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Review Article

17β-Estradiol and Inflammation: Implications for Ischemic Stroke

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ABSTRACT: Although typically associated with maintenance of female reproductive function, estrogens mediate physiological processes in nearly every body tissue, including the central nervous system. Numerous pre-clinical studies have shown that estrogen, specifically 17-beta-estradiol (17β-E2), protects the brain from ischemic injury following stroke. There are multiple mechanisms of 17β-E2's neuroprotection, including activation of several neuroprotective pathways in the brain, but 17β-E2 also mediates the local and systemic immune response to ischemic stroke. This review summarizes the immune response to stroke, sex differences in stroke pathophysiology, and the role of estrogen as an immunomodulator. This review will focus almost entirely on the role of 17β-E2; however, there will be a brief review and comparison to other forms of estrogen. Understanding the immunomodulatory action of estrogens may provide an opportunity for the use of estrogens in treatment of stroke and other inflammatory disease.

Key words: estrogen, stroke, ischemia, inflammation, immune response, neuroprotection

Stroke and the Immune Response

Ischemic stroke is characterized by an abrupt deprivation of blood flow, oxygen, and nutrients to the brain that quickly leads to cell death in the core of the ischemic brain region (1). Initiation of the ischemic cascade begins within minutes of ischemia onset and involves several events, such as increased oxidative stress and mitochondrial dysfunction, resulting in apoptosis and a progressive loss of cells in the penumbra that may continue hours and days after stroke (2). The ischemic cascade also includes a robust inflammatory response triggered by damage-associated molecular patterns (DAMPs) released from dying cells (3). DAMPs trigger activation of local microglia and promote recruitment of local and circulating leukocytes. This milieu of immune cells secrete pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα, that act locally in the brain or enter the systemic blood circulation to trigger a systemic immune response (4). A balanced immune response is essential for tissue repair and functional recovery after stroke. Systemic inflammation has also been associated with an increased risk of ischemic stroke, with nearly 30 percent of strokes occurring in patients with a current or recent infection (5). This pro-thrombotic state leads to an increased production of vascular adhesion molecules, such as the selectin family proteins and ICAM-1, and subsequent accumulation of cells along endothelial cells and vessel blockage (6). Conversely, some experimental studies suggest that infections or immune stimulation may

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actually have a protective role following stroke by increasing neurogenesis, reducing the strength of the inflammatory response, and producing free radical scavengers; however, the majority of evidence suggests that inflammation increases stroke risk and contributes to ischemic lesion progression, and thus, immune cells and cytokines may be therapeutic targets for stroke prevention and treatment.

Sex differences in Ischemic Stroke Incidence and Severity

Although lifetime risk of stroke is higher for men than for women, women tend to have more severe strokes, more stroke deaths, and increased post-stroke functional deficits than men (7). From ages 19-30, and again from ages 45 to 54, women have an increased stroke risk compared to men. One plausible explanation for the increased risk of stroke during these time periods is alterations in estrogen status. The first increase in stroke incidence is likely due to a high rate of childbirth between the ages of 19-30. During pregnancy, maternal estrogen levels rise due to an increased estrogen production by the placenta. Following childbirth, estrogen levels decrease rapidly, while still remaining elevated compared to pre-pregnancy levels. These rapid decreases and changes in estrogen levels result in many health issues, including an increased risk of ischemic stroke. The second increase in stroke incidence is likely due to the menopausal transition. Typically occurring near age 50, menopause is the cessation of reproductive fertility in women due to a decreased production of circulating sex hormones, such as estrogen and progesterone (6). This transition into reproductive senescence and long-term estrogen deprivation is accompanied by symptoms that diminish a woman's quality of life, including hot flashes, night sweats, insomnia, weight gain, and osteoporosis. Women who undergo menopause before age 42 have a doubled lifetime risk of stroke compared to women undergoing menopause after 51 years of age (8). Evidence also suggests that a decreased length of time between menarche and menopause, or overall estradiol exposure, increases the risk of ischemic stroke (9). Sex differences in stroke indicate a role of estrogen as a mediator of ischemic stroke incidence, and as such, estrogens may also serve as a therapeutic intervention in ischemic stroke.

Estrogen Structure and Function

Estrogens are lipophilic steroid hormones classically associated with regulating female reproductive function. Estrone (E1), estradiol (E2), and estriol (E3) are the three forms of estrogen synthesized from cholesterol by the ovaries, and to a lesser extent, the adrenal glands (10). However, the role of estrogens is not limited to maintenance of female reproductive function. Estrogens are distributed via the blood to a variety of tissues, including the cardiovascular, immune, and central nervous systems (11), and due to their lipophilic nature, estrogens can easily diffuse across cellular membranes, as well as the blood-brain barrier, to elicit their effects.

Estrogens can elicit their actions through interaction with an estrogen receptor (ER). Currently, there are three known subtypes of ERs: ERα, ERβ, and G-protein coupled receptor 30 (GPR30/GPER), and all three ERs are distributed in the brain (10). There are two ligand-dependent ER signaling pathways: the genomic (classical) pathway and the non-genomic pathway (12,13). In the genomic pathway, both ERα and ERβ serve as ligand-activated transcription factors. Once an ER binds estrogen, the active ER can form a homodimer (ERα/ERα or ERβ/ERβ) or heterodimer (ERα/ERβ) and translocate into the nucleus. The ligand-bound ER dimer can bind to estrogen response elements (ERE) in the promoter sequence of target genes, and once bound, the ligand-bound ER can recruit transcription factors or other co-regulatory proteins to the promoter. The pool of co-regulator proteins and transcription factors present in a cell dictate the specific genomic action of the ligand-bound ER. In addition to the genomic pathway, estrogens can signal through a non-genomic mechanism that occurs within seconds to minutes. A ligand-bound ER dimer can remain in the cytoplasm (ERα or ERβ) or at the plasma membrane (GPR30) and function as a signaling molecule through activation of protein kinases and phosphatases.

Estrogen is Neuroprotective in Ischemic Stroke

17-beta-estradiol (17β-E2), an isomer of estradiol with a hydroxyl group at the 3-beta and 17-beta position, is the most potent estrogen in circulation (10). 17β-E2 has been shown to be neuroprotective in-vivo using models of focal cerebral ischemia. Neuroprotection has been demonstrated in ovariectomized female mice, rodents, and gerbils (14-19), in both young and middle-aged animals (20). Ovariectomized female animals are the most widely-used model of a post-menopausal woman. Removing the ovaries terminates ovarian production of estrogen, similar to the diminished estrogen production seen in post-menopausal women. This model also allows researchers to remove endogenous estrogen to control the concentration of estrogen in the blood that is resupplied to evaluate estrogen's therapeutic potential. Gibson et al. (2006) compiled an extensive review of all experimental studies to date that have examined the effects of estrogen treatment on cerebral ischemia. Using a simplistic definition, pre-treatment with estrogen can be viewed as prevention of stroke, while post-treatment may be

considered a potential treatment for ischemic stroke. Overall, estrogens reduced lesion volume in a dose-dependent manner when administered up to a week before or up to four hours after transient or permanent cerebral ischemia (21).

The Women's Health Initiative

Despite the prevailing amount of experimental evidence supporting the neuroprotective role of estrogens, data from clinical trials does not agree. Also, while most experimental studies have demonstrated that both pre- and post-treatment with17β-E2 reduces lesion size and infarct volume in models of focal cerebral ischemia, there have been a limited number of studies that suggest no effect or a more negative outcome as a result of 17β-E2 treatment (22, 23).

The Women's Health Initiative (WHI) began in 1993 and consisted of two double blind, placebo controlled clinical trials to determine whether estrogen alone or estrogen given with progestin would reduce the number of cardiovascular events in post-menopausal women. The estrogen-alone trial enrolled 10,739 post-menopausal women between 50 and 79 years of age with prior hysterectomy. Of the total number of participants, 5,310 individuals received conjugated equine estrogen (CEE) (Premarin®, Wyeth), and 5,429 individuals received an equal dose of placebo. The WHI estrogen-alone trial was scheduled to end between October 2004 and March 2005; however, the study was terminated in February 2004 when CEE did not affect the risk of cardiovascular disease in participants. Aside from failing to reduce the incidence of heart disease, the incidence of stroke was increased by 39 percent in the CEE group when compared to placebo (24).

Reevaluation of the surprising findings of the WHI has generated several hypotheses to account for the increased incidence of stroke among women receiving ET (25). First, the increased risk of stroke reported by the WHI is of borderline significance. Eliminating data from subjects with comorbidities, such as obesity and hypertension, may render the increased risk insignificant. Second, the relative increased of stroke as it stands is very small. The 30 percent increased risk of stroke reported from the WHI would only manifest as 1-2 more strokes per 10,000 women per year (26). Third, the age of the subjects can confound the results of the WHI. While the overall risk of stroke significantly increased, the increased risk of stroke among younger women (<50 years of age) was negligible (26). The average age of WHI participants was 63 years of age, approximately 13 years after the average onset of menopause (24). Overall, 83 percent of WHI subjects were at least 5 years into menopause. The critical window hypothesis, suggests that initiation of ET near the onset of menopause in younger women has a higher benefit/risk ratio, whereas ET in older women who have been in menopause for an extended period of time may be ineffective or harmful (26). Additionally, the route of ET administration may impact the results of the WHI. In the WHI, estrogen was given orally, and data suggests that transdermal administration may be safer and more effective than oral administration by avoiding first-pass metabolism and bioactivation (26). It is also important to consider that in experimental studies, estrogens are generally administered via intravenous or subcutaneous injection, and this difference may shed some light on the discrepancy between experimental stroke models and clinical studies, such as the WHI. Finally the estrogen formulation may also explain, at least in part, the discrepant findings. The subjects were treated with CEE during the WHI, a much different formulation than 17β-E2 used in experimental treatment. CEE consists primarily of the equine estrogens, estrone, equilin, and equilenin, each conjugated to a sulfate group.

Although reevaluations of the WHI have generated several hypotheses to account for the negative outcomes associated with ET, it is widely accepted that the increased risk of breast cancer is due to over activation of peripheral ERs, likely ERα (26). This prevailing hypothesis has driven the design of alternative ET strategies. One strategy is the use of selective estrogen receptor modulators (SERM), synthetic ER ligands that specifically activate only one ER subtype, generally ERβ. However, it is not entirely known if all of the negative side effects are mediated through ERα; therefore, the overall therapeutic value of SERMs remains unknown and has yet to be tested in a large-scale clinical trial.

17β-E2 Action in the Brain: Neuroprotection

ERα is widely distributed in the rodent and mouse forebrain (19,27). A similar distribution exists in the human brain, where ERα is distributed throughout the forebrain, hypothalamus, and hippocampus, but not in the cerebellum (27). In both rodents and human subjects, overall ERβ expression is much lower than ERα in the cerebral cortex; however, the ratio of ERβ/ERα is greater in the rodent midbrain and ERβ is expressed weakly in the cerebellum (19, 27). GRP30 is expressed in the forebrain, hypothalamus, brainstem and hippocampus in both mice and rats (28). Several genes involved in neuronal survival are located downstream of a promoter containing an ERE, and in turn, 17β-E2 has been shown to promote transcription of several genes involved in neuronal survival. Following insult, 17β-E2 administration increased expression of several proteins involved in cell survival, including phosphoinositide 3-kinase (PI3K) (29), Akt (29,30), cyclic-AMP response element binding protein (CREB) (30), Bcl-2 (30-33), Bcl-x (34),

superoxide dismutase (SOD) (35), protein phosphatase 2A (PP2A) (36), c-fos (37), and c-jun (37). In addition, 17β-E2 inhibited expression of pro-apoptotic proteins, including Fas, FADD (38), and Bax (30), subsequently inhibiting cytochrome c release (30).

17β-E2 has also been shown to increase activation of the mitogen-activated protein kinase (MAPK) pathway and increase phosphorylation of Akt (39) and CREB (30,39).17β-E2 has also been shown to increase caspase-12 activation, while inhibiting caspase-3 and caspase-8 activity (38). Raz et al. (2011) demonstrated that 17β-E2 induced ubiquitination of p53 and prevented ischemia-induced acetylation of p53 (40).

17β-E2 Action in the Brain: Immunomodulation

While a portion of 17β-E2's protective actions in stroke are mediated by induction of neuroprotective pathways in the brain, estrogen is also a powerful immunomodulator. Because the immune response following stroke dictates functional recovery and the extent of brain damage, 17β-E2 may be dually protective in stroke by also mediating the immune response.

Innate Immunity

Following brain injury or ischemia, there is a rapid local production of estrogen, indicating that the hormone may be involved in an immediate physiological response to limit tissue damage. This early production of estrogen occurs simultaneously with the upregulation of innate immune responses. Following stroke, neutrophils rapidly migrate into the ischemic brain region, becoming more rapid and excessive as the blood brain barrier becomes disrupted (5). While adequate neutrophil infiltration is necessary to activate monocytes and macrophages to scavenge cellular debris from the ischemic core, excessive neutrophil infiltration can exacerbate tissue damage, especially in the penumbra region. 17β-E2 has been shown to inhibit production of the neutrophil chemoattractants CXCL1, CXCL2 and CXCL3 in the ischemic region, preventing an excessive neutrophil response (41). 17β-E2 has also been shown to limit neutrophil adhesion to vascular endothelial cells through up-regulation of the protein Annexin A1 on the neutrophil's surface (41). Aside from preventing an excessive neutrophil infiltration into to the brain, 17β-E2 can also mediate the clearance of neutrophils from brain after a neutrophil response. Removal of apoptotic neutrophils by monocytes and macrophages is of critical importance for inflammatory resolution. 17β-E2 has been shown to induce an activated macrophage phenotype that secretes anti-inflammatory cytokines, such as IL-10 and TGFβ (3). 17β-E2 has also been shown to prevent the production and secretion of pro-inflammatory cytokines, such IL-1β, IL-6, and TNFα, through inhibition of NFkB signaling and transcription of pro-inflammatory genes (41). Overall, 17β-E2 serves as an anti-inflammatory agent that can modulate the innate immune response and inhibit a pro-thrombotic state to reduce stroke occurrence and/or limit ischemic lesion size following stroke.

Adaptive Immunity

Dendritic cells (DC) are often described as bridging the gap between the innate and adaptive immune system. Dendritic cells present antigens to T cells to stimulate the adaptive arm of the immune response, and estrogen has been shown to promote DC differentiation and MHC expression, thereby promoting a T cell response in the brain; however, 17β-E2 modulates the cytokine environment in the brain to control which subset of T cells will be recruited to the site of injury (3). Studies have shown that cytotoxic T cell (CD8+) infiltration exacerbates tissue damage following stroke, whereas cytotoxic T regulatory cells (Tregs) suppress pro-inflammatory responses and are often associated with reduced lesion volume and better recovery (41). 17β-E2 has been shown to inhibit production of pro-inflammatory, Th1 and Th17 cytokines, including IFNγ, TNFα and IL-17, as well as increase production of the anti-inflammatory cytokines, IL10 and TGFB, thereby generating a cytokine environment that favors a Treg response (42). 17β-E2 is also able to directly promote Treg proliferation through activation of the PI3K/AKT pathway (3).

Stroke Treatment: Targeting the Immune Response

There have been several attempts to target the immune system in ischemic stroke, all of which have been largely unsuccessful in clinical trials. Table 1 summarizes the therapeutic agents and their immune targets. Several of these clinical trials failed due to systemic immunosuppression or immune dysfunction. A reevaluation of these clinical trials has concluded that time of administration after ischemic stroke is critical for successful stroke treatment. For example, Enlimobab, an anti-ICAM monoclonal antibody, was unsuccessful if given immediately after stroke onset; however, several studies have shown that Enlimobab may be successful in limiting ischemic lesion volume if given between 3-6 hours of stroke onset (42).

Conclusion/ Future Directions

17β-E2 has been shown to be a powerful immunomodulator and neuroprotective molecule in

ischemic stroke. It is likely that modulation of the immune response accounts for the neuroprotective action of 17β-E2. There have been a limited number of pre-clinical studies evaluating the effects of 17β-E2 on the immune system; however, the majority of these studies have agreed that 17β-E2 reduces inflammation through inhibiting the pro-inflammatory cytokine, IL-6. IL-6 has been shown to exacerbate ischemic stroke damage and may be a therapeutically targeted immune marker in stroke. Understanding the effects of 17β-E2 also provides the opportunity to design therapeutic agents that mimic the immunomodulatory effects of estrogens, while avoiding the controversial off-target effects of estrogen on the reproductive system, etc. The clinical potential of using estrogens or analogs that target pro-inflammatory pathways following ischemic stroke remains to be determined.

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