Neuroprotective Effects of Female Gonadal Steroids in Reproductively Senescent Female Rats

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Background and Purpose—Young adult female rats sustain smaller infarcts after experimental stroke than age-matched males. This sex difference in ischemic brain injury in young animals disappears after surgical ovariectomy and can be restored by estrogen replacement. We sought to determine whether ischemic brain injury continues to be smaller in middle-aged, reproductively senescent female rats compared with age-matched males and to test the effect of ovarian steroids on brain injury after experimental stroke in females.

Methods—Four groups of 16-month old Wistar rats (males [n=9], untreated females [n=9], and females pretreated with 17β-estradiol [25-μg pellets administered subcutaneously for 7 days; n=9] or progesterone [10-mg pellets administered subcutaneously for 7 days; n=9] were subjected to 2 hours of middle cerebral artery occlusion with the intraluminal filament technique, followed by 22 hours of reperfusion. Physiological variables and laser-Doppler cerebral cortical perfusion were monitored throughout ischemia and early reperfusion. In a separate cohort of males (n=3), untreated females (n=3), females pretreated with 17β-estradiol (n=3), and females pretreated with progesterone (n=3), end-ischemic regional cerebral blood flow was measured by [14C]iodoantipyrine autoradiography.

Results—As predicted, infarct size was not different between middle-aged male and female rats. Cortical infarcts were 21±5% and 31±6% of ipsilateral cerebral cortex, and striatal infarcts were 44±7% and 43±5% of ipsilateral striatum in males and females, respectively. Both estrogen and progesterone reduced cortical infarct in reproductively senescent females (5±2% and 16±4% in estrogen- and progesterone-treated groups, respectively, compared with 31±6% in untreated group). Striatal infarct was smaller in the estrogen- but not in the progesterone-treated group. Relative change in laser-Doppler cerebral cortical perfusion from preischemic baseline and absolute end-ischemic regional cerebral blood flow were not affected by hormonal treatments.

Conclusions—We conclude that the protection against ischemic brain injury found in young adult female rats disappears after reproductive senescence in middle-aged females and that ovarian hormones alleviate stroke injury in reproductively senescent female rats by a blood flow–independent mechanism. These findings support a role for hormone replacement therapy in stroke injury prevention in postmenopausal women. (Stroke. 2000;31:161-168.)

Key Words: cerebral ischemia ■ estrogens ■ menopause ■ progesterone ■ stroke

The role of hormone replacement therapy in altering stroke incidence and outcome in postmenopausal women remains unclear.1 Studies in young adult animals have demonstrated favorable effects of ovarian hormones on brain injury after experimental cerebral ischemia. Female rats sustain lower mortality and less neuronal damage after cerebral ischemia than males.2-3 The protection against ischemic brain injury and its related mortality found in intact female rats disappears after ovariectomy2,4 and can be restored by estrogen replacement.5-9 These studies, however, were performed on surgically ovariec-
tomized young females rather than animals whose reproductive function has naturally abated. Furthermore, the role of proges-
terone has not been evaluated in protection from stroke found in females. Stroke is a disease of the predominantly older popula-
tion, and stroke in young women before menopause is rare. However, the incidence increases dramatically after meno-
pause. Thus, in this study, we tested the hypotheses that (1) the protection against ischemic brain injury found in young adult female rats over age-matched males is lost in middle-aged, reproductively senescent female (RSF) rats and (2) female gonadal hormones, estrogen and progesterone, are protective against ischemic brain injury in RSF rats.

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, and

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the protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University. Retired breeder Wistar rats were obtained at 8 to 9 months of age (Harlan, Indianapolis, Ind) and were housed at the Johns Hopkins School of Medicine Animal Housing Facilities for another 7 to 8 months. At 16 months of age, animals within 4 experimental groups (males, untreated females, and females treated with 17β-estradiol (estrogen group; 25-μg pellet administered subcutaneously for at least 7 days) or progesterone (progesterone group; 10-mg pellet administered subcutaneously for at least 7 days) were subjected to middle cerebral artery (MCA) occlusion for 2 hours. At the end of ischemia, blood flow was reinstituted, and the rat was allowed to recover for 22 hours for assessment of tissue infarction. In a separate cohort of 16-month-old rats, animals within 4 experimental groups, as described above, were infused with [14C]iodoantipyrine (IAP) and decapitated at the end of 2 hours of vascular occlusion for measurement of end-ischemic regional cerebral blood flow (rCBF). Hormone treatment was initiated from 7 to 14 days before arterial occlusion. A 3-week-release tablet (Innovative Research) containing 25 μg 17β-estradiol or 10 mg progesterone was inserted subcutaneously through a small incision on the back of the neck to the animals under brief halothane anesthesia (1% to 2% via snout mask-enriched air).

MCA occlusion was performed as previously described. Briefly, rats were anesthetized with halothane as described above and instrumented with a femoral artery catheter for monitoring arterial blood pressure and measurement of blood gases. Rectal and temporalis muscle temperatures were measured with thermoralis muscle temperatures were measured with thermal probes and were controlled at 37±0.5°C with heating lamps. A 2- to 3-mm diameter burr hole was drilled in the right parietal bone 2 mm posterior and 6 mm lateral to bregma for placement of the laser-Doppler probe and assessment of cerebral cortical perfusion (LDP) (model MFB3D, Moor Instruments Ltd). The head of the rat was stabilized, and the rat was mounted on a modified stereotaxic frame, as previously described. The probe was positioned during the control period over an area devoid of visible blood vessels, and its position was not changed throughout the experiment. The right common carotid artery was exposed through a lateral neck incision, carefully separated from the vagus, and ligated. The external carotid artery was ligated, the occipital branch was cauterized, and the pterygopalatine artery was exposed and ligated. An occluding filament (4-0 monofilament nylon surgical suture with a heat-rounded tip) was advanced into the internal carotid artery until the filamen t was withdrawn, which was associated with a rapid restoration of blood flow (rCBF). The brain was harvested and divided into 7 equally sections at 2-mm thickness. Slices were incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) in saline for 30 minutes at 37°C. The rostral and caudal surfaces of each slice were photographed with a digital camera, and images were transferred to a computer and analyzed with image analysis software (Inquiry, Loats Associates). The infarcted area, which was identified by the lack of TTC staining, was measured on the rostral and caudal surfaces of each slice and numerically integrated across the thickness of the slice to obtain an estimate of infarct volume in each slice. Volumes from all 7 slices were summed to calculate total infarct volume over the entire ipsilateral hemisphere. Infarct volume was measured separately in the cerebral cortex and striatum and expressed as a percentage of the volume of the ipsilateral structure.

End-ischemic rCBF was measured in a nonsurvival cohort of Wistar rats with the use of quantitative autoradiography with [14C]IAP, as described previously. Animals were instrumented with femoral vascular catheters, and the MCA was occluded as in the previous cohort. At 2 hours of MCA occlusion, arterial blood pressure and blood gases were measured, then 40 μCi of [14C]IAP (New England Nuclear) in 0.8 mL of isotonie saline was infused intravenously for 45 seconds. During infusion, fifteen 20-μL samples of free-flowing arterial blood from the femoral artery catheter were collected in heparin-coated sample tubes. With the filament in place and the laser-Doppler indicating ischemic status, the rat was decapitated 45 seconds after the start of infusion. The brain was quickly removed and frozen at −50°C in 2-methylbutane on dry ice. Each brain was cryostatically sectioned into 20-μm-thick coronal sections at −20°C and thaw-mounted onto cover glasses. Sections were apposed for 1 week to film (Kodak, Bio-Max MR) with 10C standards. The concentration of [14C]IAP in blood samples was determined by liquid scintillation spectrometry (model 3801, Beckman) after decolorization with 0.2 mL of tissue solubilizer (Solunene, Packard Instruments Co). Autoradiographic images representing 3 different coronal levels (+2.2, +0.2, and −1.8 mm from the bregma) to 6 images each) were digitized, and rCBF was determined with the use of image analysis software (Inquiry, Loats). Rates of rCBF were calculated as previously described. Two methods of analysis were used to determine rCBF. First, cerebral blood flow (CBF) was measured by sampling 0.1-mm2 squares within gray matter of regions most vulnerable to MCA occlusion, the parietal somatosensory cerebral cortex and lateral striatum. Flow rates were averaged within 6 to 9 consecutive brain slices from each of 3 coronal levels. In the second method, areas perfused with predetermined intervals of CBF were isolated by digital image scanning and summed to construct a histogram distribution of brain tissue over incremental ranges of blood flow rates. Areas were averaged among 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 CBF interval.

All values are reported as mean±SEM unless otherwise indicated. Physiological parameters and LDP were subjected to 2-way ANOVA. Differences in infarct size and autoradiographic rCBF were determined by 1-way ANOVA. Post hoc comparisons were made with Newman-Keuls test. The criterion for statistical significance was P<0.05.

Results
At 16 months of age, male rats had greater body weight than age-matched females (722±40 g in males versus 420±21, 397±7, and 441±18 g in untreated females, estrogen group, and progesterone group, respectively; n=9 each group). Physiological variables are summarized in the Table. Since there were no differences between survival (n=9 in each group) and nonsurvival animals (n=3 in each group), physiological variables from all animals were pooled together (n=12). The mean arterial blood pressure was equivalent in all groups and was maintained at baseline values throughout ischemia. Baseline arterial pH, PaCO2, PaO2, and hemoglobin and glucose concentrations were equivalent among all groups and were maintained at values similar to baseline during ischemia. Plasma concentration of 17β-estradiol at the time the animals were killed 24 hours after MCA occlusion was 13.8±3.1 pg/mL in estrogen-treated males (n=8) compared with 6.5±2.3 pg/mL in untreated females (n=6). Plasma progesterone was 43.3±20.2 ng/mL in progesterone-treated females (n=8) compared with 24.8±8.7 ng/mL in untreated females (n=6). In male rats, plasma concentrations of estrogen and progesterone were 3.6±1.3 pg/mL and 4.7±1.2 ng/mL, respectively. Figure 1 traces the dynamics of LDP throughout ischemia and early reperfusion. Baseline LDP was not different among groups and averaged 491±90, 472±64, 387±74, and 371±49 arbitrary units in male, untreated female, estrogen, and progesterone groups, respectively (n=9 in each group).
LDP averaged 40±2%, 33±3%, 32±2%, and 33±4% during the 2-hour occlusion period in male, untreated female, estrogen, and progesterone groups, respectively (n=9 in each group). On withdrawal of the occluding filament, LDP signal was restored to 85±14%, 76±12%, 92±9%, and 77±10% in male, untreated female, estrogen, and progesterone groups, respectively (n=9 in each group).

Figure 2 compares infarct size in the cerebral cortex and striatum between middle-aged males and females. Infarct size was similar in males and females at all 7 coronal levels in both the cerebral cortex and striatum (n=9 in each group).

Figure 3 demonstrates the effects of estrogen and progesterone treatments on cortical and striatal infarcts in RSF rats. Cortical infarct was reduced from 31±6% of ipsilateral cerebral cortex in untreated females to 5±2% and 16±4% by estrogen and progesterone treatments, respectively (n=9 in each group). Striatal infarct was reduced by estrogen from 43±5% of ipsilateral striatum in untreated females to 24±6% (n=9 in each group). Striatal infarct was unaffected by progesterone treatment and averaged 46±6% of ipsilateral striatum (n=9).

Figure 4 illustrates rCBF rates within distinct areas of brain most vulnerable to MCA occlusion, the parietal somatosensory cerebral cortex and the lateral striatum. Blood flow rates in these regions within ischemic or control hemispheres were not different among groups (n=3 in each group) and averaged 22±17, 42±13, 36±19, and 37±22 mL/100 g per minute in the ischemic parietal cortex and 21±17, 18±10, 19±12, and 9±5 mL/100 g per minute in the ischemic lateral striatum of male, untreated female, estrogen, and progesterone groups, respectively. Figure 5 is a histogram of brain tissue volume distribution over 10-mL/100 g per minute increments of rCBF. The amount of tissue within the ischemic hemisphere perfused at flow rates between 0 to 100 mL/100 g per minute was equivalent between males and females and was not affected by hormonal treatments.

**Discussion**

The main findings of this study are as follows: (1) unlike previous reports of sex-specific stroke outcome in young adult animals, middle-aged male and female rats sustain similar brain damage after experimental stroke; (2) 17β-estradiol reduces cortical and striatal infarcts in RSF rats; (3) progesterone reduces only cortical injury in RSF rats; and (4) tissue perfusion during ischemia was unaffected by hormonal treatment. This is the first study to examine sex-related differences in outcome from ischemic stroke in naturally aging, reproductively senescent animals and the first report of beneficial hormone replacement in an animal model of postmenopausal female brain. These data suggest that a lack of reproductive steroids in the middle-aged female is important to stroke outcome. Furthermore, chronic hormone replacement to physiologically relevant plasma levels protects ischemic brain by mechanisms other than preservation of end-ischemic CBF.

Average longevity in rat is approximately 2 to 3 years of age, depending on strain, sex, and genetic background. Female rats mature sexually during early adulthood (by 2 to
3 months of age) and begin to display 4 to 5 day estrous cycles. Regular cyclicity is evident until middle age, when female rats undergo a transition to irregular cyclicity and finally to acyclicity by 12 to 18 months of age. On average, fertility is lost by 10 to 12 months, and the animal is termed "reproductively senescent."

Reproductive senescence in rodents has been used to study neuroendocrine changes associated with menopause in women. We chose to study RSF rats at 16 months to distinguish the role of waning ovarian hormones in ischemic injury without the confounding effects of advanced aging on brain morphology or cardiovascular function.

Our present finding that RSF rats sustain similar brain injury after stroke compared with age-matched males is in contrast with our previous finding that ischemic brain injury is smaller in young adult females than in age-matched males. The difference between our previous and present findings is likely related to loss of female reproductive function in RSF rats, since infarct size in middle-aged females (31±6% in cortex and 43±5% in striatum) is larger than the infarct observed in young adult females in our previous study (9±3% in cortex and 20±5% in striatum). The protection against ischemic brain injury found in young adult female rats compared with age-matched males is likely a function of ovarian hormones since the protection is lost after surgical ovariectomy and can be partially restored by estrogen replacement. The larger infarct in RSF rats compared with young adult females is not a function of age alone since infarct size in males was not different between these 2 age groups (26±7% in cortex and 42±2% in striatum in young adults and 21±5% in cortex and 44±7% in striatum in middle-aged rats). Aging (>20 months) exacerbates cerebral infarction in rodent models of focal ischemia, but the effect on neuronal susceptibility to global ischemia in rodents is inconsistent and may be affected by strain, sex, genetic background (presence of hypertension), experimental conditions, and location of affected neurons (hippocampus versus cortex/striatum).

Figure 2. Cortical and striatal infarcts after 2-hour MCA occlusion in middle-aged male (M) (n=9) and female (F) (n=9) Wistar rats. Infarct was identified in 2-mm-thick brain sections by TTC staining at 22 hours of reperfusion and expressed as a percentage of ipsilateral cerebral cortex (left) or striatum (right). AP indicates anterior-posterior. No differences were observed between the 2 groups.

Figure 3. Effects of estrogen (25-μg pellet, administered subcutaneously for 7 days; n=9) and progesterone (10-mg pellet, administered subcutaneously for 7 days; n=9) treatments on total cortical and striatal infarcts after 2-hour MCA occlusion in RSF Wistar rats (n=9). Infarct was identified in brain sections by TTC staining at 22 hours of reperfusion and expressed as a percentage of ipsilateral cerebral cortex or striatum. *Statistically significant difference compared with untreated group (P<0.05).

Figure 4. CBF rates in representative areas within gray matter of the parietal somatosensory cerebral cortex and lateral striatum in ischemic and contralateral hemispheres of male (n=3), untreated female (n=3), estrogen-treated female (n=3), and progesterone-treated female (n=3) rats. Blood flow rates were measured at the end of ischemia by [14C]IAP autoradiography. No differences were observed among treatment groups. Abbreviations are defined in Figure 1 legend.
Our findings are in agreement with the observations that neuronal susceptibility to the cytotoxic effects of N-methyl-D-aspartate (NMDA) antagonists is age dependent only in females. The observation is also consistent with the finding that vulnerability of hippocampal neurons to transient forebrain ischemia increases with age in hypertensive female rats. Finally, these findings are in agreement with the epidemiological observation that stroke risk, which is low in premenopausal women, rises after menopause to equal that of men.

Treatment with 17β-estradiol in this study reduced cortical and striatal infarcts in RSF rats. This is consistent with our previous findings and that of others that estrogen replacement reduces ischemic brain injury in surgically ovariectomized young female rats. Our demonstration of the neuroprotective effect of estrogen in this study is novel and important in that RSF rats correspond to and model a clinically relevant age group in humans, postmenopausal women, in which the neuroprotective effect of estrogen is most relevant as stroke injury is concerned.

The ability of estrogen to attenuate cerebral infarction in this study is unlikely to be related to the ability of estrogen to enhance tissue perfusion during ischemia since perfusion of brain regions affected by MCA occlusion was not different in estrogen-treated versus untreated females. Both region-specific CBF and CBF distribution across the hemisphere were similar among treatment groups (Figures 3 and 4). We and others have previously demonstrated that exogenously administered estrogen exerts a flow-independent neuroprotective effect in young ovariectomized rats. Our results, however, do not exclude the possibility that blood flow differences during the reperfusion period may play a role in mediating the beneficial effect of the hormone against cerebral ischemia. Estrogen has been shown to ameliorate post-ischemic hyperemia after global incomplete ischemia in female rabbits.

The mechanism(s) of estrogen-mediated neuroprotection is unknown but is likely multifactorial. Possible mechanisms include attenuation of oxidative injury via its antioxidant activity, prevention of intracellular calcium accumulation, inhibition of NMDA-induced excitotoxicity, enhanced expression of neurotrophin receptors, possibly by activation of mitogen-activated protein kinase signaling pathways, and modulation of antiapoptotic Bcl-2 gene expression. At least part of the neuroprotective effects of estrogen are likely receptor mediated since female estrogen receptor-α knockout mice display smaller infarcts after MCA occlusion compared with wild-type controls. Importantly, it is not known whether the mechanisms of ischemic injury and neuroprotection are similar in young adult and RSF rats. The possibility of an interaction between aging-related neurodegeneration, ischemic vulnerability, and estrogen-mediated effects is quite feasible and underscored by a recent report indicating that the responsiveness of dentate granule cells to aging and estradiol is sexually dimorphic.

Progesterone treatment was associated with smaller infarct in the cerebral cortex but not in striatum. The cortical protection is consistent with the demonstration that progesterone reduces damage after traumatic brain injury in ovariectomized rats and after focal ischemia in male rats and global ischemia in ovariectomized cats. A very limited number of observational studies have been conducted to evaluate the effect of progestins alone on stroke risk in humans; however, the addition of progesterin did not attenuate the cardioprotective effects of postmenopausal estrogen therapy. In an ongoing randomized trial for the secondary prevention of coronary heart disease in postmenopausal women, an early increase, but a long-term decrease, in risk of coronary heart disease was associated with the use of estrogen plus progestin therapy.

Our data demonstrate that progesterone is beneficial in experimental stroke, but in a more restrictive manner than estrogen. The mechanism of the observed cortical protection is unclear at present but is not related to preservation of tissue perfusion. Both the magnitude and distribution of rCBF during ischemia were unaltered by progesterone treatment. Others have shown that progesterone-treated rats exhibit better behavioral recovery and less edema and secondary neuronal loss after traumatic brain injury than untreated controls. Brains of progesterone-treated rats contain less β-aminobutyric acid receptor channel activity and expression and attenuate excitatory neuronal responses, which allow for the anxiolytic and antiepileptic properties of progesterone. Finally, progesterone is synthesized in brain by glia and enhances neurite outgrowth.
and axonal regeneration.48 The differing scope of protection by progesterone versus estrogen (lack of striatal protection by progesterone) may be related to different ischemic mechanisms in striatum versus the cerebral cortex or to differences in regional expression of progesterone versus estrogen receptors. This hypothesis would be consistent with in vitro reports that, in contrast to 17β-estradiol, progesterone fails to protect neuronal cultures from cell death caused by the neurotoxins amyloid β-protein, hydrogen peroxide, and glutamate.29 Whether combined estrogen and progesterone treatment has additive neuroprotective effect over any treatment alone has not been tested. The 2 steroids could also interact at the receptor level since estrogen and estrogen plus progesterone have been shown to upregulate progesterone receptor expression in brain.49

In summary, we have demonstrated that, in contrast to the protection against ischemic injury found in young females, RSF rats sustain brain damage after experimental stroke similar to that in age-matched males. Loss of protection is presumably related to reproductive senescence since treatment with either estrogen or progesterone reduces cortical infarction. Striatal infarct, however, was reduced only by estrogen treatment. A direct neuroprotective effect of ovarian steroids is suggested by the finding that neither estrogen nor progesterone had an effect on rCBF during ischemia. These results indicate that ovarian hormones are protective in the setting of cerebral ischemia and lend support for the use of hormone replacement therapy in brain injury prevention after stroke in postmenopausal women.

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References
Studies conducted on experimental animals indicate that the extent of brain injury after experimental cerebral ischemia is greater in young adult male than female animals. This difference in extent of cerebral ischemic injury is lost after ovariectomy of the female animals and is restored with estrogen replacement. These results suggest that estrogen protects against cerebral ischemic damage in experimental animals. The results of the accompanying article by Alkayed et al extends these previous observations to an experimental model of aging, that is, older rats that have naturally lost their reproductive capacity. This study design is important in that it represents a “natural” menopause in conjunction with the natural aging process and thus may better model conditions in the aging population of women. As might be expected from observations made in ovariectomized young female animals, 1,2 the extent of neuronal damage after experimental cerebral ischemia is the same in reproductive senescent females and age-matched males. Also consistent with what might be expected from results in young animals, short-term (7-day) treatment with low-dose estrogen (picomolar range) reduces the cerebral ischemic penumbra associated carotid occlusion and reperfusion. An important additional observation in the present study is that progesterone treatment also reduces the extent of cerebral damage. However, this “protection” is restricted to the cortical areas and does not include striatal areas. Although both ovarian hormones provide some protection against neuronal damage, the accumulated evidence points to estrogen as having a more effective contribution in both female and male animals.

The mechanism by which estrogen may provide protection against ischemic damage is multifactorial but seems to be unrelated to changes in blood flow alone. 3 This observation might also be expected, given the multiple tissues in which estrogen affects function, including all components in the vascular wall (endothelium, vascular smooth muscle, neurotransmission), 4,5 as well as neuronal function within the brain 6 and nongenomic effects that might be related to scavenging oxygen-derived free radicals. 7

Although estrogen seems to limit ischemic “stroke” in experimental studies, evidence from human clinical trials is less clear. 8,9 In case-controlled epidemiological studies, neither an increase nor decrease in the incidence of stroke was observed with estrogen or estrogen plus progesterone therapy. There are considerable limitations in extrapolating effects of estrogen replacement therapy in experimental cerebral ischemia to the incidence of strokes in humans. The outcome of human studies not only needs to address the incidence of stroke but also to differentiate between hemorrhagic and ischemic stroke. Also, results from experimental studies show that estrogen did not eliminate neuronal damage associated with cerebral ischemia but rather limited its extent. Appropriate measures in human trials to determine effects of estrogen therapy on cerebral ischemia may not be only the incident rate but rather the extent (severity) of event and rate of recovery. Alternatively, estrogen as an adjunct to interventional surgical management (carotid endarterectomy) for asymptomatic carotid artery stenosis may be a more appropriate clinical circumstance for testing outcomes of estrogen in preventing cerebral ischemia. In a prospective, randomized, multicenter trial, women had about twice the perioperative complications as men after carotid endarterectomy and 5-year event rate (stroke) was reduced by only 7% in women compared with a 66% reduction in men. 10 The hormonal status of women, including past estrogen use, was not reported in this multicenter trial. However, based on the accompanying paper, the question arises of whether short-
term, low-dose estrogen therapy before the intervention would improve outcome following the surgical procedure in women. Therefore, observations from experimental studies that demonstrate reduction in ischemic neuronal damage with estrogen replacement therapy should not be extrapolated as evidence for estrogen-reducing risk or incidence rate only. Delineating the effects of estrogen therapy in limiting stroke in humans may be clarified only when measures other than incidence rates are carefully documented, that is, as the authors of the accompanying paper conclude, to determine whether hormone replacement therapy limits brain injury after stroke in postmenopausal women.

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