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$_2$R) is implicated to be neuroprotective in stroke, although this premise has not been directly tested. Therefore, we have examined the neuroprotective effect of AT$_2$R stimulation after intracerebroventricular administration of AT$_2$R 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$_2$R 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$_2$R 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$_2$R stimulation is neuroprotective in a conscious rat model of stroke. (Stroke. 2009;40:00-00.)

**Key Words:** AT$_2$ receptors ■ cerebral infarction ■ cerebral ischemia ■ neuroprotection ■ SHR ■ stroke

Angiotensin II binds to either the angiotensin II type 1 receptor (AT$_1$R), which is responsible for most of the physiological and pathological actions of angiotensin II, or the angiotensin II type 2 receptor (AT$_2$R), which opposes the actions of the AT$_1$R. AT$_1$R 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 AT$_2$R 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.

Animal studies have convincingly demonstrated that, even if given during the reperfusion stage of stroke, AT$_1$R 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$_1$R antagonist per se caused an increase in vascular AT$_1$R 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$_1$R may have both vascular-dependent and -independent neuroprotective actions in the setting of stroke. Integral to the neuroprotective role of the AT$_2$R is its role in neuronal regeneration. Several models of nerve injury have elegantly shown that the AT$_2$R 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$_2$R may be responsible for some of the therapeutic effects observed during AT$_1$R blockade. In this context, coadministration of an AT$_2$R antagonist negated neuroanatomical and neurological benefit of candesartan or irbesartan in the filament and cerebral artery occlusion stroke models, demonstrating that AT$_2$R activation contributed to the effect of AT$_1$R antagonists. Other indirect evidence that AT$_2$R stimulation is neuroprotective in the setting of stroke is that AT$_1$R-deficient mice have larger infarct volumes and poorer neurological outcome after stroke. Moreover, AT$_1$R antagonists are less effective in AT$_2$R-deficient mice, again

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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 Thone-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.²³²⁴ 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 (−0.8 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 (Auspep), to cause temporary occlusion of the right middle cerebral artery.²³²⁴ 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 indicators²⁴²⁵ modified to be specific for the SHR strain. Typical behaviors that were observed were continuous contralateral 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

AT₃R 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 AT₃R was counted within a 0.25-mm² site for each region.

#### Statistical Analysis

Results are presented as mean±SEM. Ligated 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, AT₃R expression, neuronal expression, and superoxide production were assessed using one-way ANOVA with individual differences assessed using Dunnett’s post-test. 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 &plusmn; 3.66 and 30.27 &plusmn; 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 &plusmn; 11% and 13 &plusmn; 7%, respectively) when compared with vehicle (68 &plusmn; 12%; \(P < 0.05\) versus vehicle). Furthermore, at 3 days poststroke, all doses of CGP42112 significantly reduced errors (22 &plusmn; 16%, 5 &plusmn; 3%, and 10 &plusmn; 10% for 0.1, 1 and 10 ng/kg/min, respectively) versus vehicle (73 &plusmn; 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 &plusmn; 4.10 NeuN-positive cells per mm²) when compared with the nonstroked, sham-operated animals (220 &plusmn; 32.90 NeuN-positive cells per mm²; \(P < 0.05\); Figure 3). The loss of neuronal expression in the vehicle-

Table. Effect of Various Treatments on SBP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pretreatment SBP, mm Hg</th>
<th>72 Hours Poststroke SBP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (no stroke; n=6)</td>
<td>201±8</td>
<td>205±6</td>
</tr>
<tr>
<td>Vehicle (n=6)</td>
<td>194±10</td>
<td>191±9</td>
</tr>
<tr>
<td>CGP42112 (0.1 ng/kg/min; n=6)</td>
<td>172±8</td>
<td>187±5</td>
</tr>
<tr>
<td>CGP42112 (1 ng/kg/min; n=9)</td>
<td>171±12</td>
<td>180±10</td>
</tr>
<tr>
<td>PD123319 (36 ng/kg/min; n=10)</td>
<td>197±8</td>
<td>194±6</td>
</tr>
<tr>
<td>CGP42112 (1 ng/kg/min)+PD123319</td>
<td>189±5</td>
<td>185±5</td>
</tr>
</tbody>
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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) AT₂R 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); AT₂R 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).
treated group extended to the matched region corresponding to the infarct region on the contralateral hemisphere. Importantly, CGP42112 (1 ng/kg/min) ameliorated the loss of NeuN-immunopositive cells (82.75 ± 40.17 NeuN-positive cells per mm²) in the ipsilateral infarcted region. Coadministration of PD123319 with CGP42112 reversed the CGP42112-induced increase in neuronal survival in the ipsilateral hemisphere. PD123319 itself did not alter neuron number compared with vehicle-treated stroked rats.

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 AT₁R 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 during ET-1 washout. Moreover, the severity of stroke can be controlled using a predetermined scale of behavioral indicators, which correlates with the degree of neuronal damage in this model.

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.32,33 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.12 Furthermore, the doses of CGP42112 were at least 1000-fold less than what we have previously used for examining vasodilatation after intravenous administration.32,33 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 AT1R stimulation may play a beneficial role.4–7

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 dendrite 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. In the present study, the loss of the AT2R in the infarct area is also most likely to be a consequence of a loss of neuronal cells in the ischemic area, which has been confirmed by NeuN staining. CGP42112 increases neuronal survival and therefore also preserves AT2R expression specifically in the ischemic area. It is interesting to note that some loss of neuronal cells in the vehicle-treated group also extends to the matched region on the contralateral noninfarcted hemisphere. The mechanism through which this occurs is presently unclear; however, previous findings have indicated significant changes in areas remote from the ischemic lesion.

Figure 5. AT2R expression. A, Effect of AT2R agonist CGP42112 1 ng/kg/min (CGP; n=9) and vehicle (V)-treated SHR (n=7), and sham (S)-operated (n=6) SHR on AT2R-immunopositive cells in infarcted cortical (open columns) and noninfarcted (filled columns) regions on the ipsilateral hemisphere and corresponding regions on the contralateral hemisphere. Mean data ±SEM; *P<0.05 versus corresponding region in vehicle-treated group (one-way ANOVA). B–D, Immunohistochemical sections from SHR that were either (B) sham-operated nonstrusted or treated with (C) vehicle or (D) AT2R agonist CGP42112 1 ng/kg/min depicting AT2R immunoreactivity (brown).
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\(_2\)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\(_2\)R by reversing all the neuroprotective changes with the coadministration of PD123319. Neuroprotective mechanisms are likely to involve AT\(_2\)R-mediated increased neuronal survival in addition to a well-documented AT\(_2\)R-mediated vasodilator role, leading to preservation of neuronal function and AT\(_2\) receptor expression while having a small inhibitory effect on superoxide production. These studies provide proof-of-principle for a role of AT\(_2\)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\(_2\)R as a potential therapeutic target in the treatment of stroke, particularly using AT\(_2\)R agonists.

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Disclosures
None.

References


Supplemental Materials and Methods

Surgical Procedures
Male 15- to 16-week-old (270 to 320 g) SHR (Animal Resources Centre, Perth, Western Australia) were anesthetized with a ketamine (75 mg/kg; Sigma)/xylazine (10 mg/kg; Troy Laboratories) with additional doses administered as required. A 23-gauge stainless steel guide cannula was stereotaxically implanted into the piriform cortex 2 mm dorsal to the right middle cerebral artery according to a procedure 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 ICV cannula was also stereotaxically implanted into the left lateral ventricle (−0.8 mm anterior, +1.5 mm lateral, and −3.2 mm ventral relative to bregma). The ICV cannula was attached to a drug-filled osmotic minipump (Alzet model 2ML2) through polyvinyl chloride tubing (0.50 mm inside diameter; Dural Plastics) and was buried subcutaneously. The guide canulas were secured to the skull with dental acrylate cement and the incision closed with sutures. The animals were housed individually and were allowed a 5-day recovery period before the induction of stroke. 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 CGP 42112 1 ng/kg/min, underwent surgery without stroke induction. These sham groups acted as surgical and histological controls, respectively.

Systolic Blood Pressure
SBP was measured using a noninvasive tail-cuff apparatus (AD Instruments). At least 3 measurements were averaged on the day 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 vasoconstrictor, ET-1 (Auppep), 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; ET-1 (20 pmol/μL in saline) was administered at a rate of 0.2 μL every 30 seconds until the animal exhibited typical stroke behavior. Approximately 4 μL of ET-1 was infused to each animal with no significant difference in the dose of ET-1 administered between treatment groups. 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 beam was constructed from wood and consisted of a 1.5-m long central section, which tapered from 9 cm to 1.5 cm from one end to the other. Underhanging ledges 1.5 cm in width were positioned 1.0 cm below the upper surface of the beam. The beam was placed at a 30° angle of incline with the highest point being the narrowest end, which led directly into the animal’s home cage. Rats were required to traverse the beam from the widest end to the narrowest, most difficult end. Animals were trained to traverse the beam on the day before the pretreatment assessment. 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 area of damage was measured by tracing around the area of infarct in each section using a computerized image analysis system (MCID M4 image analyzer; Imaging Research Inc). 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. Frozen coronal cryostat sections (16 μm) were postfixed in paraformaldehyde (4%). Slides were washed in Tris-buffer (Sigma, pH 7.4) for 30 minutes and then incubated in a humidified chamber in 10% goat serum, 0.5% BSA, and 0.3% Triton X-100 in Tris buffer for 1 hour. Sections were then washed in Tris buffer and incubated overnight in a 1/500 dilution of mouse monoclonal anti-NeuN IgG antibody (Chemicon). Adjacent sections were incubated with the antibody-diluting solution as a negative control. The next day, sections were washed in Tris buffer and incubated for 2.5 hours with the fluorescence labeled secondary antibody, Alexa 488 (Invitrogen). After the incubation period, sections were washed once again in Tris buffer, dried, and cover-slipped using antifade mounting reagent (Vector shield) to prevent signal deterioration. The sections were then imaged using a fluorescence imaging microscope. The number of NeuN-positive cells 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 using analySIS software (Soft Imaging System).

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). The intensity of fluorescence was quantified within a 1-mm² site in the infarcted and noninfarcted region of the ipsilateral hemisphere and 2 matched regions in the contralateral hemisphere using analySIS software (Soft Imaging System).

Angiotensin 2 Receptor Immunohistochemistry
AT-R expression was assessed as previously described by Roulston et al.30 Frozen coronal cryostat sections (16 μm) were acetone-fixed. Slides were washed in Tris buffer (Sigma, pH 7.4) before incubation in a humidified chamber in 10% goat serum in Tris buffer for 30 minutes. This was followed by 30-minute incubation with peroxidase blocker (DAKO) to block nonspecific binding. Sections were then...
incubated overnight in a 1/250 dilution of rabbit polyclonal anti-AT$_2$ IgG antibody (Santa Cruz). Adjacent sections were incubated with a rabbit immunoglobulin fraction (DAKO), used at the same antibody protein concentration, as a negative control. The next day, sections were washed in Tris buffer $\times 3$ and incubated for 2 hours with a peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulins. After the 2-hour incubation, slides were washed in Tris buffer and then incubated with diaminobenzidine for 5 minutes. Sections were rinsed in distilled water, counterstained with hematoxylin, and cover-slipped. Sections were then imaged using a light microscope at 20$\times$ magnification. 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 AT$_2$R was counted within a 0.25-mm$^2$ site for each region.

**Statistical Analysis**

Results are presented as mean±SEM. Ledge beam and blood pressure data were analyzed using 2-way repeated measures ANOVA with individual differences assessed using Bonferroni posttest. Infarct area, AT$_2$R 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.
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Claudia A. McCarthy, Antony Vinh, Jennifer K. Callaway and Robert E. Widdop

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