Ischemic Neuroprotection With Selective κ-Opioid Receptor Agonist Is Gender Specific

Chih-Hung Chen, MD; Thomas J.K. Toung, MD; Patricia D. Hurn, PhD; Raymond C. Koehler, PhD; Anish Bhardwaj, MD

Background and Purpose—We demonstrated previously that treatment with selective κ-opioid receptor (KOR) agonist BRL 52537 hydrochloride \( ([\pm]-1-(3,4\text{-dichlorophenyl})\text{ acetyl}-2-(1\text{-pyrrolidinyl)}\text{ methylpiperidine}] \) has a long therapeutic window for providing ischemic neuroprotection, and (2) attenuates ischemia-evoked NO production in vivo in rats. Neuronally derived NO has been shown to be deleterious in the male but not in the female rodent model of focal ischemic stroke. We tested the hypothesis that BRL provides significant neuroprotection from transient focal ischemia in male but not in female rats.

Methods—Halothane-anesthetized adult male and female Wistar rats (250 to 275 g) were subjected to 2 hours of middle cerebral artery occlusion (MCAO) by the intraluminal suture technique. Adequacy of MCAO and reperfusion was monitored with laser-Doppler flowmetry over the ipsilateral parietal cortex. In the first experiment, male and female rats were treated in a blinded randomized fashion with vehicle saline or 1 mg/kg per hour BRL infusion started at the onset of reperfusion and continued for 22 hours. In the second experiment, ovariectomized (OVX) female rats were treated with vehicle or BRL. Infarct volume in the cortex and caudoputamen (CP) complex was assessed by triphenyl tetrazolium chloride staining at 72 hours after MCAO.

Results—Infarct volume (percentage of ipsilateral structure; mean±SEM) was attenuated significantly in male rats with BRL treatment (cortex 23±5%; CP 44±6%; n=15) compared with vehicle-treated male rats (cortex 38±4%; CP 66±4%; n=15) but not in female rats (BRL—cortex 26±6; CP 55±8%; vehicle—cortex 26±5; CP 62±5%; n=10 each). Neurologic deficit score was improved in BRL-treated male rats but not in female rats. Infarct volume was not different in OVX female rats treated with vehicle or BRL.

Conclusions—These data: (1) demonstrate that this dose of selective KOR agonist provides ischemic neuroprotection in male but not female rats, (2) demonstrate that the lack of protection by BRL is not attributable to circulating ovarian hormones, and (3) highlight the importance of using animal models of both sexes in preclinical studies of experimental ischemia. (Stroke. 2005;36:1557-1561.)

Key Words: cerebral ischemia, focal ■ gender ■ neuroprotection ■ receptors, opioid ■ reperfusion

Although some in vivo studies demonstrated that KOR agonists modulate dopaminergic neurotransmission in the substantia nigra, neostriatum, and the mesolimbic system,\(^9\)-\(^{11}\) we did not observe modulation of ischemia-evoked acute release of dopamine or its metabolites.\(^8\) We have demonstrated previously that BRL 52537 attenuates ischemia-evoked NO production in the striatum in vivo and have postulated that this may account for its neuroprotective action,\(^6\) although the precise signaling of this interaction remains unclear.

It is well known from epidemiologic studies that gender plays a major role in the incidence and prevalence of ischemic stroke.\(^{12}\) In addition, mounting evidence suggests gender-specific responses to brain injury after experimental ische-

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mia. Animal studies indicate that endogenous sex steroids influence outcome after ischemic stroke. Estrogen has been widely shown to protect brain after ischemic stroke, and these observations have been consistent across animal models and genetic strains. In addition to endogenous neuroprotection from estrogen, emerging data suggest that excitotoxic pathways may have a differential effect in males versus females. For example, neuronally derived NO has been shown to be deleterious in the male but not in the female rodent model of focal ischemic stroke. Previous preclinical studies on ischemic neuroprotection have paid little or no attention to these innate gender-specific differences in stroke outcome.

In this study, we tested the hypothesis that the highly selective KOR agonist BRL 52537 hydrochloride [(±)-1-(3,4-dichlorophenyl) acetyl-2-(1-pyrrolidinyl) methylpiperidine] acts in a sex-specific manner to provide ischemic neuroprotection in a well-characterized model of transient focal ischemia. We further tested the hypothesis that BRL attenuates ischemic experimental stroke in the female in the absence of neuroprotection conferred by circulating female sex steroids.

Materials and Methods

MCAO Model

All experimental protocols were approved by the institutional animal care and use committee and conformed to the National Institutes of Health guidelines for the care and use of animals in research. All techniques are as described previously. In brief, adult Wistar rats (250 to 275 g) were anesthetized with halothane (1.0% to 2.0%) in air and allowed to ventilate spontaneously. Concentration of inspired oxygen was adjusted between 25% and 30% to maintain PaO2 between 100 and 150 mm Hg. With aseptic surgical techniques, the right femoral artery was cannulated to monitor arterial blood pressure and arterial blood gases. The femoral vein was cannulated, tubed subcutaneously, and exteriorized for vascular access and drug administration. Rectal temperature was maintained throughout surgical procedures, during ischemia, and until emergence from anesthesia. Transient focal ischemia (2 hours) was produced by MCAO using an intraluminal suture technique in combination with laser-Doppler flowmetry (LDF; Moor Instruments Ltd.; Model MBF3D) as described previously with modifications.

Experimental Groups

All experiments were performed in a blinded, randomized fashion. In the first series of experiments (n = 62), male and female rats were treated with continuous intravenous infusion of vehicle (saline) or 1 mg/kg per hour BRL 52537 (Tocris) at onset of reperfusion and continued for 22 hours. All infusions were at a rate of 0.3 mL per hour.

In a second series of experiments (n = 19), ovariectomy was performed as described previously in female rats at the age of 10 to 12 weeks. Briefly, under halothane anesthesia (1% to 2% via snout mask in O2-enriched air), the ovary was accessed through a lateral abdominal incision, and the ovarian artery and vein were clamped with a fine surgical hemostat and ligated. The ovary was then resected, surgical wounds closed, and the animal allowed recovery for 1 week. Rats were then randomized to treatment with continuous intravenous infusion of vehicle or 1 mg/kg per hour BRL 52537 at onset of reperfusion and continued for 22 hours. Plasma estrogen levels were determined before MCAO and at the completion of the experimental protocol by radioimmunoassay, as described previously.

In both series of experiments, rats were allowed to emerge from anesthesia at 30 minutes of reperfusion. After treatments for 22 hours, the femoral venous catheter was removed and the vein was ligated. Rats were housed in separate cages at room temperature (22°C to 24°C) during emergence from anesthesia and thereafter until they were euthanized. Neurologic examination and rectal temperature were within normal physiological ranges in all animals at baseline and during MCAO and early reperfusion (Table 1). LDF-determined cerebral perfusion was not different in various treatment groups during MCAO and early reperfusion (Table 1). LDF-determined cerebral perfusion was not different in various treatment groups during MCAO and early reperfusion (Table 1). LDF-determined cerebral perfusion was not different in various treatment groups during MCAO and early reperfusion (Table 1). LDF-determined cerebral perfusion was not different in various treatment groups during MCAO and early reperfusion (Table 1). LDF-determined cerebral perfusion was not different in various treatment groups during MCAO and early reperfusion (Table 1). LDF-determined cerebral perfusion was not different in various treatment groups during MCAO and early reperfusion (Table 1).

Results

In the first series of experiments, mortality rate before completion of the experimental protocol (72 hours after MCAO) was as follows: 1 of 16 in male-saline controls secondary to subarachnoid hemorrhage (SAH), 1 of 17 in male-BRL group with SAH, 2 of 12 in female-saline controls (with SAH), and 3 of 17 in female-BRL group (2 with focal hemorrhages in the infarct). One rat in the male-BRL, and 4 in female-BRL groups did not achieve a reduction in LDF signal to <40% and were not included in the analysis. Thus, the final number of rats that successfully completed the experimental protocol and were included in the final analysis were: male-saline controls, n = 15; male-BRL, n = 15; female-saline controls, n = 10; and female-BRL, n = 10.

Mean arterial blood pressure, partial pressure of arterial carbon dioxide (Paco2) and oxygen (Pao2), pH, temporals, and rectal temperature were within normal physiological ranges in all animals at baseline and during MCAO and early reperfusion (Table 1). LDF-determined cerebral perfusion was not different in various treatment groups during MCAO (male-saline, 26±3%; male-BRL, 29±4%; female-saline, 28±3%; and female-BRL, 30±4%). Similarly, LDF was promptly restored on withdrawal of intraluminal suture during reperfusion in all treatment groups.

On days 3 and 4 of recovery, the median NDS in the male-BRL was significantly better than the male-saline control group (Table 2). Infarct volume (percentage of ipsilateral structure) was significantly attenuated in male rats with BRL treatment (cortex 23±5%; CP 44±6%; n = 15) compared with vehicle-treated male rats (cortex 38±4%; CP 66±5%; n = 15; Figure 1). However, in female rats, BRL treatment did...
TABLE 1. Summary of Physiological Variables at Baseline (preischemia), During Ischemia, and 30 Minutes of Reperfusion in Various Treatment Groups

<table>
<thead>
<tr>
<th></th>
<th>Male-Vehicle</th>
<th>Male-BRL</th>
<th>Female-Vehicle</th>
<th>Female-BRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP (mm Hg)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>97±2</td>
<td>97±3</td>
<td>101±3</td>
<td>99±2</td>
</tr>
<tr>
<td>Ischemia</td>
<td>96±2</td>
<td>96±2</td>
<td>97±3</td>
<td>98±3</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>94±2</td>
<td>97±2</td>
<td>98±3</td>
<td>100±3</td>
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<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>7.33±0.00</td>
<td>7.33±0.01</td>
<td>7.34±0.01</td>
<td>7.35±0.01</td>
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<tr>
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<td>7.35±0.01</td>
<td>7.35±0.01</td>
<td>7.38±0.01</td>
<td>7.39±0.01</td>
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<tr>
<td>Reperfusion</td>
<td>7.34±0.01</td>
<td>7.36±0.01</td>
<td>7.38±0.01</td>
<td>7.37±0.01</td>
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<td>Paco2 (mm Hg)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>47±2</td>
<td>44±1</td>
<td>40±3</td>
<td>46±2</td>
</tr>
<tr>
<td>Ischemia</td>
<td>43±2</td>
<td>43±3</td>
<td>41±2</td>
<td>39±2</td>
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<tr>
<td>Reperfusion</td>
<td>46±2</td>
<td>40±2</td>
<td>41±2</td>
<td>40±3</td>
</tr>
<tr>
<td>Pao2 (mm Hg)</td>
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<td></td>
<td></td>
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<tr>
<td>Preischemia</td>
<td>139±8</td>
<td>137±7</td>
<td>130±8</td>
<td>147±7</td>
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<tr>
<td>Ischemia</td>
<td>122±9</td>
<td>122±7</td>
<td>134±8</td>
<td>145±10</td>
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<tr>
<td>Reperfusion</td>
<td>120±8</td>
<td>127±6</td>
<td>129±6</td>
<td>131±7</td>
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<tr>
<td>Temporalis muscle temperature (°C)</td>
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<tr>
<td>Preischemia</td>
<td>36.2±0.0</td>
<td>36.1±0.1</td>
<td>36.1±0.1</td>
<td>36.2±0.1</td>
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<tr>
<td>Ischemia</td>
<td>36.2±0.1</td>
<td>36.1±0.1</td>
<td>36.0±0.1</td>
<td>36.1±0.1</td>
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<tr>
<td>Reperfusion</td>
<td>36.2±0.1</td>
<td>36.3±0.1</td>
<td>36.1±0.1</td>
<td>36.3±0.1</td>
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<tr>
<td>Rectal temperature (°C)</td>
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<tr>
<td>Preischemia</td>
<td>36.5±0.1</td>
<td>36.4±0.1</td>
<td>36.3±0.2</td>
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<tr>
<td>Ischemia</td>
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<td>37.4±0.2</td>
<td>37.2±0.1</td>
<td>37.7±0.2</td>
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<tr>
<td>Reperfusion</td>
<td>37.8±0.2</td>
<td>37.7±0.3</td>
<td>37.6±0.2</td>
<td>38.0±0.2</td>
</tr>
<tr>
<td>Blood glucose (g/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>108±6</td>
<td>101±8</td>
<td>111±10</td>
<td>121±11</td>
</tr>
<tr>
<td>Ischemia</td>
<td>106±6</td>
<td>102±7</td>
<td>111±8</td>
<td>123±11</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>99±6</td>
<td>105±7</td>
<td>112±7</td>
<td>124±12</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MABP indicates mean arterial blood pressure.

TABLE 2. Daily NDS in Various Treatment Groups

<table>
<thead>
<tr>
<th></th>
<th>Male-Vehicle</th>
<th>Male-BRL</th>
<th>Female-Vehicle</th>
<th>Female-BRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>9.0 (7.0–9.0)</td>
<td>7.0 (7.0–9.0)</td>
<td>7.0 (7.0–7.5)</td>
<td>7.0 (7.0–8.0)</td>
</tr>
<tr>
<td>Day 2</td>
<td>9.0 (9.0–9.0)</td>
<td>9.0 (7.0–9.0)</td>
<td>9.0 (7.0–9.0)</td>
<td>8.0 (7.0–9.0)</td>
</tr>
<tr>
<td>Day 3</td>
<td>9.0 (7.0–9.0)</td>
<td>6.0 (5.0–9.0)*</td>
<td>9.0 (8.0–9.0)</td>
<td>7.5 (7.0–9.0)</td>
</tr>
<tr>
<td>Day 4</td>
<td>8.0 (7.0–9.0)</td>
<td>6.0 (4.0–7.0)*</td>
<td>7.5 (5.75–9.0)</td>
<td>7.0 (6.0–8.0)</td>
</tr>
</tbody>
</table>

Values are presented as median (25% to 75%). *P<0.05 vs corresponding vehicle.

not significantly reduce infarct volume compared with female controls (saline controls: cortex 26±6%; CP 55±8%; BRL: cortex 26±5%; CP 62±5%; n=10 each; Figure 2).

In the second series of experiments, there were no differences in physiological variables between the 2 experimental groups at baseline or during MCAO and early reperfusion (data not shown). Similarly, LDF-determined, intraischemic cerebral perfusion was not different in ovariectomized (OVX) female rats with postischemic vehicle (35±1%) and BRL (36±1%) treatment. Three BRL-treated and 2 vehicle-treated rats died before completion of the experimental protocol. Two rats were excluded because ovariectomy did not result in a reduction in plasma estrogen levels. Estrogen levels in rats that successfully completed the protocol (n=6 per group) were similar before MCAO (OVX-vehicle 4.8±1.6 pg/mL; OVX-BRL 4.4±2.2 pg/mL) and at the end of the experiment (OVX-vehicle 4.3±2.1 pg/mL; OVX-BRL 2.2±2.8 pg/mL) in the 2 treatment groups.

There were no differences in daily NDS (data not shown), and infarct volume (72 hours after MCAO) was similar in the 2 experimental groups in the cortex (OVX-vehicle 20±7%; OVX-BRL 19±7%) and CP complex (OVX-vehicle 54±3%; OVX-BRL 60±6%; Figure 3).

Discussion

This study demonstrates 2 important findings. First, intravenous administration of selective KOR agonist BRL 52537 confers ischemic neuroprotection that is specific to the male animal. Second, lack of ischemic neuroprotection seen in...
female rats is not attributable to an interaction between the KOR agonist and female sex steroids. One mechanism by which BRL reduces ischemic damage in male animals is through reduction of early NO toxicity. However, we have shown recently that ablation of neuronal NO synthase (nNOS) activity does not alter ischemic outcome in female brain. Our data support that the sex-specific efficacy of BRL is related to its potential to reduce NO toxicity and speculate that the compound would be best used as an anti-ischemic therapy for the male.

Ischemic Neuroprotection With BRL 52537

KOR agonists have been of interest as potential therapy for ischemic neuroprotection for several years. As in our previous studies, we used BRL 52537 hydrochloride, a water-soluble agent that is highly specific for the KOR (e.g., K_i = 0.24 nmol/L; K_i = 1560 nmol/L). We have shown that BRL 52537 provides significant ischemic neuroprotection when the onset of treatment is delayed for up to 6 hours of reperfusion after 2 hours of MCAO in the rat. This observed neuroprotection with BRL is without alteration in core body temperature and without any significant effects on physiological parameters evaluated within our study. Furthermore, prolonged treatment (4 days) does not result in gross neuropathology or myelin injury in naive nonischemic rats.

Data from several previous studies support that antixcitotoxic mechanisms may be important in the neuroprotection provided by KOR agonists in cerebral ischemia. In vitro studies have demonstrated that KOR agonists modulate glutamate toxicity via inhibition of presynaptic glutamate release, possibly by closing N-type Ca^{2+} channels and inhibiting excitatory postsynaptic potentials by attenuating presynaptic Ca^{2+} influx. Others have demonstrated attenuation of glutamate release with graded ischemia in experimental stroke with a KOR agonist, as well as modulation of the inhibitory neurotransmitter \( \gamma \)-aminobutyric acid. In contrast to previous in vitro studies, neuroprotective doses of BRL did not alter the acute release of dopamine or its metabolites in the ischemic striatum in vivo in our study. In keeping with previous in vitro studies, we demonstrated that neuroprotective doses of BRL attenuate ischemia-evoked striatal NO production and have postulated that the consequent reduction in early NO toxicity may represent a mechanism for the neuroprotective action in ischemic stroke of this KOR agonist.

Gender-Selective Ischemic Neuroprotection With BRL 52537

Epidemiologic studies have highlighted that there are gender differences in the incidence and prevalence of human ischemic stroke. Mounting evidence from laboratory-based studies indicate gender-specific responses to many forms of brain injury, and endogenous sex steroids have been implicated in this differential outcome after experimental ischemic stroke. Although the role of exogenous estrogens in stroke prevention is unproven, the ability of the steroid to reduce stroke sensitivity (i.e., damage once an ischemic insult has occurred) has been widely and uniformly demonstrated. Progesterone is also a neuroprotectant in ischemic injury. It is conceivable that the neuroprotective actions of BRL are obscured in the female because they already have smaller infarct volumes compared with male rats. However, the present study shows that BRL protects only the male brain.
and performs no better in females with or without native ovarian sex steroids. Therefore, the sex specificity of BRL 52537 attenuates stroke damage in male but not female rats subjected to transient focal ischemia. The precise mechanism by which protection is sex specific is not yet elucidated, but the lack of protection in females is not dependent on presence or absence of female sex steroids. Preclinical studies of ischemic neuroprotection have not been stratified by sex in examining experimental stroke outcome. Our study highlights the importance of using animal models of both sexes in experimental studies of ischemic neuroprotection. Furthermore, there is a potential that some therapeutic agents could be effective in one sex only, a characteristic that would be most important in designing optimum clinical trials for these new drugs.

Acknowledgments

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