LY353381.HCl, a Selective Estrogen Receptor Modulator, and Experimental Stroke

Mark I. Rossberg, MD; Stephanie J. Murphy, VMD, PhD; Richard J. Traystman, PhD; Patricia D. Hurn, PhD

Background and Purpose—The impact of postmenopausal estrogen replacement therapy on stroke prevention and stroke severity remains controversial. Previously we have shown that cerebral tissue infarction volume sustained after middle cerebral artery (MCA) occlusion is smaller in female than in male animals. This protection is lost after ovariectomy but is restored by 17β-estradiol replacement. However, the therapeutic range for estradiol is suboptimal, since only doses resulting in a narrow range of plasma levels are protective in brain. The present study tested the hypothesis that a benzothiophene analogue and selective estrogen receptor modulator, LY353381.HCl (LY), reduces tissue infarction after MCA occlusion in estrogen-deficient, ovariectomized female rats.

Methods—Ovariectomized female Wistar rats received LY 10 mg/kg (n = 16) or an equivalent volume of vehicle (n = 14) by gavage for 5 to 8 days. Subsequently, each animal was anesthetized with halothane (1.2%) and treated with 2 hours of MCA occlusion by the intraluminal filament technique and 22 hours of recovery. Infarction volumes in the cerebral cortex and caudoputamen were determined by 2,3,5-triphenyltetrazolium chloride staining and digital image analysis. End-ischemic regional cerebral blood flow (CBF) was measured in separate animal cohorts by quantitative [14C]iodoantipyrine autoradiography.

Results—Caudoputamen infarction was reduced by LY treatment (49±6% versus 64±4% of ipsilateral caudoputamen in LY and vehicle groups, respectively; P<0.05). Cerebral cortical infarction was not different in the LY compared with vehicle group (7±3% versus 13±4% of ipsilateral cerebral cortex, respectively). Intra-ischemic blood pressure, arterial blood gases, and temporalis muscle temperature were controlled and equivalent between treatment groups. Averaged laser-Doppler flow during MCA occlusion was 36±3% of baseline in the LY group versus 29±2% in the vehicle group. However, end-ischemic CBF or blood flow distribution within the MCA territory was not altered by LY treatment. Cortical or caudoputamen tissue volumes with end-ischemic CBF <20 mL/100 g per minute were similar in both groups.

Conclusions—we conclude that LY confers neuroprotection from focal cerebral ischemia in caudoputamen in ovariectomized female rats. The mechanism of protection is not linked to preservation of ischemic cerebral blood flow, as determined by end-occlusion quantitative autoradiography. (Stroke. 2000;31:3041-3046.)

Key Words: cerebral infarction ■ cerebral ischemia, focal ■ estrogens ■ hormones ■ stroke ■ women

Cerebrovascular disease and stroke rates increase significantly in women after menopause. Although there is strong evidence that estrogen replacement therapy reduces postmenopausal risk of cardiovascular disease and osteoporosis, the effect of estrogen replacement therapy on stroke risk and outcome is unproven. There is an abundance of evidence for estrogen-derived neuroprotection in animal and in vitro models of experimental stroke. The steroid likely acts through multiple cellular mechanisms, including preservation of cerebral blood flow (CBF) and vasodilatory capacity, as well as through direct neuronal protection. We and others have demonstrated extensive reduction of stroke damage by 17β-estradiol treatment in male, ovariectomized female, and reproductively senescent rats. In female animals, the therapeutic range for estradiol was suboptimal in that a narrow range of plasma levels was useful in reducing ischemic injury. Furthermore, estradiol replacement has not consistently resulted in protection equivalent to that observed in reproductively active females with cyclic endogenous steroid production.

A real concern for women who hesitate to begin estrogen replacement therapy, but who have known stroke risk factors, is the risk of estrogen to increased risk of thromboembolism and breast and endometrial cancer. Additionally, while the risk of endometrial cancer can be reduced by coadministration of progestin with estrogen, it is unclear whether combined therapy alters the efficacy of estrogen in...
cardiovascular disease or stroke. Thus, the development of a new class of drugs, selective estrogen receptor modulators (SERMs), has been pursued to enhance estrogen agonist activity in the cardiovascular system and in bone, while causing either antagonist or no effect in breast and endometrium. LY353381.HCl (LY), or 2-(4-methoxyphenyl)-3-[4-[2-(1-piperidinylethoxy)phenoxyl]benzo[b]thiophene-6-ol hydrochloride, is a novel benzothiophene analogue with structure and in vivo SERM activity that is similar but not identical to raloxifene (for structural modifications, see References 31 and 32). It prevents ovariectomy-associated weight gain, serum cholesterol elevation, and bone mineral loss, while it preserves estrogen antagonist effects on the uterus. This new SERM is of interest because of its enhanced potency relative to previously studied nonsteroidal template compounds, its excellent oral bioavailability, and its penetration into brain.31,32 The purpose of this study was to determine potential neuroprotective effects of LY in experimental middle cerebral artery (MCA) occlusion in ovariectomized female rats. Additionally, we measured regional CBF during MCA occlusion to assess potential effects on preservation of intraischemic CBF.

Materials and Methods

This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research; all protocols were approved by the Animal Care and Use Committee of Johns Hopkins University. All procedures are as previously published.18 Sexually mature, female Wistar rats (aged 8 to 12 weeks; 200 to 300 g body wt) underwent aseptic bilateral surgical ovariectomy under halothane anesthesia 1 week before their randomization into 1 of 2 treatment groups (LY 10 mg/kg suspension or vehicle). The primary investigator was blinded to treatment group assignments. Rats were treated for 5 to 8 days with daily oral gavage administration (volume 0.5 mL/100 g body wt). To facilitate atraumatic drug delivery, rats were anesthetized with a brief stun dose of 5% halothane in an oxygen/air mixture. Vehicle contained purified water, 0.085% wt/vol povidone, 0.28% wt/vol polysorbate 80, and 1.506% anhydrous granular lactose.

Focal cerebral ischemia was accomplished by modification of the intraluminal filament method for proximal MCA occlusion.18 In brief, each animal was anesthetized with halothane (5% induction, then 1% to 1.5%) via nose cone with supplemental oxygen and air. A femoral artery was cannulated for blood pressure monitoring and arterial blood gas sampling. Rectal and temporalis muscle temperatures were measured and controlled with use of a heating lamp. The rat was placed on a stereotaxic frame equipped with a snout mask, a laser-Doppler flowmetry (LDF) probe holder, and a modification to allow for rotation about the longitudinal axis of the rat. The probe was positioned in its cranial window for semicontinuous measurements during vascular occlusion and the first 15 minutes of reperfusion. The right common carotid artery was exposed via a lateral neck incision, separated from the vagus nerve, and ligated. The external carotid artery was ligated, the occipital branch was cauterized, and the pterygopalatine artery was exposed and ligated. A 4.0 monofilament surgical suture with a heat-rounded tip was advanced through the common carotid artery and advanced into the internal carotid artery until an abrupt reduction in LDF signal was observed. The filament was secured in place. LDF was recorded at 5, 15, 30, 60, 90, and 120 minutes of MCA occlusion. Then the suture was withdrawn to initiate reperfusion, which was confirmed by LDF. Wounds were closed, and the animal was awakened. At 22 hours of reperfusion, the rat was reanesthetized with halothane, and the brain was harvested and sliced into seven 2-mm-thick coronal sections for 2,3,5-triphenyltetrazolium chloride staining. Infarction volumes were measured with the use of digital photography and image analysis software (SigmaScan Pro, Jandel). The infarcted area was numerically integrated across each section and over the entire ipsilateral hemisphere. Cortical and caudoputamen infarction was analyzed as a percentage of the volume of that ipsilateral cortex and caudoputamen, respectively.

End-ischemic regional CBF was measured in additional cohorts of Wistar rats that did not survive by quantitative autoradiography with $[^{14}]$Clodantapryline (IAP), as described previously.18 Femoral vascular catheters were placed, and the MCA was occluded as in the previous cohorts. At 2 hours of MCA occlusion, arterial blood pressure and blood gases were measured, then 40 μCi of $[^{14}]$Clodanapryline (New England Nuclear) in 0.8 mL of isotonic saline was infused intravenously over 45 seconds. During infusion, fifteen 10-μL samples of free-flowing arterial blood from the femoral artery catheter were collected in heparin-coated sample tubes. With the filament still in place and LDF confirming the ischemic status, the rat was decapitated 45 seconds after the start of infusion. One postcapituation arterial blood sample was collected. The brain was quickly removed and frozen at −50°C in 2-methylbutane on dry ice. Each brain was sectioned by cryostat into 20-μm-thick coronal sections at −20°C and thaw-mounted onto cover glasses. For 1 week, sections and $^{14}$C standards were apposed to film (Kodak, Bio-Max MR). The concentrations of $[^{14}]$Clodanapryline in blood samples was determined by liquid scintillation spectrometry (model 3801, Beckman) after decolorization with 0.2 mL of tissue solubilizer (Soluene-350, Packard Instruments Co). Autoradiographic images representing 5 different coronal levels (+2.2, +0.2, −1.8, −3.8, and −5.8 mm from bregma, 6 to 9 images each) were digitized, and regional CBF was determined with the use of image analysis software (Inquiry, Loats Associates). Rates of regional CBF were calculated as previously described.18,24

Two methods of analysis were used to evaluate end-ischemic CBF within MCA territory ipsilateral and contralateral to occlusion. First, CBF was measured by sampling 0.08-mm² squares within gray matter of those regions most vulnerable to MCA occlusion, the frontal and parietal lobes of cerebral cortex and the medial and lateral aspects of caudoputamen. Flow rates were then averaged from squares assayed from 6 to 9 consecutive brain slices at each of 3 coronal levels (+2.2, +0.2, and −1.8 mm from bregma). In the second method, areas categorized by predetermined intervals of CBF were isolated by digital image scanning and summed to construct a histogram distribution of brain tissue over incremental ranges of CBF. Areas were averaged from 3 images from each of 5 coronal levels (+2.2, +0.2, −1.8, −3.8, −5.8 mm from bregma) and then were numerically integrated to obtain an estimate of tissue volume for each incremental range of CBF.

All values are reported as mean±SE. Physiological parameters and LDF over the ischemic and early reperfusion interval were analyzed by 2-way ANOVA. When there was a significant effect of group, post hoc comparisons were made between groups at each time point with 1-way ANOVA and subsequent Newman-Keuls test. Differences in infarct size, mean residual laser-Doppler flow, and autoradiographic regional CBF among groups were determined with 1-way ANOVA. The criterion for statistical significance was $P<0.05$.

Results

Oral gavage feedings were tolerated over 7 days with no loss of body weight. LY treatment did not alter body weight compared with vehicle (data not shown). Physiological data in animals treated with MCA occlusion are summarized in the Table. Mean arterial pressure, arterial blood gas, blood glucose, and hemoglobin concentration were similar between groups during MCA occlusion and early reperfusion. Figure 1 shows cortical and caudoputamen infarction volumes as percentages of the ipsilateral cerebral cortex and caudoputamen, respectively, for LY- (n=16) and vehicle-treated
Summary of Physiological Variables

<table>
<thead>
<tr>
<th>Group</th>
<th>Time Interval</th>
<th>MAP, mm Hg</th>
<th>pH</th>
<th>PacO₂, mm Hg</th>
<th>PacO₂, mm Hg</th>
<th>Hb, g/100 mL</th>
<th>Glucose, g/100 mL</th>
<th>Temporals Muscle Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (n=14)</td>
<td>Baseline</td>
<td>84±2</td>
<td>7.4±0.01</td>
<td>48±1</td>
<td>135±4</td>
<td>13.3±0.3</td>
<td>85±8</td>
<td>36.5±0.2</td>
</tr>
<tr>
<td></td>
<td>During ischemia</td>
<td>85±2</td>
<td>7.39±0.01</td>
<td>50±1</td>
<td>129±4</td>
<td>13.2±0.2</td>
<td>91±8</td>
<td>36.4±0.2</td>
</tr>
<tr>
<td></td>
<td>Reperfusion</td>
<td>82±2</td>
<td>7.37±0.01</td>
<td>54±1</td>
<td>119±3</td>
<td>12.9±0.3</td>
<td>79±5</td>
<td>36.5±0.2</td>
</tr>
<tr>
<td>LY (n=16)</td>
<td>Baseline</td>
<td>89±2</td>
<td>7.38±0.01</td>
<td>50±1</td>
<td>127±5</td>
<td>13.5±0.2</td>
<td>89±2</td>
<td>36.1±0.2</td>
</tr>
<tr>
<td></td>
<td>During ischemia</td>
<td>90±3</td>
<td>7.37±0.01</td>
<td>52±1</td>
<td>128±5</td>
<td>13.4±0.2</td>
<td>90±3</td>
<td>36.1±0.2</td>
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<tr>
<td></td>
<td>Reperfusion</td>
<td>84±2</td>
<td>7.36±0.01</td>
<td>54±1</td>
<td>123±4</td>
<td>13.3±0.2</td>
<td>84±2</td>
<td>36.1±0.2</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MAP indicates mean arterial blood pressure; Hb, plasma hemoglobin concentration. See text for description of groups.

(n=14) rats. Infarction volume in caudoputamen was smaller in LY- than in vehicle-treated rats (49±6% versus 64±4% of ipsilateral caudoputamen, respectively). Cortical injury was not different between groups (7±3% in LY- versus 13±4% in vehicle-treated rats). In all animals, intraschismic cortical LDF signal was sharply reduced during MCA occlusion, then stabilized at approximately 25% to 35% of baseline signal through occlusion (Figure 2). There were no differences between LDF over the ischemia/reperfusion period between the 2 treatment groups (P=0.09), nor was there an interaction between time and treatment group. When LDF during occlusion was examined independently of reperfusion, only the 90-minute time point was significantly different between treatment groups (P=0.03). Averaged LDF over the entire 2 hours of occlusion was 36±3% in LY- and 29±2% of baseline signal in vehicle-treated rats.

To evaluate cortical and subcortical perfusion during MCA occlusion, absolute CBF and blood flow distribution within the MCA territory were quantified in an additional animal cohort (LY, n=4; vehicle, n=3). End-ischismic regional CBF in frontal and parietal cortex was not different in LY- and vehicle-treated groups (Figure 3). Blood flow to medial and lateral sections of caudoputamen was also similar in both treatment groups. Furthermore, when brain volume was partitioned into blood flow increments throughout the ischemic hemisphere, there was no difference in blood flow distribution during vascular occlusion (Figure 4). LY treatment did not alter the distribution of tissue volume into low flow zones (eg, <20 mL/100 g per minute), which represent the infarct core.

Discussion

The results of this study demonstrate 2 important findings. First, preischemic treatment with LY conferred neuroprotection from MCA occlusion in caudoputamen. Second, the mechanism of protection is not linked to preservation of regional CBF during vascular occlusion, as assessed by end-occlusion quantitative autoradiography. Cortical and caudoputamen tissue volumes with severely ischemic flow rates (<10 to 20 mL/100 g per minute) were not decreased by drug treatment, suggesting that LY did not recruit tissue from core infarction zones into the vascular penumbra. These data suggest that this novel class of SERM may be efficacious in reducing histological damage from focal cerebral ischemia when given as a pretreatment. Optimization of dose response and of treatment duration will be required in future studies to fully evaluate the efficacy of LY in experimental stroke.

SERMs represent a new class of compounds that possess tissue-specific estrogen receptor binding and elicit agonist or antagonist effects in different target tissues. The precise mechanisms by which SERMs such as tamoxifen or raloxifene act as estrogenic versus antiestrogenic molecules are actively under investigation and unclear at present. Various compounds within the class differ by relative binding affinity for known estrogen receptor subtypes (α versus β) and by chemical structure, which determines the unique conformation of receptor/ligand complex. Putative molecular mechanisms by which a SERM acts as an estrogen agonist include interaction with coactivator proteins within DNA transcription complexes, engagement of alternative docking sites within estrogen response elements in genes, or differential binding to the β receptor subtype. In rats, LY is an orally active benzothiophene compound that potently prevents ovariectomy-induced effects on body weight, serum cholesterol, and bone density. It is effective in bone mineral preservation, serum cholesterol reduction, and reduction of atherosclerotic pathology, while it maintains estrogen antagonist activity in uterus. To our knowledge, this is the first study to demonstrate a potential neuroprotective effect of a SERM in cerebral ischemia.

Our present finding of reduced infarction volume limited to caudoputamen in LY-treated animals differs from the protection we have previously observed with 17β-estradiol in this same model. Chronic pretreatment with 17β-estradiol at...
physiologically relevant doses reduces damage to cortex as well as caudoputamen in both young ovariectomized and reproductively senescent female rats. This regional difference may be related to true differences in neuroprotective potential of the 2 agents or may be a function of a less than optimal dosing regimen for LY. The optimal dose of LY for either agonist or antagonist activity within the central nervous system is not known. Our rationale for dose (10 mg/kg daily) and route (by gavage) was based on previous work demonstrating potency and efficacy in other responsive targets such as uterus and bone. The regionally restricted efficacy of LY to caudoputamen versus cortex may also be a reflection of the small amount of cortical damage exhibited in animals regardless of treatment assignment. Because damage was small in cortex relative to caudoputamen, treatment effects would be difficult to demonstrate even with the reasonably robust animal cohort size used in this study. However, we cannot exclude the possibility that LY interacts differently with available estrogen receptor subtypes in cortex relative to 17β-estradiol. Both α and β estrogen receptor subtypes have been identified in rat cortex.

We investigated effects of LY on CBF in the contralateral, nonischemic, and ipsilateral hemispheres to determine whether the compound enhanced regional perfusion. Contralateral blood flow was unchanged by chronic LY treatment compared with vehicle, suggesting a lack of basal CBF augmentation under conditions of halothane anesthesia. Both absolute CBF and CBF distribution were also similar in vehicle- and drug-treated animals, demonstrating that mitigation of intraschismic blood flow defects was not a primary mechanism by which LY reduced injury. This finding is similar to our previous observations with 17β-estradiol in this ischemic model, in which exogenous estrogen appears to exert flow-independent neuroprotection. It should be noted that these data during MCA occlusion do not exclude possible CBF enhancement during reperfusion. Nevertheless, the present results indicate that for an equivalent reduction of CBF during vascular occlusion, LY was efficacious in salvaging tissue within the caudoputamen at 24 hours after MCA occlusion.

Whether SERMs act directly on neurons or glia to provide ischemic protection is not known. It seems likely that multiple mechanisms are engaged, in much the same manner as 17β-
estrogen is hypothesized to act in neuroinjury. Accumulating evidence implicates estrogen receptor-independent antioxidant properties and free radical scavenging, receptor-dependent reduction of calcium current inflow, inhibition of glutamate and N-methyl-D-aspartate toxicity, and preservation of protective gene products such as bcl-2. Recent studies suggest that the neuroprotection of estrogen in experimental stroke is not mediated via estrogen subtype α receptors. The importance of the β subtype in neuronal survival has been suggested and is under active investigation.

In summary, we have demonstrated that pretreatment with LY reduces caudoputamen infarction volume at 24 hours in a standard model of MCA occlusion in ovariectomized female rats. The protection is not dependent on augmentation of ischemic CBF and enhanced vasodilation during vascular occlusion. However, effects on recovery of CBF during reperfusion cannot be excluded. Further study is warranted to explore the potential clinical utility of LY in neuroinjury and to more fully elucidate its mechanisms of cerebral protection.

Acknowledgments

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Because estrogen has significant neuroprotective effects in
experimental stroke, there is considerable interest in its use
to diminish the incidence of stroke and limit its severity.
Estrogen also potentially increases the risk of thromboembo-
lishis and of carcinoma of the breast and endometrium. These
potential side effects may be avoided by using selective
estrogen receptor modulators. These drugs enhance estrogen
agonist activity in the cardiovascular system and in bone, but
they do not have the adverse effects on breast and endome-
 trium that estrogen itself has.

The article by Rossberg and colleagues examined the effect
of a selective estrogen receptor modulator, LY353381, in
experimental ischemic stroke. The authors found that this
experimental drug decreased infarct size in the caudoputamen
but did not reduce infarct size in the cerebral cortex. This
effect differs from that of estrogen, which in earlier experi-
ments reduced infarct size in both locations.

It is not known whether this represents a less-potent
effect of LY353381 than estrogen or whether this is the
result of the experimental conditions. It should be noted
that the percentage reduction of the infarct size in the
cerebral cortex was not significant, although numerically it
was greater than that in the caudoputamen (50% versus
23%, respectively). The absence of statistical significance
might be related to the fact that infarct size in this model
in the cerebral cortex is small: 13%, versus 64% in the
caudoputamen). Also, a single dose of the drug was used,
which may not have been optimal.

Accordingly, although these initial results are encouraging,
additional work is necessary, using different doses of the drug
and also probably testing it in different models of ischemic
stroke.

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