Original Article

Valsartan Improves the Lower Limit of Cerebral Autoregulation in Rats

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The effects of angiotensin II type 1 receptor blockers (ARBs) on cerebral blood flow (CBF) autoregulation have not been fully clarified. Thus, we examined the acute effect of valsartan, the most selective ARB, on CBF autoregulation in spontaneously hypertensive rats. Intravenous administration of valsartan (0.3 mg/kg) reduced the mean arterial pressure (MAP) from 184 ± 5 (mean $\pm$ SEM) to 174 ± 5 mmHg ($p < 0.001$) without affecting CBF as measured by laser-Doppler flowmetry. The lower limit of CBF autoregulation (the MAP at which the CBF was 80% of the baseline value) in the valsartan-treated group (122 ± 3 mmHg) was significantly lower than that in the control group (135 ± 4 mmHg, $p < 0.05$). Reverse transcribed-polymerase chain reaction and immunohistochemical staining demonstrated that both angiotensin II type 2 receptors and angiotensin II type 1 receptors (AT1Rs) were expressed in endothelial and smooth muscle cells of the rat cerebral arteries. These results suggest that specific inhibition of AT1Rs in the cerebral circulation causes the leftward shift of the lower limit of autoregulation. (*Hypertens Res* 2006; 29: 621–626)

Key Words: angiotensin, receptor, valsartan, cerebral blood flow, autoregulation

Introduction

Angiotensin II type 1 receptor blockers (ARBs) appear to be effective at preventing cardiovascular organ damages in hypertensives (1–4). Although angiotensin converting enzyme inhibitors (ACEIs) have been reported to shift the cerebral blood flow (CBF) autoregulation curve towards lower blood pressure levels (5–10), the effects of ARBs on autoregulation have not been well clarified. For instance, losartan has been reported to shift the autoregulation curve towards higher blood pressure levels (11), but candesartan has been reported to shift the curve towards lower levels (12). In another report, losartan was demonstrated to lower blood pressure without changing cerebral perfusion in hypertensive patients (13).

Moreover, the effect of captopril, an ACEI, on CBF differs according to whether the drug is administered intravenously

or intracerebroventricularly (5), suggesting that angiotensin II on the intraluminal surface may have a different effect on cerebral circulation than angiotensin II on the abluminal surface. Thus, intravenously injected ARBs may principally elicit their effects from outside of the blood-brain barrier. On the other hand, chronic treatment, particularly chronic treatment with lipophilic candesartan, may exert its effects from outside as well as inside the blood-brain barrier.

Although valsartan and losartan are hydrophilic, valsartan is known as a more selective ARB than losartan or candesartan (14), and has a higher affinity for angiotensin II type 1 receptors (AT1Rs) than losartan (15). Therefore, we examined the acute effect of valsartan on the lower limit of CBF autoregulation in spontaneously hypertensive rats (SHR). Previous reports suggested that angiotensin II receptors are expressed in the rat cerebral arteries (16, 17). To investigate whether an ARB would be able to inhibit the response of cerebral vessels to angiotensin II from the intraluminal side, that

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Table 1. Physiological Variables

	Control (<i>n</i> =7)		Valsartan (<i>n</i> =7)	
	At rest	After i.v.	At rest	After i.v.
Age (month old)	5		5	
Body weight (g)	374±5		369±5	
Body temperature (°C)	37.0±0.0	37.0±0.0	37.1±0.0	37.1±0.0
Hematocrit	0.46±0.01	0.47±0.01	0.47±0.01	0.47±0.01
pH	7.43±0.01	7.44±0.01	7.44±0.01	7.44±0.01
PaCO ₂ (mmHg)	39.4±0.6	38.7±1.0	37.9±0.7	38.1±0.9
PaO ₂ (mmHg)	111±5	118±5	115±3	115±3
Blood glucose (mmol/l)	7.5±0.3	7.3±0.2	7.3±0.1	6.6±0.3 ^{†,‡}
MAP (mmHg)	185±3	186±3	184±5	174±5 ^{#,*}
ΔCBF (%)		−1±1		0±1

Values are mean±SEM. CBF, cerebral blood flow; i.v., intravenous administration; MAP, mean arterial pressure. ΔCBF = [CBF (baseline after administration of agent) − CBF (at rest)]/CBF (at rest) × 100 (%). [†]*p*=0.076 compared with control, [‡]*p*=0.05 compared with valsartan (at rest), [#]*p*=0.071 compared with control, ^{*}*p*<0.001 compared with valsartan (at rest).

is, whether the effects of an ARB on cerebral vessels would be mediated by inhibition of angiotensin II receptors on endothelial cells, we also examined the expression of angiotensin II receptor subtypes in endothelial and smooth muscle cells of the rat basilar artery.

Methods

Animal Preparation

This study was performed under the control of the Guidelines for Animal Experiments of the Graduate School of Medical Sciences, Kyushu University.

Twenty male SHR (342–388 g, 5 months old) were used in the autoregulation (*n*=14) and the preliminary experiment (*n*=6). Under amobarbital anesthesia (100 mg/kg, i.p., followed by 20 mg/kg, i.v., every 1 h), the femoral arteries on both sides and the right femoral vein were cannulated: one artery for continuous recording of mean arterial pressure (MAP), the other for controlled bleeding and blood sampling, and the vein for administration of drugs. Depth of anesthesia was evaluated by applying pressure to a paw or the tail and observing changes in heart rate or blood pressure. Additional anesthetic was administered when such changes occurred. The rats were intubated and mounted on a stereotaxic headholder in a sphinx position. Respiration was assisted by a mechanical ventilator (Rodent Ventilator model 683; Harvard Apparatus, South Natick, USA) with room air and supplemental oxygen. We used a heating pad to keep the rectal temperature constant at 37°C. CBF in the right parietal cortex was continuously monitored by laser-Doppler flowmetry (ALF21; Advance Co. Ltd., Tokyo, Japan) through a burr hole in the skull (7, 18). A laser-Doppler probe was placed above the dura mater approximately 4 mm posterior and 2 mm lateral to the bregma.

In Vivo Experimental Protocol

In the preliminary experiment, we determined the dose of valsartan (a gift from Novartis, Basel, Switzerland) using 6 SHR. First, using 2 rats, we confirmed the bolus intravenous injection dose of angiotensin II (100 ng/kg) that caused an elevation in MAP of 10 mmHg or more. Second, using 4 rats, we examined the dose of valsartan that blocked the blood pressure elevation induced by exogenous angiotensin II.

In the autoregulation experiment, the animals were divided into two groups (*n*=7 in each group). In the valsartan group, we administered valsartan, dissolved in saline, 15 min prior to the controlled reduction of systemic arterial pressure. In the control group, rats were injected with saline as a vehicle. Thirty minutes after preparation, we started the experimental protocol. Arterial gas-parameters were determined at the resting periods (before the administration of valsartan), before hypotension, and at the end of controlled hypotension. After the measurement of baseline MAP and CBF, arterial blood was withdrawn from the femoral artery to decrease systemic arterial pressure in a stepwise manner (10 mmHg per step) (7, 19–21). After stabilization of the arterial pressure for at least 3 min, CBF was measured at each pressure level. We defined the lower limit of CBF autoregulation as the MAP at which the CBF was 80% of the baseline value. CBF at each pressure level (every 10 mmHg step) was expressed as a percentage of the baseline value in each rat. On the assumption that the MAP-CBF relationship between 2 adjacent points was linear, we determined the MAP at which the CBF was 80% of the baseline value (21). The calculated lower limits of CBF autoregulation were averaged among the rats in each group, and the two group averages were compared.

Cell Culture

Rat basilar arterial endothelial cells and smooth muscle cells

Fig. 1. Mean arterial pressure (MAP) and cerebral blood flow (CBF) in the parietal cortex. In the valsartan group, rats were treated with valsartan (open circles, $n=7$). Rats in the control group were treated with vehicle (closed circles, $n=7$). Rhombi indicate the level of MAP at which CBF was 80% of the baseline value. The level of MAP in the valsartan group (open rhombus: 122 ± 3 mmHg) was significantly lower ($*p < 0.05$) than that in the control group (closed rhombus: 135 ± 4).

were collected from the basilar artery of male Sprague-Dawley rats aged 4 to 6 weeks as described previously (22) and cultured with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, USA). Both types of cells were used before the 7th passage.

Reverse Transcribed–Polymerase Chain Reaction

Reverse transcribed (RT)–polymerase chain reaction (PCR) for angiotensin II receptors was performed as described previously (23). Briefly, total RNA from endothelial or smooth muscle cells was prepared with TRIzol reagent (Invitrogen Corporation). One μ g of total RNA was reverse transcribed by avian myeloblastosis virus reverse transcriptase (Roche, Basel, Switzerland) in a total volume of 20 μ l. Using 1 μ l of the product as a template, PCR was performed with gene-specific primers derived from published cDNA sequences of angiotensin II type 1a receptor (AT1aR; GenBank accession no. [X62295](#)), type 1b receptor (AT1bR; GenBank accession no. [M87003](#)) and type 2 receptor (AT2R; GenBank accession no. [NM012494](#)) receptors. Primers for AT1a were 5'-GAG AGAAGACTTTGAAGGAG-3' (forward) and 5'-GGAGTT GAACAGAACAAGTG-3' (reverse), and the PCR product

Fig. 2. Expression of angiotensin II receptor subtypes in rat basilar arterial endothelial cells and smooth muscle cells as detected by reverse transcription–polymerase chain reaction. E, endothelial cells; S, smooth muscle cells; AT1a, angiotensin II type 1a receptor; AT1b, angiotensin II type 1b receptor; AT2, angiotensin II type 2 receptor.

was 249 bp in length. Primers for AT1b were 5'-TCCAGG AAGCTACCCATCTG-3' (forward) and 5'-TCTGGGACA GAATCACAGCT-3' (reverse), and the PCR product was 240 bp in length. Primers for AT2 were 5'-GTTCCCTT GTTTGGTGTAT-3' (forward) and 5'-CATCTTCAAGAC TTGGTCAC-3' (reverse), and the PCR product was 282 bp in length. Primers for GAPDH, a house-keeping gene, were 5'-TGAACGGGAAGCTCACTGG-3' (forward) and 5'-TCC ACCACCCTGTTGCTGTA-3' (reverse), and the PCR product was 307 bp in length. The PCR reaction mixtures were first denatured at 94°C for 5 min and then amplified for 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and elongation at 72°C for 20 s, followed by 7 min of extension reaction at 72°C. Ten μ l of the RT-PCR product was fractionated by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide stain.

Immunohistochemical Staining of the Rat Middle Cerebral Artery

Two male SHR were anesthetized with pentobarbital (50 mg/kg i.p.) and decapitated. The brain was quickly removed and 2-mm thick coronal sections were incubated with 4% formaldehyde (Wako Pure Chemical, Osaka, Japan) for a day. The immunohistochemical staining was performed by the avidin-biotin-peroxidase method using a kit (Histofine SAB-PO(R) Kit; Nichirei Bioscience Inc., Tokyo, Japan). In order to block endogenous peroxidase, the slices were incubated for 30 min with methanol containing 0.3% hydrogen peroxide, followed by blocking with phosphate buffer solution containing 5% bovine serum albumin (5% BSA-PBS) in skim milk for 60 min at room temperature. The slices were then incubated with

Fig. 3. Expression of angiotensin II receptor subtypes in the rat middle cerebral artery as detected by immunohistochemical staining. The expressions of angiotensin II receptor AT1R and AT2R are shown in a and b, respectively. The bar indicates 50 μ m.

the rabbit monoclonal antibody against AT1R or AT2R (Santa Cruz Biotechnology, Santa Cruz, USA) diluted (AT1R 1:400 or AT2R 1:50, respectively) in 5% BSA-PBS in skim milk overnight at 4°C. The sections were then washed with phosphate buffer solution containing 0.1% *p*-(1,1,3,3-tetramethylbutyl)phenol ethoxylate (Triton-X100; Polysciences Inc., Warrington, USA), and incubated for 30 min with the biotinylated anti-rabbit polyclonal antibody, followed by incubation with avidin-biotin-horseradish peroxidase complex. Staining was developed with 3,3'-diaminobenzidine tetrahydrochloride (Histofine DAB Substrate Kit; Nichirei Bioscience Inc.).

Statistical Analysis

The results were expressed as the means $\pm$ SEM. Statistical analyses were performed with paired *t*-test or unpaired *t*-test, as appropriate. Values of $p < 0.05$ were regarded as statistically significant.

Results

Effect of Valsartan on Cerebral Autoregulation

A 0.3 mg/kg of valsartan completely inhibited the elevation of blood pressure by angiotensin II, although 0.03 mg/kg did not. The inhibition lasted for 90 min or more in SHR.

Intravenous administration of valsartan (0.3 mg/kg) reduced MAP from 184 ± 5 to 174 ± 5 mmHg (paired *t*-test, $p < 0.001$) without affecting CBF at 15 min after injection of the agent. However, MAP after administration of valsartan was not significantly different from that of the control (Table 1). In the valsartan group, the lower limit of CBF autoregulation was 122 ± 3 mmHg, which was significantly lower (Fig. 1) than that in the control group (135 ± 4 mmHg, unpaired *t*-test, $p < 0.05$).

Expression of Angiotensin II Receptors in the Cerebral Artery

RT-PCR showed that each of the angiotensin II receptor subtypes AT1aR, AT1bR and AT2R were expressed in rat basilar artery endothelial cells. Smooth muscle cells also expressed all receptor subtypes (Fig. 2). Both AT1R and AT2R were expressed in endothelial and smooth muscle cells of the rat middle cerebral artery (Fig. 3).

Discussion

We found that valsartan shifted the lower limit of CBF autoregulation leftward in hypertensive rats without affecting the resting CBF at the dose that inhibited the angiotensin II-induced blood pressure elevation.

In previous reports, candesartan shifted the cerebral autoregulation curve towards the left (12), but losartan shifted it towards the right (11). Therefore, the effect of ARBs on cerebral circulation is still a matter of controversy. Differences in the specificity and affinity to AT1R, or in permeability through the blood brain barrier may be responsible for this diversity. Valsartan has more than 30,000 times higher selectivity of binding to AT1R than AT2R (14), and has a higher affinity to AT1R than losartan (15). The higher selectivity and affinity to AT1R might be responsible for the difference in the effects of valsartan and losartan on CBF autoregulation. A recent study demonstrated that a metabolite of losartan, EXP3179, acted as a cyclooxygenase inhibitor, and inhibited the synthesis of prostaglandin F $_{2\alpha}$ (24). Thus, losartan has another vasoactive effect that is not associated with AT1R, and this effect may be partially responsible for the differential effects on CBF between losartan and valsartan. However, the effect of each ARB on cerebral autoregulation should be confirmed by a comparison under the same experimental conditions.

Previous reports have indirectly suggested the existence of angiotensin II receptors in the middle cerebral artery by pharmacological methods (16, 17). However, the distribution of angiotensin II receptor subtypes in the rat cerebral arteries has remained unclear. In the present study, we demonstrated that each subtype of angiotensin II receptor mRNA is expressed in primary cultures of rat basilar artery endothelial and smooth muscle cells. We also revealed the presence of both AT1R and AT2R in the rat cerebral arteries by immunohistochemical staining. Thus, the acute effect of intravenously administered valsartan on the lower limit of CBF autoregulation appears to be mediated by the inhibition of AT1R at the endothelial cells of cerebral arteries.

ACEIs inhibit not only angiotensin converting enzymes but also kininase II, which catalyzes the breakdown of bradykinin (25). Bradykinin is one of the most potent cerebral vasodilators (26). We previously demonstrated that the effect of acute administration of captopril on the lower limit of CBF autoregulation is mediated, in part, by bradykinin (21). ARBs are known to have no direct effects on kininase II activity or the action of kinin. Recent studies, however, demonstrated that bradykinin and nitric oxide were involved in AT2R-mediated vasodilation of mesenteric arterial segments (27) or systemic hypotension in rats (28). A previous study demonstrated that overexpression of AT2R activated the vascular kinin system and resulted in vasodilation (29). Although the function of AT2R and its mechanisms need to be further elucidated, the compensatory stimulation of AT2R by inhibition of AT1R may have a beneficial effect on the lower limit of cerebral blood flow autoregulation.

In addition to ACEIs, calcium antagonists are reported to shift the lower limit of the cerebral autoregulation curve to lower pressure levels (30). The proposed mechanism is that the calcium antagonists dilate larger cerebral arteries and thereby inducing compensatory constriction of smaller arteries or arterioles (5). Such vasoconstriction leads to augmentation of the vasodilatory reserve capacity, and thereby shifts the lower limit of autoregulation leftwards. ARBs may also work by the same mechanism.

In conclusion, in the present study, acute intravenous injection of valsartan significantly shifted the lower limit of CBF autoregulation to lower blood pressure levels in SHR. Our results suggest that selective angiotensin II receptor blockers are beneficial for cerebral circulation.

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