can contribute to the increased expression of endothelial nitric oxide synthase leading to enhanced vasodilatation through endogenous kinins. Our results are consistent with the observations.

![Figure 2](image-url) Effect of AT1 receptor antagonism on AT1 and AT2 receptors in brain microvessels. Results were obtained by qRT-PCR and Western blot. AT1 receptor mRNA and protein were increased in SHR compared with WKY rats and were not affected by candesartan treatment. Conversely, AT2 receptor mRNA and protein were decreased in SHR. After treatment with the AT1 receptor antagonist, AT2 receptor mRNA and protein were increased in SHR to values not different from those of WKY rats (n=4 to 6). *P<0.05 versus WKY-vehicle; #P<0.05 versus SHR-vehicle, ##P<0.01 versus SHR-vehicle. There is only 1 single band for AT receptor below 50 kDa with PC12 cell lysate as positive control. The AT2 receptor was detected at the size around 45KD with KNRK cell lysate as positive control.

![Figure 3](image-url) Immunohistochemical localization of ACE and Aogen in cerebral microvessels and cerebral arteries. Figures are representative stainings by immunocytochemistry in brain sections from a WKY rat (see Methods). Results were repeated in at least 3 different animals. ACE (A) and Aogen (B and C) were located in the endothelial cells of brain microvessels (A and B) and cerebral arteries (C). Arrows point to the positive staining for ACE and Aogen in brain microvessels <50 µm in diameter and a cerebral artery. Bar is 20 µm.
that activity and expression of AT1 and AT2 receptors are inversely correlated and probably in physiological balance. The feedback between AT1/AT2 receptors have been confirmed in AT2 receptor knockout mice, where absence of AT2 receptors correlates with increased AT1 receptor binding and mRNA in hypothalamus, adrenal gland and kidney.27–29

The hypothesis of AT1/AT2 receptor cross-talk including activation of phosphatases by AT2 receptor stimulation suppressing mitogen-activated protein kinase activation and counteracting AT1 receptor effects30 assumed same cell localization of both receptors. However, we have found that in cerebral microvessels, whereas AT1 receptors colocalize with endothelial markers indicating an endothelial localization, AT2 receptors do not. Localization of AT1 and AT2 receptors in different cells is the norm in the brain.31 If localized to different cells, interactions between AT1 and AT2 receptors are likely to be of a hormonal or paracrine, rather than autocrine nature.

**Summary**

In conclusion, our results are evidence of an active local RAS in brain microvessels, influenced by circulating (pro)renin and Ang II, and with a predominant localization to the cerebral microvessels.
microvessel endothelium. Such a system is in clear overdrive in a model of genetic hypertension, explaining how increased AT$_{1}$ receptor stimulation results in enhanced cerebrovascular vasoconstriction, remodeling and inflammation, and how sustained AT$_{1}$ receptor inhibition normalizes cerebrovascular compliance and prevents inflammation, and suggesting a complementary role of AT$_{2}$ receptor stimulation. The apparent lack of same-cell localization of AT$_{1}$ and AT$_{2}$ receptors suggests unexplored interactions of hormonal or paracrine nature.

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