

Timing of estrogen therapy after ovariectomy dictates the efficacy of its neuroprotective and antiinflammatory actions

Shotaro Suzuki^{*†}, Candice M. Brown^{*†}, Christopher D. Dela Cruz[†], Enhua Yang[†], David A. Bridwell[†], and Phyllis M. Wise^{*†‡§}

Departments of ^{*}Physiology and Biophysics and [†]Biology, University of Washington, Seattle, WA 98195; and [‡]Department of Neurobiology, Physiology, and Behavior, University of California, Davis, CA 95616

Edited by Bruce S. McEwen, The Rockefeller University, New York, NY, and approved February 17, 2007 (received for review November 22, 2006)

Recent studies describing the seemingly contradictory actions of estrogens in ischemic stroke injury have led us to reevaluate the circumstances under which estrogen therapy (ET) provides benefits against cerebral stroke and decipher its mechanisms of action. One prominent feature that follows stroke injury is massive central and peripheral inflammatory responses. Evidence now suggests that postischemic inflammatory responses strongly contribute to the extent of brain injury, and 17 β -estradiol (E₂) may protect the ischemic brain by exerting antiinflammatory actions. In an attempt to explain recently reported dichotomous effects of E₂ in stroke injury, we tested the hypothesis that an extended period of hypoestrogenicity both prevents E₂ from protecting the brain against ischemia and simultaneously suppresses its antiinflammatory actions. We report that E₂ exerts profound neuroprotective action when administered immediately upon ovariectomy, but not when administered after 10 weeks of hypoestrogenicity. Consistently, E₂ treatment given immediately at the time of ovariectomy attenuated central and peripheral production of proinflammatory cytokines after ischemic stroke. In contrast, E₂ did not suppress production of proinflammatory molecules when it was administered after 10 weeks postovariectomy. These results demonstrate that a prolonged period of hypoestrogenicity disrupts both neuroprotective and antiinflammatory actions of E₂. Our findings may help to explain the results of the Women's Health initiative that reported no beneficial effect of ET against stroke because the majority of the subjects initiated ET after an extended period of hypoestrogenicity.

inflammation | ischemic injury | stroke | Women's Health initiative

Stroke is the third major cause of death nationwide, and each year \approx 40,000 more women than men are affected by stroke (1). This gender difference is related to both the longer life expectancy of women and the protective role of estrogens, because the incidence of stroke in women dramatically increases after menopause and the risk continues to increase with age (2). Over the past two decades, numerous basic science, observational, and retrospective studies have supported the concept that estrogen therapy (ET) of postmenopausal women protects against age-related diseases, including cardiovascular disease and neurodegenerative conditions associated with stroke (3–8). Consistently, the absence of ovarian steroid hormones after menopause makes postmenopausal women more vulnerable to both cardio- and cerebrovascular diseases compared with age-matched cycling women. Despite numerous studies demonstrating cardio- and cerebrovascular benefits of ET, recent results from the Women Estrogen Stroke Trial (WEST) and the Women's Health initiative (WHI) reported that ET afforded no benefit or increased the risk for stroke (9, 10). Interestingly, the majority of subjects in both the WEST and WHI were reported to be postmenopausal for many years before the initiation of ET. In the WHI, the mean age of the subjects was 63 years, among which 74% of the participants had never taken hormone treat-

ment previously (4, 8). Furthermore, women within 1 year of menopause were excluded from the study (11). In striking contrast, observational studies that reported cardiovascular benefits of hormone treatment examined women averaging 51 years of age, many of whom initiated hormone treatment in their perimenopausal period (1, 11, 12).

Inflammation plays a critical role in stroke, and postischemic inflammatory responses strongly contribute to the extent of ischemic brain injury (13, 14). The inflammatory cascades associated with ischemic stroke are characterized by increased production of proinflammatory cytokines and chemokines both centrally and peripherally. Numerous clinical studies have demonstrated that increases in cerebrospinal fluid (CSF) as well as plasma levels of proinflammatory cytokines after stroke positively correlate with larger infarct size and poor clinical stroke outcomes (15, 16). In addition, in experimental animal models of stroke, it is well established that ischemic injury triggers a cascade of inflammatory responses that strongly contributes to the extent of ischemic brain injury (17, 18).

Previously our laboratory has demonstrated that 17 β -estradiol (E₂) exerts profound neuroprotective actions in a model of stroke injury in which the middle cerebral artery is permanently occluded (19, 20). E₂ achieves its protective action by altering the expression of multiple genes involved in neuronal apoptotic pathways through an estrogen receptor alpha (ER α)-dependent fashion (20, 21). Accumulating evidence from basic science studies now suggests that E₂ exerts its neuroprotective action by suppressing inflammation through ER α -mediated mechanisms (22, 23). Indeed, a growing body of evidence from both preclinical and clinical studies leads to the concept that E₂'s antiinflammatory actions are at the fundamental core of its pleiotropic protective actions on multiple physiological systems (8).

The goal of the present study was to reevaluate the circumstances under which E₂ provides benefits against ischemic stroke and to decipher the mechanisms of its action in an attempt to explain reported inconsistent effects of E₂ in stroke injury. We tested the hypothesis that an extended period of hypoestrogenicity both prevents E₂ from protecting the brain against ischemic injury and simultaneously suppresses its antiinflammatory actions. We report that E₂ exerts profound neuroprotective and

Author contributions: S.S. and P.M.W. designed research; S.S. and C.M.B. performed research; C.D.D. and E.Y. contributed new reagents/analytic tools; S.S., C.M.B., C.D.D., E.Y., and D.A.B. analyzed data; and S.S. and P.M.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: CSF, cerebrospinal fluid; E₂, 17 β -estradiol; ER α , estrogen receptor alpha; ET, estrogen therapy; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; WEST, Women Estrogen Stroke Trial; WHI, Women's Health initiative.

[§]To whom correspondence should be addressed. E-mail: pmwise@u.washington.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0610394104/DC1.

© 2007 by The National Academy of Sciences of the USA

antiinflammatory actions only when administered immediately upon ovariectomy, but not when administered after a prolonged period of hypoestrogenicity.

Results

The Timing of E₂ Treatment Dictates the Efficacy of Its Neuroprotective Actions. This study included two experimental paradigms. In the first paradigm, 19-week-old C57BL/6J mice were ovariectomized and immediately implanted with capsules containing either oil or E₂ for 1 week (Fig. 1A). This paradigm of E₂ treatment allows the maintenance, without interruption, of stable levels of E₂ in serum (25 pg/ml) equivalent to low-basal circulating levels found during the estrous cycle of mice (20). Subsequently, animals underwent experimental ischemia by middle cerebral artery occlusion (MCAO). The total, cortical, and striatal infarct volumes were measured by using 2,3,5-triphenyltetrazolium chloride (TTC) staining 24 h after the onset of ischemic injury. The extent of brain injury was clearly delineated by TTC staining and was distributed throughout the injured hemisphere (Fig. 1B and C). Consistent with our previous observations, where we used young adult mice, low physiological levels of E₂ significantly reduced the total infarct volume (Fig. 1D; $P < 0.02$) and the extent of injury in both the cortex (Fig. 1E; $P < 0.05$) and striatum (Fig. 1F; $P < 0.03$) of 20-week-old mice.

In the second paradigm, 9-week-old mice were ovariectomized and 10 weeks later implanted with oil or E₂ capsules for 1 week before they underwent experimental ischemia (Fig. 2A). This paradigm of E₂ treatment led to a prolonged period of hypoestrogenicity before stable serum levels of E₂ were achieved through a delayed implantation of a Silastic capsule, which produced low-basal circulating levels equivalent to those attained in the first paradigm. Thus, mice in both paradigms were designed to undergo ischemic injury at 20 weeks of age. In contrast to animals in the first paradigm, mice in the second paradigm that experienced a prolonged period of hypoestrogenicity before restoration of basal plasma E₂ levels exhibited extensive ischemic brain injury regardless of E₂ treatment (Fig. 2B and C). E₂ did not reduce the total infarct volume when treatment was initiated after a 10-week period of hypoestrogenicity (Fig. 2D; $P = 0.697$). Specifically, E₂ failed to reduce brain infarction in the cortex (Fig. 2E; $P = 0.447$) as well as in the striatum (Fig. 2F; $P = 0.581$), compared with oil-treated counterparts.

Up-Regulation of ER α Is Disrupted After Prolonged Hypoestrogenicity.

To begin to decipher the mechanisms for a loss of neuroprotective action of E₂ after an extended period of hypoestrogenicity, we examined the expression of ER α in the ischemic brain. Previously, we have shown that the presence of ER α , but not ER β , is a prerequisite for E₂ to achieve its protective action (20). Furthermore, our previous observations have demonstrated that ischemic injury up-regulates the expression of the ER α gene on the injured side of the brain (300–450% increase compared with sham) at the levels of mRNA and protein in both rats (24, 25) and mice (25, 26), suggesting that this up-regulation of ER α mediates the ability of E₂ to protect against neuronal death. In the present study, when E₂ treatment was delayed for 10 weeks postovariectomy, ischemic injury did not up-regulate ER α protein on the ipsilateral side of the ischemic brain compared with sham animals [supporting information (SI) Fig. 5].

Immediate E₂ Treatment Attenuates Proinflammatory Responses in the Brain.

A mouse cytokine multiplex proteomic array technique was used to measure proinflammatory cytokines in the brains from both experimental paradigms. Ischemic injury dramatically increased the production of monocyte chemoattractant protein-1 (MCP-1; $P < 0.0001$ for oil-treated mice; $P = 0.0153$ for E₂-treated mice) and IL-6 ($P < 0.0001$ for oil-treated mice; $P =$

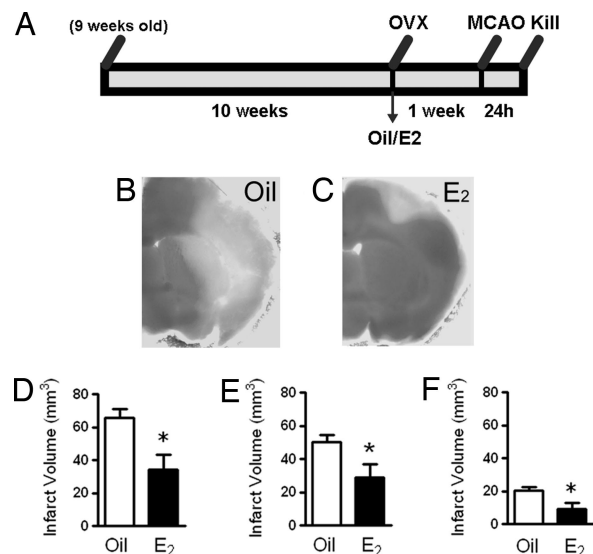


Fig. 1. Low physiological levels of E₂ protect the brain against ischemic injury. (A) C57BL/6J mice (19-week-old) were ovariectomized and immediately implanted with capsules containing either oil ($n = 8$) or E₂ ($n = 11$) for 1 week. Subsequently, animals underwent experimental ischemia by middle cerebral artery occlusion (MCAO) at 20 weeks of age and were killed 24 h after the onset of injury. (B and C) Infarct volumes were measured by using TTC staining in oil- (B) or E₂- (C) treated mice. (D–F) Immediate E₂ treatment significantly reduced the total infarct volume (D; *, $P < 0.02$) and the extent of injury in both the cortex (E; *, $P < 0.05$) and striatum (F; *, $P < 0.03$) of 20-week-old mice (unpaired two-tailed t test, mean \pm SEM, $n = 8–11$).

0.0043 for E₂-treated mice) on the injured side of the brain compared with the contralateral side (Fig. 3A and B). An immediate E₂ treatment significantly attenuated ischemia-induced up-regulation of MCP-1 ($P = 0.0093$) and IL-6 ($P = 0.0271$) on the ipsilateral side of the ischemic brain (Fig. 3A and B). In addition, E₂ prevented injury-induced down-regulation ($P = 0.0004$) of the neuroprotective VEGF (Fig. 3C; $P = 0.0024$). In striking contrast, when administered 10 weeks after ovariectomy, E₂ did not suppress the production of MCP-1 (Fig. 3D; $P = 0.285$) as well as IL-6 (Fig. 3E; $P = 0.541$) and did not cause any changes in the expression of VEGF (Fig. 3F; $P = 0.326$).

E₂ Suppresses Peripheral Cytokine Production After Ischemic Injury.

We then measured the levels of peripheral cytokines in plasma after MCAO-induced ischemic injury. In accordance with its effects on central inflammatory responses, E₂ exerted antiinflammatory actions only when administered immediately after ovariectomy (Fig. 4). E₂ suppressed plasma levels of IL-6 (Fig. 4A; $P = 0.004$), TNF α (Fig. 4B; $P < 0.05$), granulocyte macrophage colony-stimulating factor (GM-CSF; Fig. 4C; $P = 0.0002$), IL-4 (SI Fig. 6A; $P = 0.0003$), and IL-5 (SI Fig. 6B; $P < 0.001$). When E₂ was administered 10 weeks postovariectomy, E₂ did not suppress the production of IL-6 (Fig. 4D; $P = 0.737$), TNF α (Fig. 4E; $P = 0.351$), GM-CSF (Fig. 4F; $P = 0.663$), IL-4 (SI Fig. 6C; $P = 0.787$), and IL-5 (SI Fig. 6D; $P = 0.280$).

Discussion

In the present study, we tested the hypothesis that an extended period of hypoestrogenicity impairs the ability of E₂ to protect the ischemic brain and simultaneously prevents its antiinflammatory actions. Our study clearly demonstrates that low physiological levels of E₂ exert profound neuroprotective actions when administered immediately upon ovariectomy, but not after a 10-week period of hypoestrogenicity. In addition, an immediate E₂ treatment significantly attenuated both central and pe-

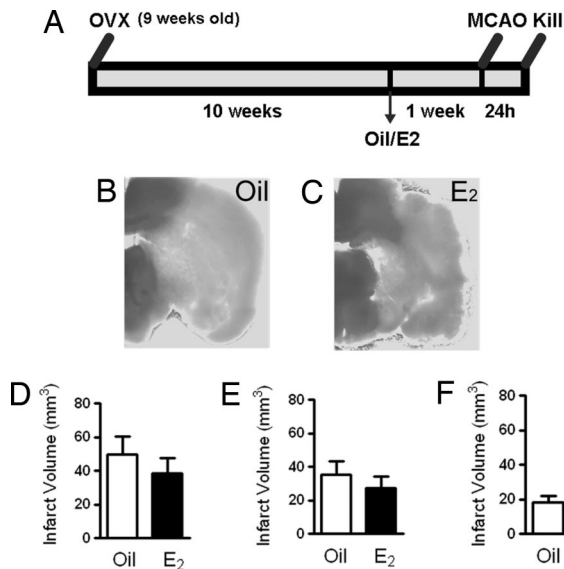


Fig. 2. Prolonged hypoestrogenicity disrupts the ability of E₂ to protect the ischemic brain. (A) Nine-week-old C57BL/6J mice were ovariectomized and 10 weeks later implanted with oil ($n = 12$) or E₂ ($n = 10$) capsules for 1 week before they underwent experimental ischemia. This paradigm of E₂ treatment leads to a prolonged period of hypoestrogenicity before stable serum levels of E₂ are restored through a delayed implantation of a Silastic capsule. (B and C) In contrast to animals that received capsules immediately at the time of ovariectomy (Fig. 1), mice in this group exhibited extensive ischemic brain injury regardless of E₂ treatment revealed by TTC staining. (D–F) A delayed E₂ treatment did not reduce the total infarct volume (D; $P = 0.697$). Specifically, after a prolonged period of hypoestrogenicity, E₂ failed to reduce brain infarction in the cortex (E; $P = 0.447$) as well as in the striatum (F; $P = 0.581$), compared with oil-treated counterparts (unpaired two-tailed t test, mean \pm SEM, $n = 10$ –12).

ripheral production of proinflammatory cytokines after ischemic stroke injury. To our knowledge, this is the first study to show that E₂ attenuates postischemic inflammatory responses in the brain as well as in the peripheral blood *in vivo*. We also showed that the ability of E₂ to suppress the production of deleterious proinflammatory molecules was obliterated when E₂ was administered after 10 weeks postovariectomy.

Controversies over the Efficacy of Estrogen Therapy. Over the past two decades, numerous observational, retrospective, and interventional studies demonstrated the cerebrovascular benefits of ET among postmenopausal women (reviewed in refs. 3–8). However, recent clinical trials including the WEST and WHI reported that ET increased the risk for stroke or afforded no benefit (9, 10). Although the majority of controlled trials only investigated the risk and benefit of ET, both the WEST and WHI also reported the effect of ET on stroke outcomes (1, 9, 27). In the WEST, women who took E₂ replacement had marginally greater neurological deficits after nonfatal stroke compared with women in the placebo group (9). In the WHI, although hormone therapy did not exaggerate stroke disability outcomes, it did not exhibit any beneficial actions (27). It is important to remember that the vast majority of women who engaged in these trials were, on average, 12 years postmenopause before the initiation of hormone therapy (4, 8). In striking contrast, in observational studies that reported beneficial effects of ET, the majority of subjects initiated hormone replacement in their perimenopausal period (11, 12). Thus, to explain reported inconsistent effects of ET in stroke injury, we tested the hypothesis that the long duration from surgically induced menopause to the initiation of

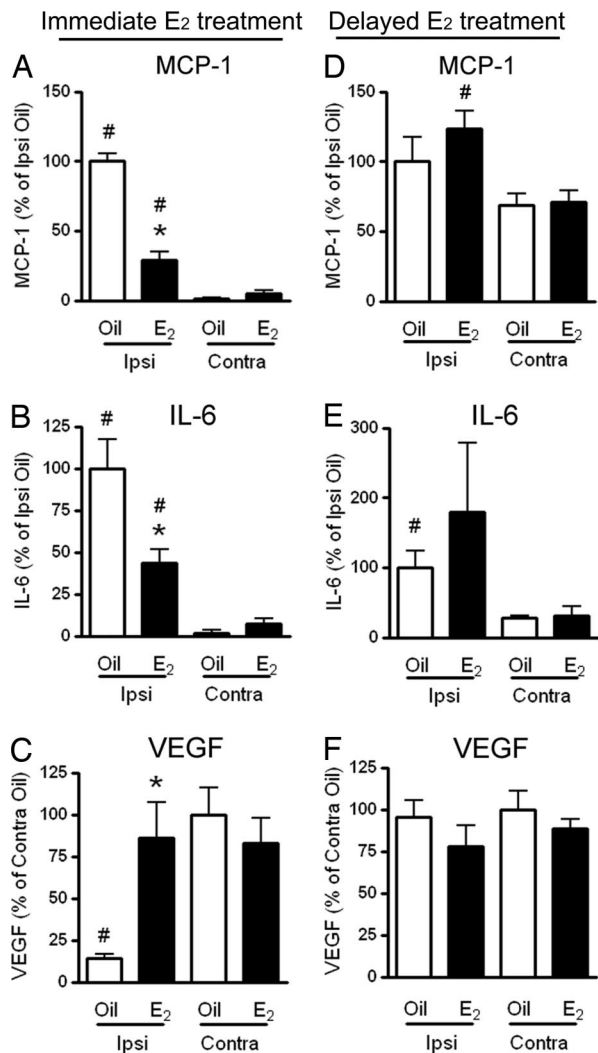


Fig. 3. Immediate E₂ treatment attenuates proinflammatory responses in the brain. In the first paradigm, mice were ovariectomized at 19 weeks of age and immediately implanted with capsules containing either oil ($n = 5$) or E₂ ($n = 8$) for 1 week. (A and B) A mouse cytokine multiplex proteomic array revealed that ischemic injury increased the expression of monocyte chemoattractant protein-1 (MCP-1; #, $P < 0.0001$ for oil-treated mice; #, $P = 0.0153$ for E₂-treated mice) and IL-6 (IL-6; #, $P < 0.0001$ for oil-treated mice; #, $P = 0.0043$ for E₂-treated mice) on the injured side of the brain compared with the contralateral side. An immediate E₂ treatment attenuated ischemia-induced up-regulation of MCP-1 (*, $P = 0.0093$) and IL-6 (*, $P = 0.0271$) on the ipsilateral side of the ischemic brain. (C) E₂ prevented injury-induced down-regulation (#, $P = 0.0004$) of the neuroprotective VEGF (*, $P = 0.0024$). In the second paradigm, mice at 9 weeks of age were ovariectomized for 10 weeks and subsequently implanted with an oil ($n = 5$) or E₂ ($n = 6$) capsule for 1 week. (D) When administered 10 weeks after ovariectomy, E₂ did not suppress ($P = 0.285$) the injury-induced production of MCP-1 (#, $P = 0.0052$). (E) Similarly, E₂ did not attenuate ($P = 0.541$) the ischemia-induced up-regulation of IL-6 production (#, $P = 0.0261$). (F) A delayed E₂ treatment caused no changes in the expression of VEGF ($P = 0.326$). In both paradigms, data represent the mean \pm SEM of five to eight animals per experimental group.

ET negates the beneficial effects of E₂ on the outcomes of experimentally induced ischemic stroke.

Dichotomous Actions of E₂ in the Ischemic Brain. Our findings clearly show that ET must be initiated immediately upon ovariectomy to exert beneficial actions on the outcomes of stroke injury. We found that a prolonged period of hypoestrogenicity entirely prevents E₂ from exerting these powerful neuroprotective ac-

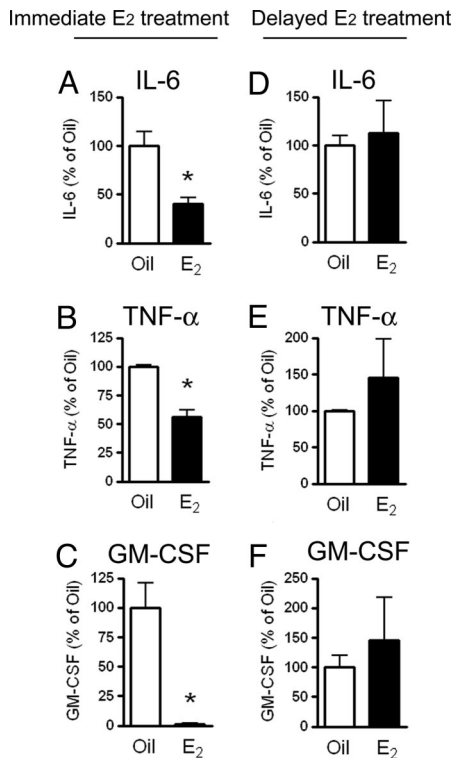


Fig. 4. E₂ exerted antiinflammatory actions only when administered immediately after ovariectomy. (A–C) Immediate E₂ treatment ($n = 9$) suppressed plasma levels of IL-6 (A; *, $P = 0.004$), TNF- α (B; *, $P < 0.05$), and GM-CSF (C; *, $P = 0.0002$), compared with oil-treated controls ($n = 8$). (D–F) When E₂ was administered 10 weeks postovariectomy ($n = 11$), it did not suppress the production of IL-6 (D; $P = 0.737$), TNF- α (E; $P = 0.351$), and GM-CSF (F; $P = 0.663$), compared with oil-treated counterparts ($n = 14$). In both series, data represent the mean \pm SEM of 8–14 animals per experimental group.

tions. Specifically, E₂ significantly decreased the size of the infarct when administered immediately upon ovariectomy, but not when administered after a 10-week period of hypoestrogenicity. Previously we have shown that E₂ exerts profound neuroprotective actions in young (2- to 3-month-old) mice (20). In the current study, we demonstrate that E₂ also effectively reduces the infarct volume in 5-month-old mice. Together our findings show that a constellation of factors mediating E₂'s protective actions is preserved in mice approaching middle age.

To begin to discern why E₂ treatment does not exert its protective action after an extended period of hypoestrogenicity, we examined the expression of ER α in the ischemic brain. Our earlier studies that used both ER α and ER β knockout mice demonstrated that ER α , but not ER β , mediates the ability of E₂ to protect the brain against ischemic injury (20). Consistently, ischemic stroke injury only up-regulates the expression of ER α without inducing ER β expression on the injured side of the brain (24–26). The dramatic reexpression of ER α after injury appears to be a recapitulation of its expression during fetal and neonatal development when E₂ plays pivotal trophic roles in neurite outgrowth, synaptogenesis, and neuronal organization. Therefore, the injury-induced reexpression and up-regulation of ER α may allow E₂ to act once again as a trophic and neuroprotective factor in the adult ischemic brain. Interestingly, we found that ischemic injury did not up-regulate ER α protein expression when E₂ did not protect the brain against stroke injury after delayed E₂ treatment. This finding is in striking contrast to our previous observation that ER α is highly up-regulated (300% to 450% increase compared with sham animals) when E₂ treatment

is given immediately at the time of ovariectomy (24–26). Taken together, these findings suggest that a prolonged hypoestrogenicity impairs the ability of the injured brain to provide signals conveying the need for the reappearance of ER α , which likely mediates the protective actions of E₂ against neuronal death.

Ischemic Stroke Induces Massive Proinflammatory Responses in the Brain. We observed in our animal model of stroke that production of MCP-1 is up-regulated on the injured (ipsilateral) side compared with the uninjured (contralateral) side of the ischemic brain. MCP-1, a member of the cysteine-cysteine chemokine family, is a potent chemoattractant factor known to play deleterious roles in the brain after stroke injury (28). It increases infiltration of inflammatory cells (i.e., macrophages, monocytes, and neutrophils) from circulation into the ischemic brain, leading to the development of ischemic brain injury (29, 30). Consistently, overexpression of MCP-1 in mice exacerbates ischemic brain injury, enhances migration of macrophages and neutrophils into the ischemic brain, and increases brain infarction (29).

We also observed that ischemic injury up-regulated IL-6 on the injured side of the brain. Although there is debate over whether an up-regulation of IL-6 after stroke simply indicates the degree of brain damage or represents an actual neuropathogenic step that leads to neuronal death, clinical studies have shown that higher IL-6 levels in stroke patients at the time of hospital entry positively correlate with further brain infarction and accompanying neurological worsening, regardless of the initial size and topography of ischemic infarction at hospital entry (13, 14). Consistent with our observation, previous studies have shown that experimental stroke induces massive up-regulation of IL-6 in mice (17, 18). IL-6 has been shown to contribute to the development of ischemic brain injury by increasing the production of acute-phase proteins and inducible nitric oxide synthase, as well as by activating the hypothalamic–pituitary–adrenal axis after stroke (31). Among stroke patients, hypercortisolism is positively correlated with cognitive dysfunction and increased mortality (32).

In addition, we observed that ischemic injury caused down-regulation of VEGF on the ipsilateral side of the brain. VEGF is an angiogenic protein with therapeutic potential in ischemic disorders, including stroke, because of its neurotrophic and neuroprotective properties (33). In experimental stroke, administration of VEGF reduces brain infarction (34) and simultaneously improves neurological outcomes (35). Thus, down-regulation of VEGF may cause a detrimental effect on the development of ischemic brain injury.

Parallelism Between the Ability of E₂ to Protect and Its Antiinflammatory Action. We found that an immediate E₂ treatment not only protected the brain, but also significantly attenuated ischemia-induced up-regulation of MCP-1 and IL-6 in the ischemic brain. In contrast, E₂ did not exert protective action and did not suppress production of proinflammatory molecules when it was administered 10 weeks postovariectomy. This striking parallelism between protective and antiinflammatory actions of E₂ suggests that E₂ protects the brain against neuronal death by inhibiting ischemia-induced proinflammatory cascades. Inflammation is known to play a critical role in stroke, and postischemic inflammatory responses strongly contribute to the extent of ischemic brain injury (13, 14). A growing body of evidence now suggests that E₂ exerts neuroprotection by exerting antiinflammatory actions through ER α -mediated mechanisms (22, 23). Consistently, E₂ down-regulates the expression of proinflammatory cytokines, including IL-6 (36), MCP-1 (37), and TNF- α (38). Although the promoter regions of IL-6, MCP-1, and TNF- α genes lack a classical estrogen response element, E₂ inhibits the expression of these genes through either NF- κ B and/or activator

protein-1 (AP-1) response elements (39). It is well established that ER α mediates E₂'s negative regulation of proinflammatory cytokines; however, only a few studies have examined the roles of ER β (38). Taken together, we hypothesize that a prolonged hypoestrogenicity disrupts the ability of the injured brain to up-regulate ER α , an essential mediator for antiinflammatory and neuroprotective actions of E₂, thus eliminating the beneficial effects of E₂ treatment.

Interestingly, an immediate E₂ treatment prevented ischemia-induced down-regulation of VEGF. Previous studies have identified a functional estrogen response element in the VEGF promoter, which mediates estrogenic regulation of VEGF production (40). Importantly, E₂ has been shown to prevent injury-induced down-regulation of antiapoptotic factor Bcl-2, a key regulator of apoptosis, both *in vivo* (24) and *in vitro* (41). Thus, E₂ may confer neuroprotection in the ischemic brain by additionally preventing down-regulation of this important protective factor. When E₂ was administered 10 weeks after ovariectomy, E₂ failed to prevent down-regulation of VEGF, further pointing to the importance of the proper timing of E₂ administration after ovariectomy in achieving powerful protective actions of this hormone.

E₂ Inhibits Postischemic Peripheral Inflammatory Responses. In addition to local brain inflammation, ischemic stroke triggers deleterious peripheral inflammatory reactions (15–18). Recent evidence demonstrates that peripheral inflammatory responses contribute to the extent of ischemic brain injury, and this communication between peripheral and central inflammation is a critical component of damaging mechanisms during stroke (13, 14). We observed that immediate E₂ treatment significantly reduced plasma levels of IL-6 and TNF α after ischemic injury. Previous studies have demonstrated that experimental stroke induces production of IL-6 and TNF α in plasma (17, 18), and our results extend these findings to show a neuroprotective action of E₂ to attenuate production of deleterious proinflammatory cytokines after stroke injury. Importantly, human stroke patients also exhibit elevated levels of plasma IL-6 and TNF α , which positively correlate with larger brain infarction and poor neurological outcomes (14, 42–44). Thus, data from both basic science and clinical studies demonstrate deleterious roles of IL-6 and TNF α in the development of ischemic brain injury. After stroke, these proinflammatory cytokines initiate a coordinated inflammatory response that includes activation of endothelium, recruitment of leukocytes and other immune cells, and up-regulation of receptors for innate immune responses (i.e., Toll-like receptors). These reactions lead to the added production of proinflammatory cytokines and contribute to both apoptotic and necrotic neuronal death (45).

We also observed that an immediate E₂ treatment significantly reduced plasma levels of GM-CSF after experimental stroke. GM-CSF is a hematopoietic growth factor with a potent proliferative effect on macrophages, microglia, and granulocytes during stroke (46). In addition, immediate E₂ treatment significantly suppressed plasma levels of IL-4 and IL-5. Clinical studies reported elevated serum IL-4 levels in human stroke patients, causing enhanced synthesis of IgE, an allergic factor that promotes platelet activation and arterial spasm in vascular disease (47). Although little is known about the role of IL-5 in ischemic brain injury, *in vitro* studies suggest that it is a potent mitogen factor for microglia (48, 49). Therefore, our results reveal a role for E₂ in the suppression of peripheral postischemic inflammatory responses. Consistent with its central antiinflammatory action, E₂ did not decrease plasma levels of any proinflammatory cytokines when it was administered 10 weeks after ovariectomy. Taken together, our findings demonstrate that the timing of E₂ treatment after ovariectomy dictates the efficacy of its powerful beneficial actions in the ischemic brain.

In conclusion, our study clearly demonstrates that E₂'s ability to protect against stroke injury correlates with its ability to suppress the injury-induced central and peripheral inflammatory responses. Indeed, a growing body of evidence leads to the concept that E₂'s antiinflammatory actions are at the fundamental core of its pleiotropic protective actions on multiple physiological systems (reviewed in ref. 8). We have also shown that the timing of E₂ reintroduction dictates both the efficacy of its neuroprotective action and its ability to suppress ischemia-induced inflammation. Together with previous studies that have raised a critical question regarding the timing and duration of hormone treatment (50, 51), the results of this study may help explain reported inconsistent actions of ET in stroke injury and demonstrate how the duration from loss of ovarian hormone production to the initiation of ET disrupts the beneficial actions of E₂. Our findings, in combination with the results of the WHI and other recent controlled clinical trials, emphasize the tremendous importance of strengthening the collaboration between basic science and clinical researchers to better design future clinical trials to take the maximum advantage of empirical and mechanism-based information and approaches.

Materials and Methods

All surgical procedures were performed in strict compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and have been approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

E₂ Therapy. Our study included two experimental paradigms. In the first paradigm, adult C57BL/6J female mice at 19 weeks of age were ovariectomized and immediately implanted s.c. with a Silastic capsule (0.062 in/0.125 in, inner/outer diameter; volume, 0.035 ml; Konigsberg Instruments, Pasadena, CA) containing either sesame oil (vehicle) or E₂ (180 μ g/ml) for 1 week. This paradigm of E₂ treatment produces stable levels of E₂ in serum (25 pg/ml) equivalent to low-basal circulating levels found during the estrous cycle of mice (20). In the second paradigm, mice at 9 weeks of age were ovariectomized to eliminate endogenous ovarian steroid production for 10 weeks and then implanted s.c. with an oil or E₂ capsule for 1 week. Mice from both paradigms then underwent ischemic injury at 20 weeks of age.

In Vivo Cerebral Ischemia. At 20 weeks of age, all mice were anesthetized with a mixture of chloral hydrate (350.0 mg/kg body weight, i.p.) and xylazine (4.0 mg/kg), and the right middle cerebral artery was permanently occluded as previously described (20). Briefly, a 5/0 size blue nylon suture was inserted into the internal carotid artery to the base of the middle cerebral artery. This occlusion led to a dramatic reduction in blood flow to the striatum and overlying cortex. In all mice, body temperature was monitored and maintained at normothermia until recovery from anesthesia.

Histologic Preparation. Brains were collected 24 h after the onset of ischemic injury ($n = 8$ –12 per experimental group). Detailed TTC staining procedures are described in *SI Materials and Methods*.

ER α Immunocytochemistry. Animals were subjected to immunocytochemistry for the detection of ER α as previously described (25, 26). Detailed procedures are described in *SI Materials and Methods*.

Central Multiplex Cytokine Analysis. A mouse cytokine multiplex proteomic array technique (Allied Biotech, Ijamsville, MD) was validated for brain tissue lysate samples and used to measure cytokine and chemokine concentrations in the ischemic brain.

All brains were collected 24 h after the onset of ischemic injury ($n = 5-8$ per experimental group). Detailed procedures for tissue processing and cytokine measurements are described in *SI Materials and Methods*.

Peripheral Multiplex Cytokine Analysis. Plasma was collected by cardiac puncture 24 h after the onset of MCAO-induced injury ($n = 8-14$ per experimental group). Cytokine and chemokine concentrations were measured in plasma by using a Lincplex 10-plex multiplex cytokine assay kit (Linco Research, St. Louis, MO) and subsequently quantified by using a Luminex100 machine (Luminex, Austin, TX). A Lincplex multiplex cytokine assay

kit (Linco Research) has been previously validated for the use of mouse plasma samples. See *SI Materials and Methods* for detailed procedures.

Data Analysis. All data are expressed as mean \pm SEM. The effect of injury or E₂ treatment was analyzed by using Student's *t* test. All differences were considered significant at $P < 0.05$.

This work was supported by National Institutes of Health Grants AG17164 and AG02224 (to P.M.W.) and the Ellison Medical Foundation (P.M.W.).

1. Bushnell CD (2006) *Semin Neurol* 26:123-130.
2. Mitka M (2006) *JAMA* 295:1755-1756.
3. Paganini-Hill A (2001) *Maturitas* 38:243-261.
4. Turgeon JL, McDonnell DP, Martin KA, Wise PM (2004) *Science* 304:1269-1273.
5. Behl C (2002) *Nat Rev Neurosci* 3:433-442.
6. McCullough LD, Hurn PD (2003) *Trends Endocrinol Metab* 14:228-235.
7. Wise PM, Dubal DB, Rau SW, Brown CM, Suzuki S (2005) *Endocr Rev* 26:308-312.
8. Turgeon JL, Carr MC, Maki PM, Mendelsohn ME, Wise PM (2006) *Endocr Rev* 27:575-605.
9. Viscoli CM, Brass LM, Kernan WN (2001) *N Engl J Med* 345:1243-1249.
10. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, et al. (2002) *JAMA* 288:321-333.
11. Miller VM, Clarkson TB, Harman SM, Brinton EA, Cedars M, Lobo R, Manson JE, Merriam GR, Naftolin F, Santoro N (2005) *J Appl Physiol* 99:381-383.
12. Harman SM, Naftolin F, Brinton EA, Judelson DR (2005) *Ann N Y Acad Sci* 1052:43-56.
13. Perini F, Morra M, Alecci M, Galloni E, Marchi M, Toso V (2001) *Neurol Sci* 22:289-296.
14. Vila N, Castillo J, Davalos A, Chamorro A (2000) *Stroke* 31:2325-2329.
15. Fassbender K, Rossol S, Kammer T, Daffertshofer M, Wirth S, Dollman M, Hennerici M (1994) *J Neurol Sci* 122:135-139.
16. Reynolds MA, Kirchick HJ, Dahlen JR, Anderberg JM, McPherson, PH, Nakamura KK, Laskowitz DT, Valkirs GE, Buechler KF (2003) *Clin Chem* 49:1733-1739.
17. Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD (2006) *J Cereb Blood Flow Metab* 26:654-665.
18. Clark WM, Rinker LG, Lessov NS, Hazel K, Eckenstein FP (1999) *Neurol Res* 21:287-292.
19. Dubal DB, Kashon ML, Pettigrew LC, Ren JM, Finklestein SP, Rau SW, Wise PM (1998) *J Cereb Blood Flow Metab* 18:1253-1258.
20. Dubal DB, Zhu B, Yu B, Rau SW, Shughrue PJ, Merchenthaler I, Kindy MS, Wise PM (2001) *Proc Natl Acad Sci USA* 98:1952-1957.
21. Rau SW, Dubal DB, Bottner M, Gerhold LM, Wise PM (2003) *J Neurosci* 23:11420-11426.
22. Vegeto E, Belcredito S, Ghisletti S, Meda C, Eterri S, Maggi A (2006) *Endocrinology* 147:2263-2272.
23. Vegeto E, Belcredito S, Eterri S, Ghisletti S, Brusadelli A, Meda C, Krust A, Dupont S, Ciana P, Chambon P, Maggi A (2003) *Proc Natl Acad Sci USA* 100:9614-9619.
24. Dubal DB, Shughrue PJ, Wilson ME, Merchenthaler I, Wise PM (1999) *J Neurosci* 19:6385-6393.
25. Dubal DB, Rau SW, Shughrue PJ, Zhu H, Yu J, Cashion AB, Suzuki S, Gerhold LM, Bottner MB, Dubal SB, et al. (2006) *Endocrinology* 147:3076-3084.
26. Suzuki S, Brown CM, Wise PM (2006) *Endocrine* 29:209-215.
27. Wassertheil-Smoller S, Hendrix SL, Limacher M (2003) *JAMA* 289:2673-2684.
28. Che X, Ye W, Panga L, Wu DC, Yang GY (2001) *Brain Res* 902:171-177.
29. Chen Y, Hallenbeck JM, Ruetzler C, Bol D, Thomas K, Berman NEJ, Vogel SN (2003) *J Cereb Blood Flow Metab* 23:748-755.
30. Kim JS, Gautam SC, Chopp M, Zaloga C, Jones ML, Ward PA, Welch KM (1995) *J Neuroimmunol* 56:127-134.
31. Allan SM, Rothwell NJ (2001) *Nat Rev Neurosci* 2:734-744.
32. Marklund N, Peltonen M, Nilsson TK, Olsson T (2004) *J Intern Med* 256:15-21.
33. Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA (2003) *J Clin Invest* 111:1843-1851.
34. Hayashi T, Abe K, Itoyama Y (1998) *J Cereb Blood Flow Metab* 18:887-895.
35. Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, Bruggen N, Chopp M (2000) *J Clin Invest* 106:829-838.
36. Galien R, Garcia T (1997) *Nucleic Acids Res* 25:2424-2429.
37. Inadera H, Sekiya T, Yoshimura T, Matsushima K (2000) *Endocrinology* 141:50-59.
38. An J, Ribeiro RC, Webb P, Gustafsson JA, Kushner PJ, Baxter JD, Leitman DC (1999) *Proc Natl Acad Sci USA* 96:15161-15166.
39. Czlonkowska A, Ciesielska A, Gromadzka G, Kurkowska-Jastrzebska I (2005) *Curr Pharm Des* 11:1017-1030.
40. Mueller MD, Vigne JL, Minchenko A, Lebovic DI, Leitman DC, Taylor RN (2000) *Proc Natl Acad Sci USA* 97:10972-10977.
41. Nilsen J, Chen S, Irwin RW, Iwamoto S, Brinton RD (2006) *BMC Neurosci* 7:74-87.
42. Zaremba J, Losy J (2001) *Acta Neurol Scand* 104:288-295.
43. Zaremba J, Skrobanski P, Losy J (2001) *Biomed Pharmacother* 55:258-263.
44. Intiso D, Zarrelli MM, Lagioia G, Rienzo F, Checchia De Ambrosio C, Simone P, Tonali P, Cioffi Dagger RP (2004) *Neurol Sci* 24:390-396.
45. Hallenbeck JM (2002) *Nat Med* 6:1363-1368.
46. St. Pierre BA, Merrill JE, Dopp JM (1996) in *Cytokines and the CNS*, eds Ransohoff RM, Benveniste EN (CRC Press, Boca Raton, FL), p 339.
47. Kim HM, Shin HY, Jeong HJ, An HJ, Kim NS, Chae HJ, Kim HR, Song HJ, Kim KY, Baek SH, et al. (2000) *J Mol Neurosci* 14:191-196.
48. Ringheim GE (1995) *Neurosci Lett* 201:131-134.
49. Liva SM, Vellis JD (2001) *Neurochem Res* 26:629-637.
50. Wilson ME, Dubal DB, Wise PM (2000) *Brain Res* 873:235-242.
51. Chen S, Nilsen J, Brinton RD (2006) *Endocrinology* 147:5303-5313.