Angiotensin AT_2 Receptor Stimulation Causes Neuroprotection in a Conscious Rat Model of Stroke

Claudia A. McCarthy, BBNS(Hons); Antony Vinh, PhD; Jennifer K. Callaway, PhD; Robert E. Widdop, PhD

Background and Purpose—The angiotensin II type 2 receptor (AT_2R) is implicated to be neuroprotective in stroke, although this premise has not been directly tested. Therefore, we have examined the neuroprotective effect of AT_2R stimulation after intracerebroventricular administration of AT_2R agonist CGP42112 in a conscious rat model of stroke.

Methods—Spontaneously hypertensive rats were treated with either CGP42112 (0.1 to 10 ng/kg/min intracerebroventricularly) alone or in combination with the AT_2R antagonist PD123319 (36 ng/kg/min intracerebroventricularly) beginning 5 days before stroke induction. A focal reperfusion model of stroke was induced in conscious spontaneously hypertensive rats by administering endothelin-1 to the middle cerebral artery through a surgically implanted cannula. Behavioral tests were used to assess the severity of neurological deficit as a result of the ischemic event. Cortical and striatal infarct volumes were measured 72 hours poststroke.

Results—Blood pressure was unaffected by treatments. CGP42112 dose-dependently reduced cortical infarct volume poststroke, and PD123319 abolished the neuroprotective effect of CGP42112. PD123319 had no effect on infarct volume alone. These results were consistent with the behavioral findings, indicating that CGP42112 reduced motor deficit on the ledged beam test at 72 hours poststroke and immunohistochemical analyses showing that CGP42112 increased neuronal survival and minimized the loss of AT_2R expression in the infarcted region.

Conclusion—Based on infarct, behavioral, and immunohistochemical data, these results indicate that centrally administered CGP42112 exhibits a neuroprotective effect, which was independent of blood pressure. Thus, for the first time, we have shown that central AT_2R stimulation is neuroprotective in a conscious rat model of stroke. (Stroke. 2009;40:1482-1489.)

Key Words: AT_2 receptors ■ cerebral infarction ■ cerebral ischemia ■ neuroprotection ■ SHR ■ stroke

Angiotensin II binds to either the angiotensin II type 1 receptor (AT_1R), which is responsible for most of the physiological and pathological actions of angiotensin II, or the angiotensin II type 2 receptor (AT_2R), which opposes the actions of the AT_1R. AT_1R antagonists are commonly used in the treatment of hypertension, one of the main risk factors for stroke.

Over the past decade, it has become apparent that the beneficial effects of AT_1R antagonists may extend beyond the control of blood pressure.

Animal studies have convincingly demonstrated that, even if given during the reperfusion stage of stroke, AT_1R antagonists improve cerebral perfusion during an ischemic event, thus reducing neuronal damage possibly by increasing cerebral vascular compliance, resulting in increased cerebrovascular perfusion during stroke.

In this context, treatment with an AT_1R antagonist per se caused an increase in vascular AT_1R in the cerebral circulation. In addition to their beneficial local vasodilator effects, it is known that sartan compounds exhibit antiapoptotic, anti-inflammatory, and antioxidant properties, indicating that blockade of the AT_1R may have both vascular-dependent and -independent neuroprotective actions in the setting of stroke. Integral to the neuroprotective role of the AT_1R is its role in neuronal regeneration. Several models of nerve injury have elegantly shown that the AT_2R has regenerative capabilities associated with restored behavioral function and anatomic innervation after sciatic nerve crush and optical axotomy as well as increased neurite extension in cell culture.

It has been speculated that the increased stimulation of the AT_1R may be responsible for some of the therapeutic effects observed during AT_1R blockade. In this context, coadministration of an AT_2R antagonist negated neuroanatomical and neurological benefit of candesartan or irbesartan in the filament and cerebral artery occlusion stroke models, demonstrating that AT_2R activation contributed to the effect of AT_1R antagonists. Other indirect evidence that AT_2R stimulation is neuroprotective in the setting of stroke is that AT_2R-deficient mice have larger infarct volumes and poorer neurological outcome after stroke. Moreover, AT_1R antagonists are less effective in AT_2R-deficient mice, again...
confirming that AT2R play a pivotal role in the effect of AT1R antagonists.21

However, to date, all investigations examining the neuroprotective nature of the AT2R as recently reviewed by Thorne-Reineke et al22 have been indirect because they invoke a diminished or reversible sartan effect during genetic or pharmacological AT2R blockade. Therefore, the main aim of the present study was to stimulate directly AT2R because we hypothesized that stimulation of the central AT2R would be neuroprotective in its own right in a conscious rat model of stroke, thus representing a new pharmacological target in stroke.

Materials and Methods

Full details can be found in the supplement material, available online at http://stroke.ahajournals.org.

Surgical Procedures

Male 15- to 16-week-old (270 to 320 g) spontaneously hypertensive rats (SHR) had a 23-gauge stainless steel guide cannula stereotaxically implanted into the piriform cortex 2 mm dorsal to the right middle cerebral artery as previously described.23,24 The stereotaxic coordinates were modified for this strain of rat (0.2 mm anterior, −4.7 mm lateral, and −7 mm ventral relative to bregma). An additional 23-gauge intracerebroventricular (ICV) cannula was also stereotaxically implanted into the left lateral ventricle (−3.8 mm anterior, +1.5 mm lateral, and −3.2 mm ventral relative to bregma) for drug administration. These studies were approved by a Monash University Animal Ethics Committee.

Treatments

Rats were randomly allocated to the following treatment groups: 0.1 (n = 6), 1 (n = 9), or 10 ng/kg/min CGP42112 (n = 6; gift from Novatis); CGP42112 (1 ng/kg/min)+PD123319 (36 ng/kg/min; n = 10; gift from Pfizer); PD123319 (36 ng/kg/min) alone (n = 10), or saline (n = 11). The ICV cannula and osmotic minipump containing these treatments were implanted 5 days before stroke and continued to deliver treatment for 3 days poststroke. Two additional sham groups, with or without CGP42112 1 ng/kg/min, underwent surgery without stroke induction. These sham groups acted as surgical and histological controls, respectively.

Systolic Blood Pressure

Systolic blood pressure (SBP) was measured before the start of ICV treatment and also 72 hours poststroke (Day 14).

Stroke Induction

Stroke was induced in conscious animals by titrating the administration of the vasocostrictor, ET-1 (Auspex), to cause temporary occlusion of the right middle cerebral artery.23,24 ET-1 was infused through a 30-gauge injector protruding 3 mm beyond the end of the previously implanted guide cannula. Stroke was characterized by behavioral indicators24,25 modified to be specific for the SHR strain. Typical behaviors that were observed were continuous contralateral or ipsilateral circling behavior; clenching, dragging, or failure to extend the forelimb contralateral to the side of ET-1 infusion; and forepaw shuffling, jaw flexing, or chin rubbing while circling. Each stroke was graded based on these predetermined behavioral changes using a scale of 1 to 4 with 1 being a mild stroke and 4 being a severe stroke. Core body temperature was monitored rectally using a digital thermometer over a 3-hour period. The experimenter was blinded to the treatments. Only rats with a stroke grade of 4, exhibiting at least 7 of the previously mentioned behaviors, were used for the purpose of this investigation, and any animal with a core temperature >40°C after stroke was euthanized.

Assessment of Motor Function

Motor coordination and limb function were assessed using the ledged beam as previously described.26 The ledged beam test was conducted before treatment (Day 0), after recovery from cannula implantation (Day 6), immediately before stroke induction (Day 11), 24 hours poststroke induction (Day 12), and 72 hours poststroke induction (Day 14). All values were compared with presurgery; therefore, each rat acted as its own control. The number of total foot steps taken to run the length of the beam was recorded as well as the number of steps taken on the ledge by each foot. The latter number was recorded as a percentage of the total number of footsteps taken and recorded as percentage error.

Quantification of Ischemic Damage

At 72 hours after stroke, rats were reanesthetized and perfused (25 mL/min) with physiologically buffered saline (0.1 mol/L; pH 7.4) through the apex of the heart. Brains were removed, frozen over liquid nitrogen, and stored at −80°C. Coronal cryostat sections (16 μm) were cut at 8 predetermined levels of the forebrain (−3.20 mm to 6.80 mm relative to bregma), slide-mounted, and stored at −80°C. The infarct area and volume were calculated using a method developed by Callaway and colleagues.27 The infarct volume was quantified by integrating the area of damage with the distance between each level.28

Neuronal Immunohistochemistry

Neuron integrity was assessed using the neuronal marker NeuN. The number of NeuN-positive cells (identified by anti-NeuN IgG antibody) was counted within a 1-mm² site in the infarcted and noninfarcted region of the ipsilateral hemisphere and 2 matched regions of the contralateral hemisphere.

Localization of Superoxide by Dihydroethidium Staining

Dihydroethidium (2 μmol/L; Invitrogen) was used as previously described29 to detect superoxide in frozen coronal cryostat sections (16 μm; −0.8 mm relative to bregma in vehicle).

Angiotensin 2 Receptor Immunohistochemistry

AT2R expression was assessed as previously described by Roulston et al.30 Images were taken from the cortical noninfarcted and infarcted regions of the ipsilateral hemisphere and 2 matched regions of the contralateral hemisphere. The number of cells immunopositive for AT2R was counted within a 0.25-mm² site for each region.

Statistical Analysis

Results are presented as mean±SEM. Ledged beam and blood pressure data were analyzed using 2-way repeated-measures analysis of variance (ANOVA) with individual differences assessed using Bonferroni posttest. Infarct area, AT2R expression, neuronal expression, and superoxide production were assessed using one-way ANOVA with individual differences assessed using Dunnett’s posttest. All statistical analyses were performed using PRISM (GraphPad), and a value of P<0.05 was considered statistically significant.

Results

Systolic Blood Pressure

There was no significant difference in SBP between any of the treatment groups, and CGP42112 did not significantly alter SBP (Table).

Infarct Volume

When compared with vehicle (112.60±21.03 mm³), CGP42112 (1 ng and 10 ng/kg/min) significantly reduced cortical infarct volume at 72 hours poststroke (16.43±5.21 and 9.34±5.30 mm³, respectively; P<0.01, P<0.001 versus vehicle). In addition, CGP42112 (10 ng/kg/min) also reduced
striatal infarct volume when compared with vehicle (12.40 ± 3.66 and 30.27 ± 4.58 mm³, respectively; P < 0.05). While the lowest dose of CGP42112 also reduced cortical damage, this result did not reach statistical significance. This neuroprotective effect observed in the animals receiving CGP42112 (1 ng/kg/min) was reversed by PD123319; however, PD123319 had no effect when administered alone (Figure 1).

Motor Function
Stroked rats treated with vehicle showed a significant increase in percentage errors on the ledged beam at 1 and 3 days poststroke indicating that the test is sensitive to stroke-induced deficit. Importantly, there was no change in the percentage errors in the sham-operated, nonstroked animals, confirming that any deficit observed was a stroke-related injury and not due to surgical procedures. CGP42112 (1 and 10 ng/kg/min) significantly reduced errors 1 day poststroke (12 ± 11% and 13 ± 7%, respectively) when compared with vehicle (68 ± 12%; P < 0.05 versus vehicle). Furthermore, at 3 days poststroke, all doses of CGP42112 significantly reduced errors (22 ± 16%, 5 ± 3%, and 10 ± 10% for 0.1, 1 and 10 ng/kg/min, respectively) versus vehicle (73 ± 9%; P < 0.05 to 0.01). PD123319 negated the improvements in performance observed in CGP42112-treated rats while having no effect when administered alone. There was no change in percentage error in the sham group at any of the times tested (Figure 2).

Neuronal Expression
The number of neurons as indicated by NeuN-immunopositive labeling was significantly lower in the cortical infarct region of the ipsilateral hemisphere in vehicle-treated rats after stroke (8.14 ± 4.10 NeuN-positive cells per mm²) when compared with the nonstroked, sham-operated animals (220.00 ± 32.90 NeuN-positive cells per mm²; P < 0.05; Figure 3). The loss of neuronal expression in the vehicle-

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Figure 1. Infarct area. A–E, Histological sections showing typical infarcted (darker area) and noninfarcted regions from SHR that were either (A) sham-operated nonstroked or treated with (B) vehicle, (C) AT₁R agonist CGP42112 1 ng/kg/min, (D) CGP42112 (CGP) 1 ng/kg/min + PD123319 36 ng/kg/min, or (E) PD123319 alone. Mean data±SEM for infarct volume 72 hours poststroke in (F) cortical and (G) striatal regions on the ipsilateral side are shown for vehicle (n=11); CGP42112 at 0.1 ng/kg/min (n=6), 1 ng/kg/min (n=9), and 10 ng/kg/min (n=6); AT₁R antagonist PD123319 alone (n=10), and in combination with CGP 1 ng/kg/min (n=10). *P<0.05, **P<0.01, ***P<0.001 versus vehicle (one-way ANOVA).
Superoxide Production

Superoxide production was elevated globally after stroke induction except for in the infarcted region of the ipsilateral hemisphere of the CGP42112 (1 ng/kg/min) treated group. There was no treatment effect on superoxide production on the contralateral hemisphere or in the noninfarcted region (Figure 4).

AT2 Receptor Immunohistochemistry

AT2R expression was lower in the cortical infarct region in the ipsilateral hemisphere of stroked vehicle-treated rats. CGP42112 (1 ng/kg/min) reversed the loss of AT2R expression in the infarct region (Figure 5). Additionally, CGP42112 tended to increase AT2R expression in the noninfarcted region of the ipsilateral hemisphere as well as in the contralateral hemisphere. In the absence of stroke, CGP42112 treatment had no effect on the number of AT2R-immunopositive cells (data not shown). There was no significant change in the number of AT2R-immunopositive cells in the striatal region (data not shown).

Discussion

This investigation is the first study to show directly that stimulation of the AT2R is neuroprotective against an ischemic stroke. Using a model of stroke in conscious SHRs, we demonstrated that pretreatment with the AT2R agonist CGP42112, across a nanomolar dose range, almost completely prevented cortical infarction and preserved brain function assessed by neuroanatomical and behavioral measurements, all of which was independent of changes in SBP.

Previous studies have indirectly implicated the beneficial effect of AT2R stimulation after stroke, since the reduction in infarct volume and neurological deficit observed during AT2R blockade were reversed with the coadministration of an AT2R antagonist. Furthermore, when compared with wild-type mice, AT2R knockout mice have larger infarct areas and poorer neurological outcome after stroke. Thus, to date, all findings regarding the role of the AT2R have been based on indirect studies inhibiting AT2R function by pharmacological or genetic means. Moreover, many of the previous studies use models of stroke that induce large infarcts, which are less likely to occur in a clinical setting and/or permanently occlude the middle cerebral artery, which eliminates the potentially damaging reperfusion phase of stroke.

To this end, we examined the role of the AT2R in a model of stroke that has several advantages over other models. First, rats were hypertensive, an important risk factor for stroke. Second, animals were conscious during the stroke procedure, thus avoiding the influence of anesthesia, which itself can cause neuroprotection. Third, by using a vasoconstrictor such as ET-1, the current model exhibits initial cerebral vasoconstriction, reducing perfusion, followed by a reperfusion phase of stroke. Thus, to date, all findings regarding the role of the AT2R have been based on indirect studies inhibiting AT2R function by pharmacological or genetic means. Moreover, many of the previous studies use models of stroke that induce large infarcts, which are less likely to occur in a clinical setting and/or permanently occlude the middle cerebral artery, which eliminates the potentially damaging reperfusion phase of stroke.

The current results demonstrated a dose-related neuroprotective effect of CGP42112 that was more striking in the cortex than in the striatum. Consistent with our findings, it was previously reported, using this model, that there was less neuroprotection in the striatal region when compared with the cortical region, because the striatal region is generally considered to be the core of the infarct and refractory to therapeutic intervention.

These results of the current study are remarkable for several reasons. Often, the effects of AT2R activation need to
be unmasked after AT₁R blockade, as is the case of the peripheral vasodilator effects of CGP42112 in conscious SHRs. However, we found that the AT₂R agonist alone had a striking effect on infarct volume even in the absence of AT₁R blockade and indeed was comparable in its neuroprotective effect to that of an AT₁R antagonist in the only study that has centrally administered candesartan, albeit in a different model. Furthermore, the doses of CGP42112 were at least 1000-fold less than what we have previously used for examining vasodilatation after intravenous administration. Such differences point toward increased sensitivity to the functional effects of central AT₂R activation. However, we found that the AT₂R had no tonic influence, because the AT₂R antagonist PD123319 had no effect on stroke outcome when administered alone, although when coadministered with CGP42112, PD123319 negated any neuroprotective effect, thus confirming that CGP42112 was acting via the AT₂R. Importantly, these effects occurred in the absence of a reduction in SBP, which is well known to be neuroprotective, although it is possible that local cerebral vasodilatation via AT₂R stimulation may play a beneficial role.

Our behavioral data indicated that all 3 doses of CGP42112 reduced motor deficit at 24 hours poststroke, although there was some variation in responses at this time, which may reflect the individual variation often seen in behavioral studies and the relatively small sample size. In any case, this neuroprotective effect was more striking after 72 hours. Moreover, in the current study, the lowest dose of CGP42112 improved motor performance after stroke, whereas the reduction in infarct volume failed to reach statistical significance. AT₂R signaling has been associated with many neuron-regenerative processes, including increased dendritic branch-
ing and neurite outgrowth, thus facilitating functional recovery. Importantly, the behavioral improvement mediated by CGP42112 was reversed by the coadministration of an AT2R antagonist, again suggesting that the neuroprotection was mediated via AT2R. Consistent with this notion, stroke resulted in a complete loss of neurons identified by the neuronal marker NeuN in the infarcted tissue compared with the sham group, and AT2R stimulation significantly increased neuronal survival in a PD123319-sensitive manner. CGP42112 has also been reported to bind to non-angiotensin II sites on inflammatory cells; however, this mechanism is not likely to play a major role in the current study because PD123319 reversed all of the neuroprotective effects of CGP42112 in the current study.

Previous research has shown that AT2R are neuronally expressed and that there is a time-dependent modulation in AT2R expression after stroke. CGP42112 has also been reported to bind to non-angiotensin II sites on inflammatory cells; however, this mechanism is not likely to play a major role in the current study because PD123319 reversed all of the neuroprotective effects of CGP42112 in the current study.

Oxidative stress is a major contributing factor to the pathology of stroke. AT1R stimulation causes an increase in superoxide production, whereas AT2R opposes this effect because AT2R-deficient mice had elevated levels of superoxide in the ischemic area after stroke. In the current study, stroke caused a global increase in superoxide production, and this effect was attenuated in the infarct region by CGP42112, i.e., in the same area in which AT2R expression has been preserved. While the role of the AT2R in oxidative stress is still not fully understood, the inverse relationship between superoxide production and AT2R expression suggests that the AT2R reduces oxidative stress induced during ischemia. In addition, superoxide production was increased on the contralateral noninfarcted hemisphere suggesting that stroke is a
global phenomenon. In accordance, using the same model, Miller et al.\textsuperscript{38} found that superoxide production was increased in the contralateral cerebral vessels at 24 hours poststroke.

In conclusion, we have directly stimulated AT\textsubscript{2}R using CGP42112 in a clinically relevant model of stroke and have demonstrated a striking reduction in both cortical and striatal infarct volume. These findings are consistent with markedly improved behavioral outcome induced by the striking reduction in motor deficit at both 24 hours and 72 hours poststroke. We confirmed that CGP42112 was acting through the AT\textsubscript{2}R by reversing all the neuroprotective changes with the coadministration of PD123319. Neuroprotective mechanisms are likely to involve AT\textsubscript{2}R-mediated increased neuronal survival in addition to a well-documented AT\textsubscript{2}R-mediated vasodilator role, leading to preservation of neuronal function and AT\textsubscript{2} receptor expression while having a small inhibitory effect on superoxide production. These studies provide proof-of-principle for a role of AT\textsubscript{2}R stimulation in neuroprotection, although a limitation of the present study was that CGP42112 was given as a pretreatment before stroke induction. Clearly, studies are required in which CGP42112 is given after stroke to more closely mimic the clinical situation. Collectively, these findings highlight the AT\textsubscript{2}R as a potential therapeutic target in the treatment of stroke, particularly using AT\textsubscript{2}R agonists.

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Disclosures
None.

References
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