AT₁ 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 AT₁ 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 AT₁ 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 AT₁ 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 AT₁ receptors were localized to the endothelium. There was no evidence of AT₂ receptor localization in the microvascular endothelium. In SHR, (pro)renin receptor mRNA and AT₁ receptor mRNA and protein expression were higher, whereas Aogen, ACE mRNA and AT₂ receptor mRNA and protein expression were lower than in WKY rats. Candesartan treatment increased Aogen, ACE and AT₂ receptor in SHR, and increased ACE and decreased Aogen in WKY rats, without affecting the (pro)renin and AT₁ receptors.

Conclusions—Increased (pro)renin and AT₁ receptor expression in SHR substantiates the importance of the local RAS overdrive in the cerebrovascular pathophysiology in hypertension. AT₁ receptor blockade and increased AT₂ 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 AT₁ receptor blockade.2–5 At least part of these effects are the consequence of antagonism of AT₂ receptors located in the endothelium of cerebral microvessels and the endothelium and smooth-muscle cells of small brain arterioles.5

Endothelial AT₁ 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 AT₁ 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 AT₁ 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 ± 2 versus 103 ± 1 mm Hg, vehicle-treated versus candesartan-treated, respectively; P < 0.01).

Isolation of brain microvessels was performed as described. Different groups of SHR and WKY rats, treated with candesartan or vehicle was 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/technotes/statisticalreferenceguide.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′-ACCGGCTTCTTATTTGGT-3′ (sense) and 5′-CCCTCTAATCATGGCCCTA-3′ (antisense); ACE, 5′-ATGTTACAGAAGGGCGTGGAAA-3′ (sense) and 5′-TTGTAGAAGTTCCACCGAGCA-3′ (antisense); Aogen, 5′-CATAGATTCTGTGTTGGAGAACA-3′ (sense) and 5′-AATGTCTGCTGCGCTCAT-3′ (antisense); AT1 receptor, 5′-CCTGCGCTGATGTTGCTATG-3′ (antisense) and 5′-CCACCTAGAAGTTCCACCGAGCA-3′ (sense); ACE, aminopeptidases, endopeptidases and cathepsins. 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, Ang-1, Ang-2, Ang-3, and AAS-1. 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.

**Immunohistochemistry 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 brain was immediately removed, frozen in isopentane over dry ice at −30°C, and stored at −80°C until used. We performed immunohistochemistry with the dianisobenzidine 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, purchased at the University of Bern, Bern, Switzerland) were used.

For immunofluorescence staining of Aogen, brain sections were incubated with unlabeled Aogen antibody (dilution 6:1000, University of Bern, Bern, Switzerland) and a monoclonal antibody against 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 (TONI-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 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.

**qRT-PCR and Western Blot Studies**

We found lower Aogen, ACE mRNA and AT2 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 AT2 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).
Localization of Aogen, ACE, AT₁ and AT₂ in Brain Microvessels

Figures 3 and 4 show the localization of ACE and Aogen to endothelial cells of cerebral microvessels and arteries in brain sections. Aogen was also localized to astrocytes associated with brain 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 AT₁ receptors colocализed 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 Aogen not only in microvessel-associated astrocytes but also localized to endothelial cells. 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 present data). 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,23 in isolated mesenteric resistance arteries24 and in aorta.25 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 synthase3,25,26 leading to enhanced vasodilatation through endogenous kinins. Our results are consistent with the observations in SHR.

Figure 2. 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 AT1 receptor below 50 kDa with PC12 cell lysate as positive control. The AT1 receptor was detected at the size around 45kD with KNRK cell lysate as positive control.

Figure 3. 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

Figure 4. Immunofluorescence staining of Aogen in cerebral microvessels and cerebral artery. Figures are representative stainings by immunofluorescence in brain sections from a WKY rat (see Methods). Results were repeated in at least 3 different animals. Aogen (green) and the endothelial cell marker Factor VIII (von Willebrand; red) are colocalized (yellow) in endothelial cells of brain microvessels and cerebral arteries. DAPI nuclear stain is displayed as blue. Arrows point to the positive staining for colocalization of Aogen and Factor VIII in brain microvessels <50 μm in diameter and a cerebral artery. Bar is 16 μm.

Figure 5. Immunofluorescence staining of AT1 and AT2 receptors in cerebral microvessels. Figures show immunofluorescence stainings in isolated microvessel preparations from an SHR (see Methods) and results were repeated in at least 3 different rats. AT1 receptors (green) and the endothelial cell marker glucose transporter-1 (red) were frequently colocalized (yellow). However, there was no clear colocalization of AT2 receptor (green) and the endothelial cell marker glucose transporter-1 (red). Bar is 20 μm.
microvessel endothelium. Such a system is in clear overdrive in a model of genetic hypertension, explaining how increased AT1 receptor stimulation results in enhanced cerebrovascular vasoconstriction, remodeling and inflammation, and how sustained AT1 receptor inhibition normalizes cerebrovascular compliance and prevents inflammation, and suggesting a complementary role of AT2 receptor stimulation. The apparent lack of same-cell localization of AT1 and AT2 receptors suggests unexplored interactions of hormonal or paracrine nature.

Acknowledgments

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References

### TABLE I. Gene Expression of Angiotensin System

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<th>SHR-AT1 Blocker vs SHR</th>
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We listed the major gene expression of the angiotensin system in brain microvessels from WKY rats and SHR in the microarray study. At least 2 independent experiments were performed with each using pool RNA from 5 rats. The number of the experiments for WKY-vehicle, WKY-AT1 blocker and SHR-AT1 blocker is 2 for each group and the number of the experiments for SHR-vehicle group is 3. By Affymetrix Microarray Suite 5.0 software, “detection P value” (set at P<0.05) was generated to statistically decide whether a transcript was reliably expressed (Present) or not expressed (Absent) on a GeneChip. A symbol of “/” indicated that the transcript was detected as “Present” or “Absent” in all the experiments of the group. A symbol of “+/” indicated that there is 1 “Present” and 1 “Absent” transcript detected in the group with 2 individual experiments. A symbol of “+/+” indicated that there is 1 “Present” and 2 “Absent” transcript detected in the group with 3 individual experiments, whereas “+/+/-” indicated that there are 2 “Present” and 1 “Absent” transcript detected in the group. A significant “Decrease” or “Increase” was considered as a transcript showing D or I in all comparisons between 2 groups. NC indicates no change.

**Figure I.** Immunofluorescence localization of Aogen in cerebral microvessels. Figures represent immunofluorescence staining in microvessels from brain sections of a WKY rat (see Methods). Left: Aogen (green) and the endothelial marker Factor VIII (von Willebrand; red) are colocalized (yellow). Middle: No colocalization of Aogen (green) with α-actin (red) in smooth-muscle cells. Right: Aogen (green) and GFAP (red) are colocalized in astrocytes associated with microvessels. Arrows point to the sites of colocalization in endothelial cells (left) and astrocytes associated with brain microvessels (right). Bar is 20 μm.
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