6 Gender, Sex Steroids, and Cerebral Ischemic Pathobiology

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Abstract: Biological sex is an important genetic determinant of outcome from cerebral ischemia and clinical stroke. Emerging data suggest that sex, as well as reproductive steroids, shapes ischemic cell death in brain. Female sex steroids, the estrogens and progesterone, provide robust neuroprotection in a variety of experimental settings and strongly contribute to sex-specific responses to ischemia. The purpose of this chapter is: (1) to review the importance of biological sex to ischemic outcome and mechanisms of brain injury, (2) to evaluate the role of female sex steroids as endogenous or exogenous ischemic neuroprotectants, and (3) to review most likely mechanisms by which female sex steroids act to interrupt ischemic cell death pathways.

List of Abbreviations: AP1, activator protein 1; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; CREB, cAMP-responsive element-binding protein; E14, embryonic day 14; E2, estradiol; ER, estrogen receptors; αERKO, estrogen receptor deficient knockout mice subtype α; βERKO, estrogen receptor deficient knockout mice subtype β; ERE, estrogen-response elements; GAD, glutamic acid decarboxylase; i.p., intraperitoneal; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MAPK, mitogen-activated protein kinase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NMDA, *N*-methyl-*D* aspartate; NF-κB, nuclear factor-kappa B; OVX, ovariectomized; ERK1 and ERK2, p42/p44 extracellular signal-regulated kinases 1 and 2; ONOO, peroxynitrate; PARP-1, poly-ADP ribose polymerase; PR, progesterone receptor; PKC, protein kinase C; RSF, reproductively senescent female; SHRSP, spontaneously hypertensive stroke prone; SD, Sprague-Dawley; TBI, traumatic brain injury

1 Introduction

Stroke or brain attack is a sexually dimorphic disease. Women enjoy protection from stroke relative to men, and this sex difference in risk-of-stroke persists well beyond the menopause. In animal models, stroke sensitivity as measured by tissue damage resulting from an ischemic event is also sex specific. In part, these differences are due to cellular actions of female sex steroids: the principal mammalian estrogen, 17β-estradiol and the progesterone family (Hurn and Macrae, 2000; Hurn and Brass, 2003; Murphy et al., 2003, 2004). The purpose of this chapter is: (1) to review the importance of biological sex to ischemic outcome and mechanisms of brain injury, (2) to evaluate the role of female sex steroids as endogenous or exogenous ischemic neuroprotectants, and (3) to review most likely mechanisms by which female sex steroids act to interrupt ischemic cell death pathways. New data suggest that androgens also play a role in ischemic outcome, however this emerging area of research will not be reviewed here.

2 Biological Sex Differences in Response to CNS Injury

2.1 Clinical Stroke Incidence and Outcome in Men and Women

It is now well recognized that there are biological sex-linked patterns of cerebrovascular disease and stroke. Overall incidence of stroke is higher in men compared to women in all countries and across ethnic backgrounds (Sudlow and Warlow, 1997). This sexually dimorphic epidemiology remains apparent until ages well beyond the menopausal years (Giroud et al., 1991; Sacco et al., 1998). For example, in the Northern Manhattan Stroke Study, stroke rates in women do not equalize to those of men until beyond 75 years of age (Sacco et al., 1998). Nevertheless, stroke risk increases with age in both sexes, and there is some evidence that outcome from an ischemic event is worse in aged women than in their male counterparts (Bousser, 1999). Knowledge of mechanisms of ischemic cell death and neuroprotection is important for both sexes. However, as discussed below, tantalizing new evidence suggests that these mechanisms may not be identical in male and female animals.

2.2 Sex Differences in Ischemic Outcomes: Animal Models

Early evidence in female versus male spontaneously hypertensive, genetically stroke prone (SHRSP) rats emphasized that the male phenotype is "ischemia-sensitive." In a study of 2,000 animals, Yamori et al. (1976) showed that life expectancy is longer in the female SHRSP with delayed development of cerebral hemorrhage and vascular lesions. In adult animals, experimental outcome from adult brain injury is clearly sex linked. Female rats and mice of various inbred and outbred strains experience smaller tissue damage for an equivalent insult from focal or global cerebral ischemia (Hall et al., 1991; Alkayed et al., 1998; Carswell et al., 1999; Alkayed et al., 2000; McCullough et al., 2003) and improved functional outcome (Li et al., 2004). Similarly, male animals sustain greater injury than do age-matched females after tramatic brain injury (Bramlett and Dietrich, 2001). We have explored complicated rodent models with genetic risk factors associated with human stroke, e.g., insulin-dependent genetic diabetes (Toung et al., 2000), non-insulin-dependent diabetes (Vannucci et al., 2001), and hypertension (Alkayed et al., 1998). In each genetic strain and despite deleterious complications from diabetes or hypertension, females are less sensitive to cerebral ischemia than are males. These data suggest that male animals, unlike females, must cope with a basal "ischemia-sensitive" phenotype.

2.3 Sex-Specific Cultures: Hormone-Independent Cell Death or Survival

Data from cell cultures in which background sex steroids are removed support the concept that cell death mechanisms can be sex specific. Some molecular pathways of cell death or survival diverge, depending on the genetic sex of the tissue (defined as female XX or male XY). Sex alters cell fate. For example, cultured female dopaminergic neurons (embryonic day 14, E14) tolerate exposure to toxic dopamine concentrations at the LD50 level and survive twofold relative to male cells (Lieb et al., 1995). Similarly, female neurons (E19) from cortical plate or ventricular zone have greater longevity in culture than do male cells and differentially express higher levels of phosphorylated kinases such as Akt (Zhang et al., 2003). Sensitivity to glutamate, peroxynitrate (ONOO), and staurosporine in neuronal culture (E17) is sex specific, with male neurons being more susceptible to glutamate and ONOO than females. In contrast, response to oxidants such as H_2O_2 is gender neutral (Du et al., 2004). These observations are not limited to neurons. Cell death resulting from oxygen–glucose deprivation is less in female versus male astrocytes cultured from rat or mouse at postnatal day 3 (Liu et al., 2004).

2.4 Sex-Specific Cell Death Mechanism: NO Toxicity and Activation of Poly-ADP Ribose Polymerase

Data from genetically engineered mice also suggest that molecular mechanisms of cell injury are not necessarily identical in male and female brain. When both sexes are studied, ischemic outcome in transgenic mice can be overtly gender dependent, even when the gene of interest is not linked to sexual development, e.g., inducible or neuronal nitric oxide synthase (Loihl et al., 1999; Sampei et al., 2000b; McCullough et al., 2004). For example, it is well accepted that the neuronal nitric oxide synthase (nNOS) plays an important role in initiating ischemic cell death. In large part, nitric oxide cytotoxicity involves its rapid reaction with superoxide anion, resulting in peroxynitrite formation and protein nitration. Genetic deletion or pharmacological inhibition of nNOS is neuroprotective in male animals, presumably by reduction of available nitric oxide (NO). However, loss of nNOS in female knockouts or with enzyme inhibition paradoxically increases histological infarction after middle cerebral artery (MCA) occlusion (McCullough et al., 2004). Activation of poly-ADP ribose polymerase (PARP-1) is a critical component of neuronal cell death after exitotoxic or ischemic insults in male mice or mixed cell culture unseparated by genetic sex (Eliasson et al., 1997; Goto et al., 2002). Surprisingly, loss of PARP-1 activity in female knockouts or after PARP inhibition hugely exacerbates ischemic damage after MCA occlusion (McCullough et al., 2004). While it is not clear

how these cell death pathways diverge in the male and female, these data suggest that sex matters at the molecular level in ischemic brain injury.

3 Estrogen

Estrogen has been extensively studied in the last decade, in part, because of its potential role in protecting premenopausal women from cardiovascular disease and stroke (Hurn and Macrae, 2000; Hurn and Brass, 2003). Mammalian estrogens are naturally synthesized from testosterone by aromatization, a process that occurs not only in gonads, placenta, and fat but also in brain (Naftolin, 1994; Azcoitia et al., 2003). Estradiol (E2) is the most potent and abundant of the three major human estrogens: estrone, estriol, and E2. Accordingly, it has been studied comprehensively as a model hormone. There are two stereoisomers of E2, 17α and 17β, but only 17β-estradiol binds effectively to estrogen receptors (ER).

3.1 Estradiol Signaling

Classical E2 signaling is via binding to ERs, followed by transcriptional regulation of target genes. Recent studies have also identified nontranscriptional rapid signaling effects of E2, mainly phosphorylation and activation of proteinases and kinases (Kelly et al., 2003; Maggi et al., 2004), which affect ion currents (Lagrange et al., 1996; Mermelstein et al., 1996), sensitivity of neurotransmitter receptors (Weaver Jr et al., 1997; Disshon et al., 1998), and transcriptional regulation of genes that do not carry classic estrogen-response elements (ERE) (Webb et al., 1999). These effects have, at least in part, been attributed to E2 binding to a putative membrane-bound ER (Toran-Allerand et al., 2002).

3.1.1 ER-Mediated Gene Transcription

Many physiological effects of E2 are mediated through binding to ER proteins. Two receptor subtypes, termed ER-α and ER-β, have been identified. The receptors form homo- and heterodimers upon activation (Cowley et al., 1997), and the resulting complexes then bind to ERE within the promoter of target genes and alter rate of gene transcription. Recent data suggest that ER complexes also interact with other transcription factors, e.g., nuclear factor-kappa B (NF-κB) and activator protein 1 (AP1) (Paech et al., 1997; McKay and Cidlowski, 1999). In addition, E2-induced transcription can be influenced by cofactors and coactivators, e.g., androgen receptor associated protein ARA-70 (Yeh et al., 1998). EREs have been identified in genes implicated in normal brain function and pathology, e.g., choline acetyltransferase (Miller et al., 1999), α1a-adrenergic receptor (Lee et al., 1998), oxytocin (Adan et al., 1993) and its receptor (Bale and Dorsa, 1997), preproenkephalin (Zhu and Pfaff, 1995), somatostatin (Xu et al., 1998), galanin (Kofler et al., 1995), glial fibrillary acidic protein (Stone et al., 1998), brain-derived neurotrophic factor (Sohrabji et al., 1995), transforming growth factor-α (El Ashry et al., 1996), cyclin D1 (Sabbah et al., 1999), and bcl-2 (Teixeira et al., 1995).

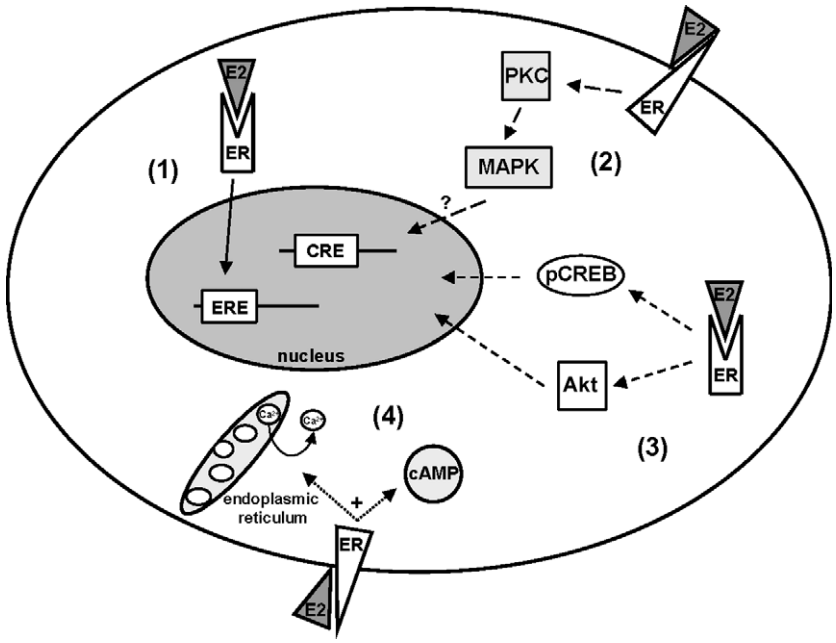
3.1.2 ER-Mediated Rapid Signaling

Recent studies have shown that E2 also elicits rapid intracellular signaling, independent of protein synthesis. These data suggest an additional action on yet unidentified membrane or cytoplasmic sites or with a putative membrane-bound ER (Toran-Allerand et al., 1999, 2002; Kelly et al., 2003). Rapid actions include modification of protein phosphorylation and levels of intracellular signaling molecules such as cyclic adenosine monophosphate (cAMP) (Minami et al., 1990) or calcium (Beyer and Raab, 1998). Activation of protein kinase C (PKC) (Ansonoff and Etgen, 1998), phosphorylation and activation of cAMP-responsive element-binding protein (CREB) (Zhou et al., 1996; Watters and Dorsa, 1998) and Akt (Singer et al., 1999),

and stimulation of the mitogen-activated protein kinase (MAPK) pathway (Singh et al., 1999) are additional important downstream factors involved in rapid signaling. Many of these effects may be caused by an interaction of E2 and ER with membrane and receptor-bound G-proteins (Mermelstein et al., 1996) (◉ *Figure 6-1*).

◘ Figure 6-1

Basic estrogen signaling pathways relevant for neuroprotection. (1) Classical gene transcription: 17β-estradiol (E2) binds to cytoplasmic estrogen receptor (ER) and increases gene transcription via estrogen response element (ERE). (2) Putative membrane regulated signaling: E2 activates protein kinase C (PKC) and mitogen activated protein kinase (MAPK) pathway after binding to a membrane-bound receptor. The current hypothesis is that this induces transcription. (3) Initiation of transcription through non-ERE transcription factors: E2 phosphorylates and activates Akt and cAMP-responsive element binding protein (CREB). (4) Intracellular increase in second messengers: E2 raises levels of second messengers cyclic adenosin monophosphate (cAMP) and calcium (Ca^{2+}). Current hypothesis is that this is a nontranscriptional mechanism. Any one of these pathways, or their combination, can eventually increase cell and tissue resistance toward ischemia



3.1.3 Non-receptor-Mediated Mechanisms

In vitro studies suggest that 17β-E2 or 17α-E2 can protect cells against a variety of stressful stimuli in ER deficient cell lines or in the presence of ER antagonists such as tamoxifen (Regan and Guo, 1997; Culmsee et al., 1999). Common stressors studied include hypoxia and glutamate or AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) toxicity. The data suggest that E2 has non-receptor-mediated effects in addition to the mechanisms outlined above. At least in part, these effects may be due to the hormone's antioxidative potential. Supraphysiologic concentrations of E2 (μM concentrations) can prevent intracellular peroxide accumulation and lipid peroxidation by inactivation of free radicals that could otherwise oxidize lipoproteins (Keller et al., 1997; Vedder et al., 1999). Both 17α-E2 and 17β-E2 possess antioxidant properties, emphasizing that the ER does not play a role (Behl and Manthey, 2000). E2 may also interact directly with neurotransmitter receptors, e.g., *N*-methyl-D-aspartate (NMDA) receptors to influence

neurotransmitter uptake or ion fluxes (Weaver et al., 1997). However, very high concentrations are required for potent antioxidation, so this mechanism may not be dominant under physiological conditions.

3.1.4 Dose Specificity

Experimental studies have used a large array of estrogen formulations and doses, not exclusively E2. Physiologic plasma levels of 17β-E2 range between 10 and 30 pg/ml in rat, for example, reaching peak levels of up to 140 pg/ml over the course of the estrous cycle (Butcher et al., 1974; Nequin et al., 1979). Chronic treatment of male or ovariectomized female animals with E2 doses that produce physiological plasma levels produces neuroprotection in a variety of animal models (Toung et al., 1998; Rusa et al., 1999; Alkayed et al., 2000). However, acute treatment strategies or treatment with supraphysiological doses (in some cases one or two orders of magnitude above the physiological range) do not always provide protection in vivo (Dubal et al., 1998; Rusa et al., 1999). The optimal dose or plasma level required for neuroprotection can differ, depending on species, injury model, duration, and timing of exposure. The requirement for physiological versus pharmacological concentrations/doses provides clues as to the dominant protective mechanism at work. For example, physiological levels are thought to act predominantly via ER signaling, whereas direct antioxidant actions would be predicted to result from supraphysiological hormone doses.

3.1.5 ERs in Brain

Not surprisingly, the highest levels of ER expression are in brain regions associated with reproductive function such as hypothalamic nuclei (Pfaff and Keiner, 1973). However, both ER-α and ER-β are also expressed at lower levels in brain regions relevant to cerebral ischemia, e.g., neocortex and hippocampus (Shughrue and Merchenthaler, 2000). At present, ER expression has been identified in a wide range of cell types under normal conditions. These include the cerebral vasculature, neurons, astrocytes, microglia, and oligodendrocytes (Azcoitia et al., 1999; Gudino-Cabrera and Nieto-Sampedro, 1999; Mor et al., 1999). Although sex differences have not been widely studied, ER mRNA is expressed to a higher extent in brains of adult females as compared to males (Shughrue et al., 1992). The importance of ERs, and of the specific ER subtype, for neuroprotective signaling remains unclear. Both ER-α and -β are differentially expressed in brain, although with some overlap (Li et al., 1997; Greco et al., 2001), suggesting that ER-mediated mechanisms of neuroprotection are possible. Current data using ER deficient knockout mice subtype α (αERKO) or subtype β (βERKO) do not fully clarify this issue. Enhanced damage after reversible MCA occlusion is not observed in gonadally intact αERKO mice of either sex relative to their respective wild type controls (Sampei et al., 2000a). In a permanent occlusion model, E2 replacement in ovariectomized αERKO females, but not βERKO females, is ineffective, suggesting that E2 signals through ER-α to reduce stroke damage (Dubal et al., 2001). Rapid E2 signaling involving phosphorylation of CREB and MAPK can be elicited in the absence of either one of the classical receptors, but not in double-knockouts (Abraham et al., 2004). The importance of ER-controlled mechanisms to neuroprotection requires further investigation.

3.2 Estradiol as a Neuroprotectant

E2's physiological signaling mechanisms have been well described, however, less is known in the context of cerebral ischemia. A large body of evidence demonstrates that the steroid protects cultured cells and brain tissue from ischemic damage. Details of these studies are summarized in ❯ *Tables 6-1* and ❯ *6-2* and discussed below.

Table 6-1

Representative in vitro studies of estradiol as a neuroprotectant

Tissue or cell type	Injury or stress	Key effects	Source
Cortical neurons	Glutamate (0.1 M)	Reduces cell death	Singer et al. (1996)
Hippocampal neurons	Glutamate (30 μM)	Reduces cell death	Weaver et al. (1997)
Rat mesencephalic neurons	Oxidative stress glutamate (1 mM), superoxide anion/H_2O_2	Reduces cell death, independent of ER, nongenomic mechanism	Sawada et al. (1998)
Dorsal root ganglion neurons	Growth-factor withdrawal	Increases cell survival; ER-dependent upregulation of bcl-*x*	Patrone et al. (1999)
Rat cortical neurons	Anoxia	Reduces cell death, ER independent	Zaulyanov et al. (1999)
Nigral neurons	Bleomycin sulfate or buthionine sulfoximine	Reduces apoptosis; ER dependent; independent of caspase-3 and JNK activation	Sawada et al. (2000)
Mouse dopaminergic neurons		Stimulates neurite outgrowth via CREB phosphorylation; ER independent	Beyer and Karolczak (2000)
Human neurons	Toxins: methamphetamine, cocaine and HIV protein	Reduces cell death; ER dependent	Turchan et al. (2001)
Purified neurons with ER-α versus astrocyte/neuronal coculture	Multiple insults	Protection only present in coculture system	Dhandapani and Brann (2003)
HT-22 cells	Iron-induced lipid peroxidation	Reduces peroxidation and increases cell survival	Vedder et al. (1999)
Murine hippocampal HT-22 cells	Oxidative stress	17β-estradiol and enantiomer ENT-E(2) reduce cell death	Green et al. (2001)
Neuroblastoma SK-ER3 cells		Increases expression of antiapoptotic nip-2; reduces apoptotic cell death; ER dependent	Meda et al. (2000)
PC-12 cells	Oxidized low density lipoprotein	17β, but not 17α-estradiol, reduces death	Berco and Bhavnani (2001)
Hypothalamic cell line		Promotes cell survival, ER-α required; Fas and FasL signaling required	Nilsen et al. (2000)
Slice, mouse hippocampus	Deafferentation of the entorhinal cortex	ER-dependent increase in sprouting of mossy fibers	Teter et al. (1999)
Slice, rat cortex	Kainic acid or potassium cyanide	Reduces cell death only at physiological levels (1–30 nM); protection lost at higher doses	Wilson et al. (2000)
Slice, rat hippocampus	Glutamate (1 mM)	Increased expression of CA3 NMDA receptor, associated with increased cell death	Sato et al. (2002)

Table 6-2
Representative in vivo studies of estradiol as a neuroprotectant

Ischemia model	Animals studied	Treatment	Follow-up	Key effects	Source
Focal (middle cerebral artery occlusion, MCAO), transient	Ovariectomized (OVX) female Sprague-Dawley (SD) rats	1-mg/kg 17β-E2 at reperfusion	23 h	Reduced infarct size	Zhang et al. (1998)
	OVX SD	E2 implant 24 h before MCAO	6 h, 24 h, 1 week	Reduced infarct size; reduced mortality	Simpkins et al. (1997)
	OVX SD	100-μg/kg E2 s.c., 2 h before MCAO	24 h	Reduced infarct size	Fan et al. (2003)
	Male Wistar	1-mg/kg Premarin i.v. at reperfusion	22 h	Reduced infarct size	McCullough et al. (2001)
	Male Wistar, intact or castrated	E2 25- or100-μg implants 1 week, or Premarin 1 mg/kg i.v. 30 min before MCAO	22 h	Reduced infarct size	Toung et al. (1998)
	OVX Wistar	E2 25- or 100-μg implants 1 week, or Premarin 1 mg/kg i.v. at MCAO	22 h	Reduced infarct size with chronic, no effect with acute treatment	Rusa et al. (1999)
	OVX Wistar	0.1- or 1-mg/kg E2 i.v. 30 min before MCAO or 20- or 200 μg s.c. for 1 week	72 h	No effect	Vergouwen et al. (2000)
	Reproductively senescent female Wistar rats	25-μg E2 implant 1 week before MCAO	22 h	Reduced infarct size	Alkayed et al. (2000)
	Male diabetic rats	25-μg E2 implant 1 week before MCAO	22 h	Reduced infarct size	Toung et al. (2000)
	Male rats, intact or castrated	E2 implant 1 week before MCAO	23 h	Reduced infarct size	Hawk et al. (1998)
	Male mice and female OVX mice	E2 implants 180 μg/ml	2 week for histology	Improved motor-function; reduced atrophy in males only	Li et al. (2004)
Focal (MCAO), permanent	Male SD	E2 local 10/30 min before or 10-μg/kg E2 i.v. 30 min before/ 30 min after MCAO	4 h	Reduced infarct size	Saleh et al. (2001a, 2001b)

	Male mice	0.3–30 μg/kg E2 s.c. or i.p. 24 or 3 h before MCAO	2 day	Reduced infarct size	Culmsee et al. (1999)
	OVX SD, young and middle aged	1-mg/ml E2 implant 1 week before ischemia	24 h	Reduced infarct size	Wise and Dubal (2000)
	OVX SD, young or middle aged (3–4 or 9–12 months)	180- or 1000-μg/ml E2 implant 1 week before MCAO	24 h	Reduced infarct size	Dubal and Wise (2001)
	OVX spontaneously hypertensive rats	200-μg/kg/week estradiol valerate s.c., 3 week	3 day	Reduced infarct size	Fukuda et al. (2000)
	OVX SD	180- or 1000-μg/ml E2 implant 1 week before/at MCAO	24 h	Reduced infarct size with chronic, no effect with acute E2	Dubal et al. (1998)
Global (4 vessel occlusion)	OVX SD	E2 s.c. 24 h before ischemia	96 h	Reduced cell loss	He et al. (2002)
	OVX Wistar	25-μg E2 implant 1 week before ischemia	1 week	No protective effect	Harukuni et al. (2001)
Global (right carotid artery occlusion plus hypotension)	OVX SD	0.2-mg/kg/day E2 i.p., 2 week before ischemia	72 h	Reduced cell loss	Wang et al. (1999)
	OVX SD	0.1-, 0.5-, or 5-mg/kg/day E2 i.p., 2 week	72 h	Reduced cell loss at low doses	Pelligrino et al. (1998)
	Diabetic OVX SD	0.1-mg/kg/day E2 i.p., 1week	72 h	Increased cell loss, reduced function	Santizo et al. (2002)
Global (bilateral carotid artery occlusion)	OVX C57BL/6J mice	25-μg E2 s.c. 2 week before ischemia	72 h	Reduced cell loss	Horsburgh et al. (2002)
	OVX C57BL/6J mice	ER-α agonist PPT 2 mg/kg/day or ER-β agonist DPN (8 mg/kg/day) s.c., 1 week before ischemia	72 h	Reduced cell loss with ER-β, but not ER-α agonist	Carswell et al. (2004)
	Male gerbils	0.36-mg E2 s.c. 2 week before ischemia	7 day	Reduced cell loss	Jover et al. (2002)
	Male gerbils	3-, 10-, or 30-μg E2 i.c.v. or 4 mg/kg i.p. 1 h before ischemia	7 day	Reduced cell loss with higher doses	Chen et al. (1998, 2001)
	Male gerbils	0.05 or 0.025 μg/d E2 icv from 2 h before ischemia	7 d	Reduced cell loss	Sudo et al. (1997)

3.2.1 Effects of E2 In Vitro

Results from in vitro studies of experimental cell damage, e.g., excitotoxicity or oxidative stress, largely demonstrate protective effects. E2 is neurotrophic at physiologic concentrations (1–30 nM), stimulating neurite outgrowth and increasing survival of cultured neurons (Beyer and Karolczak, 2000; Meda et al., 2000). The presence of E2 in the media broadly protects primary cultures and neuronal cell lines against noxious stimuli, including hypoxia, glutamate or NMDA toxicity, oxidants, and many toxins. However, concentration–response relationships are complex and difficult to interpret among the studies. At supra-physiological concentrations, protection is ER independent and largely attributable to the hormone's antioxidative potency. Alternatively, ER-dependent regulation of apoptosis inhibiting genes is uncovered in the presence of physiologic E2 concentrations (Patrone et al., 1999; Meda et al., 2000). E2-mediated rapid signaling at physiologic concentrations contributes to altered gene expression (Beyer and Karolczak, 2000). It seems likely that E2 acts by different mechanisms in specific cell types, and accordingly, results in organotypic slices are less consistent than culture. In slice preparations, physiological E2 concentrations can reduce (Wilson et al., 2000) or exacerbate (Sato et al., 2002) cell death.

3.2.2 Effects of E2 In Vivo

Despite the complexity of E2's actions, most animal studies report profound neuroprotection. Chronic E2 replacement to physiologically relevant plasma levels reduces infarct size after transient and permanent focal ischemia in ovariectomized female rodents (see ❯ *Table 6-2* for representative studies). This effect is also seen in male animals and in complex disease strains like the SHRSP rat. Effects of high E2 doses are less clear, whether given as an acute treatment before or after focal cerebral ischemia (Dubal et al., 1998; Rusa et al., 1999). Similarly, studies of E2 in global cerebral ischemia demonstrate robust neuroprotection. Harukuni et al. (2001) observed surprisingly increased cell death in E2-treated, ovariectomized rats after four-vessel occlusion. However, E2 levels in treated animals were low in this study, suggesting that higher levels of circulating estrogen might be necessary for neuroprotection in this model. Effects of E2 on vascular behavior during the insult may be important as E2 can increase intraocclusion cerebral blood flow (He et al., 2002). However, there seems to be a ceiling for this effect (Pelligrino et al., 1998). Coexisting pathologic conditions, such as diabetes, in some settings can enhance undesirable actions of E2, exacerbating ischemic damage (Santizo et al., 2002).

To date, despite overall encouraging data, transfer of the positive experimental results to the clinic is hampered by study limitations: (1) experimental studies have mainly been performed in small rodents and are needed in higher order species; (2) most studies have focused on short-term histological outcomes and only few data are available for neurological/behavioral outcome parameters; (3) precise dose and duration data for any one model are limited.

3.2.3 Effects on Peri-Ischemic Cerebral Blood Flow

In vivo neuroprotection is, in part, related to E2's vadodilatory actions and the steroid's ability to promote optimal endothelial function (Mendelsohn and Karas, 1994; White et al., 1995). Under basal conditions, cerebral blood flow is higher in premenopausal women than in men or in older women (Davis et al., 1983; Shaw et al., 1984), suggesting a tonic role in the vasculature. Estrogen can also influence blood flow during ischemic stress and during reperfusion (Hurn et al., 1995; Alkayed et al., 1998; Pelligrino et al., 1998).

3.2.4 Effects on Postischemic Inflammation

Recent studies suggest that E2 exerts an anti-inflammatory effect after cerebral ischemia, by reducing the number of active microglia (Lei et al., 2003), by decreasing intravascular leukocyte adhesion and

migration into brain (Santizo et al., 2000), and by suppressing endothelial expression of adhesion molecules (Nathan et al., 1999; Mori et al., 2004). Moreover, E2 can suppress reactive gliosis and expression of the inflammation promoting transcription factor NF-κB after cerebral injury (Wen et al., 2004). Taken together, it appears that E2 can restrict cellular damage in the proinflammatory mileau of ischemia/reperfusion.

3.3 Exemplary Cellular Mechanisms of Protection

Significant work is now focused primarily on identification of genomic and nongenomic mechanisms that explain E2's neuroprotective properties in vitro and in vivo. One known genomic mechanism is through elaboration of antiapoptotic protooncogene bcl-2. Bcl-2 is well established for protective effects in brain injury of all types (for review, see Graham et al., 2000; Mattson et al., 2000). Under basal conditions and in the setting of ischemia, E2 enhances bcl-2 expression, presumably by transcriptional actions through one or more EREs with the bcl-2 promoter (Teixeira et al., 1995). E2 upregulates bcl-2 expression in several brain regions of cycling female rats (Garcia-Segura et al., 1998). E2 increases the expression of bcl-2 mRNA and protein in the peri-infarct region after transient MCA occlusion in rats, accompanied by a decrease in infarct size (Alkayed et al., 1998; Dubal et al., 1999; Alkayed et al., 2001). Moreover, bcl-2 overexpressing mice are protected from the exacerbation of cerebral ischemic injury ordinarily caused by ovariectomy in wild-type female mice (Alkayed et al., 2001).

Nongenomic signaling may also contribute significantly to E2-conferred resistance to insult and enhanced survival of neuronal cells. For example, rapid activation of the MAPK pathway has been implicated in several paradigms of cell death in vitro. E2 elicits rapid tyrosine phosphorylation and activation of MAP kinases ERK1 and ERK2 (p42/p44 extracellular signal-regulated kinases 1 and 2) in ER expressing cells. Subsequently, primary cortical or hippocampal neuronal cultures are protected from glutamate (Singer et al., 1999; Mize et al., 2003) as well as β-amyloid toxicity (Fitzpatrick et al., 2002). Additional studies are needed to completely understand the relevance of this vital signaling pathway in vivo.

Nonspecific mechanisms of protection are likely dominant at supraphysiologic levels of hormone exposure. High-dose E2 protects murine cortical cultures during oxidative stress from hemoglobin or sodium azide, and protection is independent of either the ER or of protein synthesis (Regan and Guo, 1997). Furthermore, E2 in large doses (0.3–30 mg/kg) can reduce infarct size in mice in an ER-independent fashion that is not blocked by tamoxifen (Culmsee et al., 1999). Protective effects of high-dose E2 may have clinical relevance for short-term treatment of patients after ischemic events who could benefit the plethora of receptor-mediated plus non-receptor-mediated hormonal effects. However, due to adverse effects of chronic exposure to large E2 doses, this approach has little relevance for hormone-replacement therapy.

3.4 Summary

Estrogen is a powerful neuroprotective agent in a variety of experimental models of cerebral ischemia. Protection is conveyed both by genomic and nongenomic mechanisms leading to increased cell survival after ischemia (❯ *Figure 6-2*, ❯ *Table 6-3*). Whereas the experimental data are clear, the translation to humans is less certain at present. Despite repeated observational studies that estrogen/progestin therapy reduces cardiovascular disease and stroke risk in women, new prospective and randomized trials (e.g., Women's Health Initiative) do not support the long-term use of hormone replacement therapy. There are no trials to date that test the use of E2 or estrogen formulations in acute stroke therapy to ameliorate injury. We can extrapolate from the experimental data that the extent of protection E2 provides will be related to dose and cell specificity, as well as the presence of confounding diseases or ER polymorphisms. Future research should focus on unraveling the molecular mechanisms conveying estrogen's protective effects. In addition, the exact contribution of progesterone requires study if we are to understand hormone replacement therapy and stroke.

Figure 6-2
Estrogen's potential mechanisms of neuroprotection

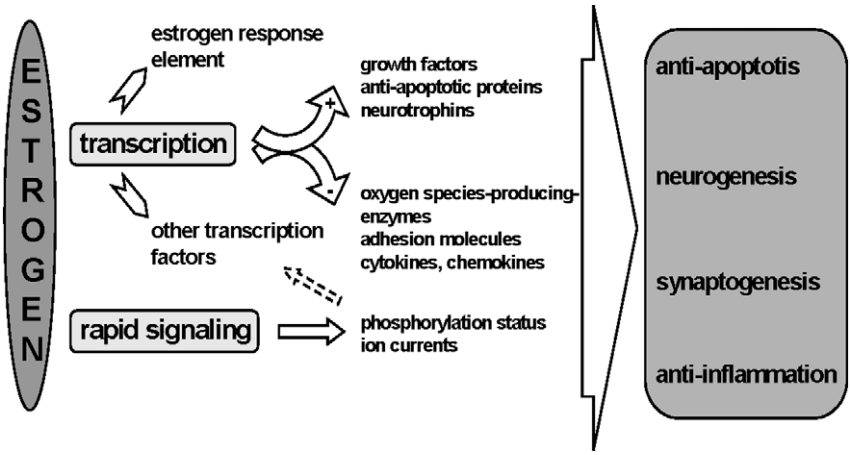


Table 6-3
Summary of potential estrogenic mechanisms of neuroprotection

Protective mechanism	References
Preservation of intraischemic blood flow	Alkayed et al. (2000)
Improvement of postischemic reperfusion	McCullough et al. (2001)
Antioxidant activity	Regan and Guo (1997)
Amelioration of excitotoxicity	Singer et al. (1999)
Upregulation of bcl-2 expression in injured area	Dubal et al. (1999), Alkayed et al. (2001)
Increase of neurite outgrowth, spine density, and cell viability in culture	Brinton et al. (1997), Murphy et al. (1998)
Increase of neurotrophic factors	Toran-Allerand (1996)
Reduction of leukocyte adhesion after transient global ischemia	Santizo et al. (2000)
Supression of microglial activation	Bruce-Keller et al. (2000)
Reduction of reactive gliosis	Garcia-Segura et al. (2001)
Increase of neuronal stem cell proliferation	Tanapat et al. (1999)

4 Progesterone and Neurosteroids

Progesterone, precursors, and metabolites can be generated locally from cholesterol in the brain as neurosteroids; formed from in situ metabolism of bloodborne precursors; or synthesized by the adrenal glands, placenta, or gonads. Pregnenolone is the immediate precursor of progesterone. Progesterone itself is partly transformed into 5α-pregnane-3, 20-dione (5α-dihydroprogesterone) and 3α-hydroxy-5α-pregnan-20-one (3α,5α-tetrahydroprogesterone or allopregnanolone). In the rat, plasma progesterone can range from basal levels of 2–18 ng/ml to approximately 120–130 ng/ml in pregnancy, with intermediate values of 40–90 ng/ml during late proestrus (Wiest, 1970; Butcher et al., 1974; Sutter-Dub et al., 1974; Nequin et al., 1979). The effects of these neurosteroids in brain range from neuroendocrine control of reproduction and sexual behavior, regulation of neurotrophicity, and modulation of such behaviors as anxiety, stress, sleep, and memory.

Neurosteroids exert their actions on target brain cells through genomic and nongenomic actions. Progesterone classically binds to a selective intracellular receptor. There are two forms of the progesterone

receptor (PR), a large molecular form B and a smaller form A. Once activated, the PR interacts with a specific progesterone-response element within the promoter region of target genes. Other transcription factors, e.g., c-Jun or c-Fos, can also modulate PR transcriptional activity (Herrlich and Ponta, 1994). The PR appears in rat brain close to birth and increases during development in a region-specific manner (Kato et al., 1984). PR has been identified in neurons of the hypothalamus, cortex, amygdala, and cerebellum as well as in glial cells (Jung-Testas et al., 1984). Nongenomic mechanisms of action of progesterone and related neurosteroid metabolites in brain include: (1) intercalation into phospholipid bilayers; (2) binding to specific membrane receptors; (3) modulation of ion channels or ionic ATPase enzymes; (4) interactions with ligand-gated ion channels such as the $GABA_A$ and NMDA receptors; and (5) associations with G-protein–coupled receptors for neurotransmitters like the sigma or oxytocin receptors (for review, see El-Etr et al., 2000).

4.1 Progesterone and Outcome from Cerebral Ischemia

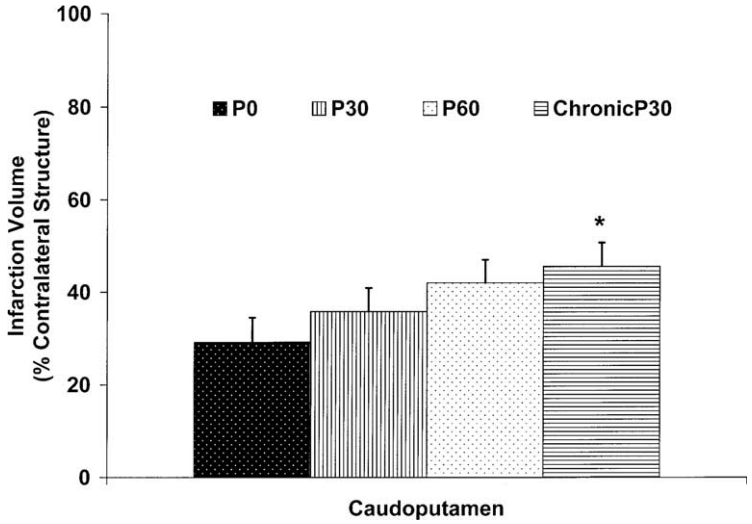
The effects of exogenous progesterone have been studied in rodent models and in higher order gyrencephalic animals such as cats. In transient focal and global cerebral ischemia models (*Table 6-4*), progesterone is neuroprotective in progesterone-deficient rodents [males, OVX females, and reproductively senescent females (RSF)]. Chronic progesterone administered before transient focal ischemia in OVX rats

Table 6-4
Exogenous progesterone in cerebral ischemia: animal studies

Gender/species	Effect on ischemic injury	Proposed mechanism	References
Male C57BL/6 mice	Reduced lesion volume, transient focal model	None tested	Gibson and Murphy (2004)
Male Sprague-Dawley rats	Reduced edema, permanent focal model	Not due to reduced blood–brain barrier permeability	Betz and Coester (1990a, 1990b)
Male Wistar rats	Reduced histological damage and improved neurological deficits, transient focal model	None tested	Jiang et al. (1996)
Male Wistar rats	Reduced infarction volume and improved functional outcome, transient focal model	None tested	Chen et al. (1999)
Male spontaneously hypertensive rats	Reduced lesion size and neurological deficits, transient focal model	None tested	Kumon et al. (2000)
Female OVX Wistar rats	Chronic preischemic treatment increased striatal infarction, transient focal model	None tested	Murphy et al. (2000)
Female OVX Wistar rats	Peri-ischemic treatment reduced cortical injury, transient focal model	Blood flow independent mechanism	Murphy et al. (2002)
RSF Wistar rats	Reduced cortical infarction, transient focal model	Blood flow independent mechanism	Alkayed et al. (2000)
RSF Wistar rats	No effect, transient focal model	None tested	Toung et al. (2004)
Female OVX cats	Reduced cell loss, transient global model	None tested	Gonzalez-Vidal et al. (1998), Cervantes et al. (2002)

Figure 6-3

Caudate-putamen infarction volume (% contralateral) at 22 h of reperfusion following 2-h middle cerebral artery (MCA) occlusion in ovariectomized female Wistar rats. Animals were treated with intraperitoneal injections: 0 (P0, $n = 14$), 30 (P30, $n = 14$), or 60 (P60, $n = 12$) mg/kg progesterone 30 min before ischemia or with 30-mg/kg progesterone (Chronic P30, $n = 16$) daily for 7–10 days before ischemia. *Significantly different from P0 ($p < 0.05$); values are means + SEM. Adapted from Murphy et al. (2000)



exacerbates caudoputamen infarction (*Figure 6-3*), but no mechanism of injury was tested in this study (Murphy et al., 2000). Because there are few studies of long-term progesterone exposure, the treatment duration requirement is not clear. Effective dose and duration may differ between sexes at various ages, suggesting that neuroprotective mechanisms are not necessarily identical in male and female animals throughout maturity. No studies are available to clarify effects of progesterone precursors and metabolites on ischemic outcome. However, in a rodent model of penetrating brain injury, pregnenolone and pregnenolone sulfate inhibits gliotic tissue formation (Garcia-Estrada et al., 1999). In traumatic brain injury models, allopregnanolone enhances behavioral recovery and decreases neuronal loss (Djebaili et al., 2004; He et al., 2004).

4.2 Comparison to Estrogen as a Neuroprotectant

Clinical and experimental inquiries have focused heavily on estrogen as a preventative agent in injured brain, but progesterone in some injury models has shown promise as a postinjury therapy in both males and females (for review, see Stein et al., 1999; Roof and Hall, 2000; Stein, 2001; Vink et al., 2001; Stein and Hoffman, 2003). Progesterone is a more attractive therapy for men, as chronic estrogen administration presents undesirable side effects and consequences. The use of estrogen alone (unopposed estrogen) in women is also problematic due to increased risk of uterine cancer (Grady et al., 1995). Therefore, estrogen is typically administered in combination with a progestin. Accordingly, progesterone may be more desirable in future clinical applications than estrogen.

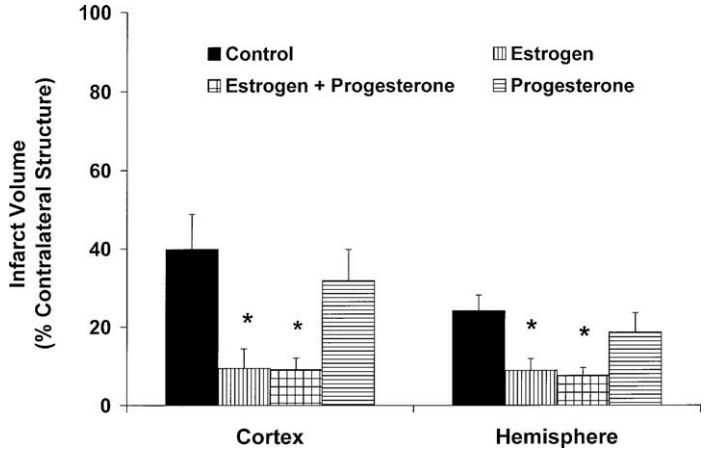
4.3 Combined Hormone Treatments and Outcome in Brain Injury Models

The emphasis on individual female sex steroids in experimental studies makes it difficult to evaluate interactive roles of estrogen and progestins. A single study has examined combined hormone administration

in cerebral ischemia (Toung et al., 2004). In this study, combined treatment in RSF rats was evaluated after reversible MCA occlusion. Subcutaneous estradiol initiated 7 or more days prior to occlusion combined with intraperitoneal (i.p.) progesterone injections at 30 min preischemia and through early reperfusion were compared to each steroid administered alone. Cortical, caudoputamen, and total infarct volumes were assessed by 2,3,5-triphenytetrazolium chloride staining and digital image analysis at 22 h reperfusion. Combined hormone administration and chronically administered 17β-estradiol alone reduced cortical and total brain ischemic injury (*Figure 6-4*) while progesterone alone did not reduce injury. These results suggest that in ischemic RSF rat brain, combined hormone administration reduces infarction volume and that progesterone does not attenuate estrogen's effect.

Figure 6-4

Cortical and total hemispheric infarction volume (% contralateral structure) at 22 h of reperfusion following 2-h MCA occlusion in reproductively senescent female (RSF) rats ($n = 10$/group). Animals were treated with no hormone (control), 25-μg estrogen subcutaneous implant (estrogen), 25-μg estrogen implant + 5 mg/kg progesterone i.p. (estrogen + progesterone), or 5-mg/kg progesterone i.p. (progesterone). All hormone implants were placed 7 days prior to MCA occlusion, and progesterone injections were given 30 min before occlusion, at initiation of reperfusion and at 6 h of reperfusion. *$p < 0.05$B compared to control; values are means + SEM. Adapted from Toung et al. (2004)



4.4 Mechanisms of Neuroprotection

While mechanisms of ischemic neuroprotection have not been evaluated, studies of traumatic brain injury (TBI) suggest potential protective mechanisms. For example, progesterone decreases cerebral edema (Roof et al., 1993; Roof et al., 1996; Roof et al., 1997; Galani et al., 2001; Wright et al., 2001; Grossman et al., 2004) in TBI models. Effects on cerebral edema may be partly mediated by progesterone's antilipid peroxidation capacity (Roof et al., 1997; Stein et al., 1999; Roof and Hall, 2000). Progesterone and allopregnanolone lower caspase-3 activity in TBI (Djebaili et al., 2004), suggesting these neurosteroids can decrease cell loss by decreasing caspase-3–dependent apoptotic mechanisms in this setting. Anti-inflammatory mechanisms are also important (Arvin et al., 1996; Garcia-Estrada et al., 1999; Grossman et al., 2004). Progesterone has direct effects on GABA neurotransmission by altering GABA conduction (Majewska, 1992), enhancing $GABA_A$ receptor numbers (Weiland and Orchinik, 1995), increasing $GABA_A$ agonist binding affinity (Jussofie et al., 1995) and number of binding sites (Juptner et al., 1991), and by enhancing glutamic acid decarboxylase (GAD) mRNA (Weiland, 1992; Grattan et al., 1996). Progesterone moderates neuronal responsivity to excitatory amino acids (Smith et al., 1987; Smith, 1991), thus ameliorating excitotoxic injury.

PRs have been implicated in vascular injury mechanisms. In carotid artery injury, OVX progesterone receptor knockout mice sustain more pronounced vascular injury than wild-type animals, independently of exogenous progesterone availability (Karas et al., 2001). The study suggested that PRs regulate vascular injury response in a complex manner and can augment or attenuate the degree of injury. In brain, progesterone's neuroprotection may be influenced by regional and temporal expression of PR subunits/subtypes. Finally, progesterone's actions in injured brain may be mediated, in part, by locally produced metabolites such as 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone (allopregnenolone) (di Michele et al., 2000). In TBI in vivo (Djebaili et al., 2004; He et al., 2004) and excitotoxic injury in vitro (Lockhart et al., 2002; Ciriza et al., 2004), allopregnanolone provides neuroprotection similarly to progesterone.

4.5 Summary

Progesterone, its precursors, and its metabolites are produced endogenously within brain. These neurosteroids act by either altering specific gene transcription after binding to PRs or acting directly on the neuronal or glial plasma membrane. In the experimental setting, progesterone has an overall neuroprotective effect. However, the optimal dose and duration may differ between sexes at various ages, suggesting that steroid mechanisms are not necessarily identical in males and females as these animals mature. Little research has been done on combined estrogen and progesterone treatment in in vivo brain injury models, but one study suggests that combined hormone administration reduces infarcts and that progesterone does not attenuate estrogen's effect.

5 Conclusions and Emerging Hypotheses

Clinical and experimental evidence emphasize that many forms of brain injury are sexually dimorphic. Females are less vulnerable to cerebral ischemia, while the male phenotype is "ischemia sensitive." Novel evidence suggests that this dimorphism is present not only in the intact brain but also shapes the behavior of cultured XX versus XY cells. These differences may not be solely under hormonal control. Nevertheless, female sex steroids at physiological concentrations likely play a role in the female's endogenous neuroprotection. Both estrogen and progesterone can improve outcome from experimental brain injury. However, these pleiotrophic hormones have diverse cellular targets and the potential for competing mechanisms of action, leading to a "good, bad or null" profile. Future studies will harness the potential of these steroids as we demystify their mechanisms in brain protection.

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