REVIEW

Estrogen and Cerebrovascular Physiology and Pathophysiology

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ABSTRACT—Numerous studies have uncovered a wide variety of estrogen effects with apparent cardiovascular benefits, the most recognized ones being vasodilation, anti-atherogenesis, diminished post-ischemic inflammation and anti-oxidant effects. This article provides an overview of the influence of estrogen on the cerebral vasculature, under physiologic and pathophysiologic conditions, and covers both acute and chronic effects. The discussion is primarily focused on the vasodilatory and anti-inflammatory actions of estrogen, since those particular estrogen influences have received the greatest attention in studies published to date. With respect to vasodilation, although some consideration is given to the role of other vasodilating mechanisms and factors, the emphasis is mostly placed on the endothelial isoform of nitric oxide synthase, eNOS, which has emerged as a clear target of estrogen. Some consideration is given to recent findings that suggest that estrogen can stimulate eNOS activity by decreasing the expression of the eNOS inhibitor caveolin-1. With regard to the ability of estrogen to counteract inflammation, potential mechanisms by which estrogen limits the post-ischemic leukocyte adhesion, and the expression of the inducible NOS, are discussed.

Keywords: Brain, Caveolin, Estrogen receptor, Inflammation, Leukocyte adhesion, Nitric oxide

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1. Foreword

It is well-established that the incidence of cardiovascular disease, including stroke, and related mortality are lower in

females prior to menopause, when compared to males and post-menopausal females. It is quite likely that the protection in pre-menopausal females relates to higher levels of circulating estrogens, principally 17β-estradiol (E₂). The identification of the specific mechanisms involved in E₂-related cardiovascular defense has been the objective of numerous experimental studies, as summarized in recent

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excellent reviews (1, 2). The results of such investigations have uncovered a wide variety of E_2 effects with apparent cardiovascular benefit, some of the best-known actions being vasodilation, anti-atherogenesis, diminished post-ischemic inflammation and anti-oxidant effects.

The major objective of the present paper will be to provide an overview of estrogen's influence on the cerebral vasculature. In comparison to the periphery, the brain has received very little attention in this regard. Nevertheless, the information gained from studies in non-cerebral vascular tissue does provide a template for discussing possible estrogen actions in brain vessels, and a reference point for comparison to the limited currently available data regarding estrogen and the cerebral circulation. However, cerebral vessels exist in an environment that is quite different from other vascular tissues. The presence of an extensive neuronal and glial influence has a profound effect on cerebrovascular function and reactivity to endogenous substances, including estrogen. In particular, astrocytes and astrocyte-derived factors not only may regulate cerebral hemodynamics, but also may play a major role in establishing the unique phenotype of cerebrovascular endothelial cells. This is exemplified by the presence of endothelial cell tight junctions, which form the blood-brain barrier and limits the entry of blood-borne substances into the brain; *and* by the apparent resistance of cerebral endothelium to inflammatory reactions (e.g., leukocyte adhesion and diapedesis). Moreover, general and segmental differences in vascular smooth muscle and endothelial cell receptor populations, ion channels and expression of proteins related to control of vessel function, compared to other vascular beds, also contribute to the "unique" character of the cerebral vasculature. Thus, one must be cautious when applying information gained from studies in non-cerebral tissues to any discussion of estrogen and vascular function in the brain.

With such caveats in mind, this review begins with a somewhat generic summary of basic cellular mechanisms of estrogen action. This is followed by a more specific discussion of estrogen's influence on vasodilating function, with a major emphasis on the endothelial isoform of nitric oxide synthase (eNOS). A consideration of estrogen's purported ability to act as a counter-inflammatory agent is provided, with some attention given to post-ischemic leukocyte adhesion and regulation of inducible NOS (iNOS) expression. In addition, some discussion of acute versus chronic effects is presented.

2. Basic cellular mechanisms of estrogen action

The "classical" mode of estrogen action is via a stereoselective activation of intracellular receptors. Thus, 17β -estradiol (but not 17α -estradiol) binds to its receptor

forming a complex that regulates nuclear transcriptional activity either directly, via interactions with palindromic sequences within gene promoters (i.e., estrogen response elements) or indirectly through other transcriptional modulators. There are two structurally distinct estrogen receptors, ER- α and ER- β . Based upon protein and/or mRNA analyses, these receptors appear to be expressed in a wide variety of tissues, including the brain and vascular cells (2–4). Differences exist in the patterns of distribution of the two receptor types in the brain, vascular tissues and other structures, although co-expression of both receptors within the same cells has been noted. The basic receptor complex exists either as a homodimer (i.e., α/α or β/β) or a heterodimer (α/β), with each form possessing unique transcriptional properties. There are some estrogen-related experimental findings that cannot be explained through the classical ERs, but appear to involve genomic effects nonetheless. This raises the possibility that a yet unidentified ER and/or estrogen-modulated pathway for the regulation of gene product expression may exist (5).

The preceding paragraph describes a genomic process of estrogen action that is likely to require hours of estrogen exposure for changes in protein expression to be realized. However, estrogen also can elicit acute physiologic effects. One of the earliest acute actions of estrogen to be identified was its ability to promote vasodilation (6). Acute responses to estrogen, like vascular relaxation, that occur within seconds to minutes of estrogen administration cannot be attributed to genomic mechanisms. Current evidence points to the possibility of a cell surface receptor that, in endothelial cells, may be linked to increased Ca^{2+} entry (7) *and*, in vascular smooth muscle cells, may be coupled to intracellular cAMP generation (8, 9), stimulation of Ca^{2+} -dependent K^+ channels (10, 11) or blockade of voltage-dependent Ca^{2+} channels (12). It is uncertain, at this time, as to whether that cell surface target of estrogen action indeed represents a novel receptor (see, for example, ref. 13) or is a "classical" ER localized to the plasma membrane (7, 14, 15). Recent findings would appear to support the latter. That is, evidence indicated that the acute actions of estrogen are actually mediated through ER- α and/or ER- β , but via a different signal transduction route from that utilized to influence gene transcription. That conclusion is based upon findings that the acute reactions to estrogen can be blocked by pretreatment with classical ER pure antagonists, such as ICI 182,780, *and* that such responses do not occur when 17α -estradiol is given (16). These findings may also be consistent with a process whereby a stereoselective plasma membrane estrogen binding protein (13) is linked to a classical ER in the cytoplasm.

3. Estrogen and vasodilation

The discussion of the principal mechanisms involved in the vasodilating influence of estrogen has been organized according to acute versus chronic estrogen actions. Acute estrogen effects are a function of estrogen's ability to act as a direct vasodilating agent. Chronic estrogen actions relate to the steroid's capacity to *facilitate* vasodilation through either influencing the transcription of proteins that participate directly in vasodilation or via modulating the expression and/or activity of other proteins with indirect involvement in vasodilation.

3.1. Acute effects

3.1.1. Non-cerebral tissues

Based upon the results of multiple studies, it would appear that the acute vasodilating effects of estrogen can occur through both endothelium-dependent and -independent (i.e., direct vascular smooth muscle) mechanisms. With respect to endothelium-derived factors, two possibilities have been considered: increased generation of eNOS-derived NO and enhanced synthesis of vasodilator prostanoids.

In cultured endothelial cells harvested from a variety of non-cerebral sources, many reports have indicated that acute estrogen treatments, at physiologically relevant concentrations, promote increased NO generation (7, 14, 17–19). Acute E₂-induced increases in blood flow in vivo or relaxation of vessels ex vivo have been reported to be NO-dependent in some (20–22), although not all (23–27), relevant studies. Rather than directly increasing endothelial NO production, acute E₂ administration may also act to potentiate responses to the eNOS-dependent vasodilators like acetylcholine. This “permissive” action of E₂ has been demonstrated in the coronary circulation of postmenopausal human females (28).

Like the body of experimental information regarding NO-related mechanisms, the results of some studies support a role for vasodilator prostanoids in the acute actions of estrogen (29, 30), while others do not (25, 27, 31). Of some additional interest, many of the studies on peripheral vascular tissue, reporting prostanoid- and/or NO-dependence, also found that the acute E₂ response could be blocked by pretreatment with an estrogen receptor blocker (7, 14, 17–19, 22). This suggests that the acute activation of the pathways leading to increased NO and prostanoid generation are linked to classical ERs, although the mechanisms involved in that linkage are unknown.

Another potential site for acute estrogen actions is the vascular smooth muscle cell. Evidence for direct actions of estrogen on non-cerebral vascular smooth muscle has been obtained in isolated cell systems and in endothelium-denuded vessel preparations. Some reports have revealed

a stereoselective effect of estrogen (9, 27, 32) on vascular smooth muscle that may (9, 32) or may not (25, 27, 33) involve classical estrogen receptors. Other publications point to direct vascular smooth muscle relaxant effects of estrogen that variously depend upon: a) increases in intracellular cAMP (9, 26) or cGMP (26); b) activation of the cGMP-dependent kinase (34); c) potentiation of Ca²⁺-dependent (11, 34) or ATP-sensitive (25) K⁺ channel activity; or d) blocking voltage-dependent plasma membrane L-type Ca²⁺ channel function (25, 33, 35).

3.1.2. Cerebral tissue

In the cerebral circulation, several studies have shown that acute estrogen administration is capable of eliciting vasodilations, but only at concentrations (micromolar) seemingly well beyond the physiologically relevant range (36–40). To our knowledge, only one preliminary study, in newborn pigs, has directly addressed whether NO plays a role in the estrogen action. Thus, Zuckerman and Son (36) found that pial arteriolar diameter increases elicited by high-dose E₂ were unaffected by NOS inhibition. In another study, Ghanam et al. (38) reported that acute E₂ applications relaxed isolated segments of rabbit middle cerebral arteries via an endothelium-independent process that appeared to involve restriction of Ca²⁺ entry in vascular smooth muscle cells – a finding consistent with a lack of any eNOS influence. In the rabbit, topical application of E₂ in μ M amounts elicited a pial arteriolar dilation that was blocked in the presence of an ER antagonist (40). However, the role of NO in that vascular response was not addressed. In summary, unlike in the periphery, where there is some evidence to indicate that NO may play a role in the acute vasodilating effects of E₂, comparable evidence in the cerebral vasculature is lacking.

Whether, as in non-cerebral tissues, prostanoids may contribute to the acute vasodilatory actions of E₂ in brain has not been extensively explored. Indeed, only one preliminary report has addressed that issue. In that study, the vasodilating actions of high-dose E₂ on pial arterioles of neonates was found to be reduced in the presence of indomethacin (36), suggesting some role for prostanoids in that response.

One common feature in the studies cited above regarding the acute cerebrovascular effects of E₂, is that pharmacologic (i.e., micromolar) doses were required to elicit the effects observed. Thus, the physiologic relevance of these data might be called into question. However, the picomolar to nanomolar amounts of circulating E₂ found in intact females refers to the free, unbound steroid content. As much as 98% of the total E₂ content is protein-bound (41), with 50–60% of that fraction bound to albumin and the remainder to sex hormone-binding globulin (42). Since E₂ binds to albumin with low affinity (but high capacity), albumin-

bound estrogen is often included in the bioavailable fraction (43). Therefore, the physiologically relevant vasoactive threshold concentration for E_2 action may actually be 1–2 orders of magnitude greater than indicated by the free steroid level. As a consequence, the results of studies employing isolated vessel or local application in vivo approaches should not be dismissed simply because the E_2 doses used to elicit a response exceeded the range of E_2 concentrations seen in the circulation of intact females. On the other hand, since E_2 -induced cerebral vasodilation appears to involve an ER (40), one must also consider physiologic relevance in terms of the E_2 concentrations capable of activating the ER mediating the vascular response. In vitro estimates of E_2 binding to its receptor (in ER-transfected cells) indicate a high binding affinity, with the dissociation constant (K_d) lying in the subnanomolar to nanomolar range (44). This would appear to be more in line with concentrations of unbound E_2 measured in the circulation and favor nanomolar E_2 as the physiologically relevant level, at least when considering ER-mediated actions. However, the results of a recent study could be interpreted to suggest that much higher estrogen concentrations may be needed to activate the ER in the cerebral circulation in vivo. Thus, although topically-applied E_2 was found to elicit a dose-dependent pial arteriolar dilation, this only occurred in the seemingly supraphysiologic 0.1–100 μM concentration range, and those responses were prevented in the presence of pharmacologic blockade of the ER (40). Obviously, further study is needed before any explanation can be attempted regarding this wide disparity in the apparent ER sensitivities to E_2 , when comparing cerebral vessels in vivo and ER-transfected cells in vitro.

In addition to direct actions on the vessels, a number of reports exist where the acute effects of E_2 on brain neuronal function or metabolic activity have been addressed. Because changes in neuronal activity are often linked to changes in cerebral vascular tone, such publications are of some relevance to the present discussion. In a paper published more than 15 years ago, it was shown that high-dose intravenous E_2 increased metabolic activity in multiple brain regions (45). The results of subsequent studies have indicated that acute E_2 administration, either in vivo or in vitro, can antagonize the actions and/or reduce the receptor sensitivities of neurotransmitters or neuromodulators associated with *repression* of neuronal activity in a number of brain regions, by a mechanism that may involve interference with inhibitory G-protein function (46–50). Other studies have suggested that acute applications of E_2 are capable of potentiating the actions or enhancing the synthesis of neurotransmitters that promote increased neuronal activity (51–53). There is some evidence that these short-term neuronal actions of E_2 involve increased cAMP synthesis and activation of the cAMP-dependent protein kinase (48, 51, 54), are stereo-

selective (48, 54), and may (48, 49) or may not (52) involve classical ERs. The upshot of these acute neuronal effects of E_2 would be increased neuronal activity, which, in turn, is likely to be accompanied by vasodilation. In many cases, these effects of E_2 can be elicited with nanomolar doses. Thus, in the brain, the acute vasodilating actions of E_2 do not necessarily require direct interactions with vascular cells, but could occur indirectly, via actions on perivascular neurons.

3.2. Chronic effects

As a generalization, the vascular influence of chronic estrogen “exposure” is probably best characterized through a process resulting in potentiation of vasodilating capacity. To a large measure, that influence involves classical ERs, relates to genomic mechanisms (see earlier) and involves the regulation of proteins participating in vasodilation. The endothelium appears to be a major target for estrogen-related regulation of protein expression. In this regard, there are three candidates that merit consideration – eNOS and the proteins modulating its activity, cyclooxygenases, and the proteins responsible for the production of endothelium-derived hyperpolarizing factors. Of those three, eNOS clearly has received the greatest attention in the literature, including studies on cerebral vascular tissue.

3.2.1. Regulation of eNOS

3.2.1.1. Non-cerebral tissues

Physiologic evidence: The enhancement of eNOS expression and function accompanying long-term estrogen exposure was initially revealed in studies on peripheral vascular tissue. Evidence in support of this can be derived from multiple sources. For example, arteries and arterioles harvested from female experimental animals exhibit a greater endothelium-derived NO generating capacity than their male counterparts in most (e.g., refs. 55–58), but not all (e.g., refs. 59, 60) instances. The absence of unanimity among published reports could relate to species-specific factors. In fact, in mice, the reverse situation was found, with the male rather than the female aorta displaying greater NO generation (60). However, the results of that particular investigation may still support an E_2 -related effect, since ER expression in the aorta is higher in the male versus the female mouse (60). Other possible examples of a linkage between chronic estrogen status and eNOS include reductions in the vasodilating actions of eNOS-dependent vasodilating stimuli, like acetylcholine or shear stress (61), following ovariectomy (57, 58, 62) or after the onset of menopause (63, 64). That estrogen depletion specifically plays a key role in that diminished eNOS function is supported by results showing that the vasodilating responses could be restored with estrogen supplementation (23, 62). Consistent with these observations, chronic estrogen therapy

in male to female transsexuals has been shown to enhance NO-dependent, acetylcholine (ACh)-induced vasodilations (65, 66). In pregnant animals, increased ACh-induced vasodilatory responses, which appear to parallel the rise in circulating estrogens, have been demonstrated in carotid and uterine arteries (67).

Regulation of eNOS expression: In line with the functional evidence given above, prolonged exposure to E_2 has been reported to be accompanied by increased eNOS protein or mRNA expression in peripheral vascular endothelium (68, 69) and in cultured endothelial cells (70–72), via a process mediated by classical ERs (70). Thus, there is extensive evidence indicating that eNOS is a target for E_2 in the periphery.

3.2.1.2. Cerebral tissue

Physiologic evidence: In the cerebral circulation, recent data (summarized in Table 1) would indicate that estrogen has an influence on eNOS similar to that seen in peripheral vascular tissue. Thus, long-term estrogen exposure in rats was found to promote a *reduced myogenic tone*, in subsequently isolated brain arteries, via an endothelium and NO-dependent process (73, 74). In another report, Palmon et al. (75) observed a direct correlation between microvascular cGMP levels in rats and prolonged increases in plasma E_2 – a finding that could be explained by, but does not prove, increased endothelial NO generation. Based upon

such results, one might expect that intact and E_2 -treated ovariectomized female rats would exhibit higher resting cerebral blood flow (CBF) levels compared to untreated ovariectomized rats. Curiously, no significant differences in global or regional CBF values have been noted among these groups in findings published to date (1, 76–78). Insofar as resting CBF is determined by multiple factors unrelated to NO, and factors capable of counteracting NO influence, the absence of any meaningful differences in basal CBF levels is not altogether surprising. On the other hand, chronic estrogen replacement therapy in postmenopausal human subjects was accompanied by a reduction in basal cerebrovascular resistance (79). Furthermore, resting CBF and CBF reactivity to vasodilating stimuli have been reported to be greater in female versus male subjects [reviewed by Hurn and McCrae (1)]. Unfortunately, no studies to date have addressed whether those observations are in any way related to NO.

A perhaps more striking example of the linkage between chronic estrogen status and eNOS function can be taken from studies where, instead of basal tone, responses to *agonist-stimulated* eNOS-derived NO release were examined. Results from our laboratory revealed that chronic estrogen depletion, via ovariectomy, in rats was accompanied by a loss of eNOS-dependent (i.e., ACh-induced) vasodilating function in pial arterioles, but those changes could be

Table 1. Chronic estrogen “status” and possible eNOS-related changes in the brains of experimental animals

Model	Estrogen status	eNOS-related result	Reference
Rat isolated middle cerebral artery	depleted (ovariectomy)	↑ myogenic tone	73
Rat isolated middle cerebral artery	repleted (ovariectomy + E_2)	↓ myogenic tone (endothelium- and NO-dependent)	73
Rat isolated middle cerebral artery branch	depleted (ovariectomy)	↑ myogenic tone	74
Rat isolated middle cerebral artery branch	repleted (ovariectomy + E_2)	↓ myogenic tone (endothelium- and NO-dependent)	74
Rat brain microvessels	enhanced (high-dose E_2 -treated intact females)	↑ cGMP content	75
Rat pial arterioles in vivo	depleted (ovariectomy)	loss of eNOS-dependent vasodilation	80
Rat pial arterioles in vivo	repleted (ovariectomy + E_2)	restoration of eNOS-dependent vasodilation	80
Rabbit isolated middle cerebral artery	E_2 -treated, hyper-cholesterolemic males	restoration of eNOS-dependent vasodilation	38
Rat brain microvessels	depleted (ovariectomy)	↓ eNOS protein expression	82
Rat brain microvessels	repleted (ovariectomy + E_2)	↑ eNOS protein expression	82
Rat pial arterioles	depleted (ovariectomy)	↓ eNOS, ↑ CAV-1 protein expression	80
Rat pial arterioles	repleted (ovariectomy + E_2)	↑ eNOS, ↓ CAV-1 protein expression	80

prevented with 1–2 weeks of E₂ replacement (80). In a preliminary study (81), we also found that the changes in eNOS-dependent cerebral vasodilation associated with chronic estrogen status exhibited a regional selectivity, with results for the cortex and striatum, but not the hippocampus, mirroring those in pial arterioles (Fig. 1). Consistent with these findings, Ghanam et al. (38), reported that NO-dependent, ACh-induced relaxation of isolated rabbit middle cerebral arteries was enhanced when these animals were given chronic E₂ treatment prior to vessel harvest.

Regulation of eNOS expression: Additional investigations, albeit limited in number (see Table 1), have examined the effects of chronic estrogen depletion and repletion on cerebral eNOS expression. The general conclusion from these studies is that, like most of the investigations directed

toward peripheral vascular tissue (see above), chronic reductions in circulating estrogen (via ovariectomy) are associated with diminished cerebrovascular eNOS protein abundance, while E₂ replacement restores eNOS (80, 82). The E₂-related effect on eNOS appears to exhibit a rather complex regional selectivity, with pial and striatal eNOS expression being reduced by ovariectomy and restored by E₂ replacement, while in the hippocampus, no variations in eNOS were seen (Fig. 2). Those regional variations in eNOS abundance in most, but not all, cases mirrored the eNOS-related functional changes discussed in the preceding paragraph. The principal exception to this was the loss of eNOS-dependent vasodilating function in the cortex, following ovariectomy, despite no significant changes in eNOS abundance (Fig. 2). That apparent departure from

Fig. 1. eNOS-dependent vasodilating function in the cortex, hippocampus and striatum of intact, ovariectomized (OVX) and OVX + E₂-treated females. These studies, performed in fentanyl/N₂O anesthetized animals, used a 10 min i.v. infusion of the blood-brain permeant muscarinic agonist oxotremorine to elicit an eNOS-dependent vascular response (see refs. 190, 191). Changes in CBF were monitored using 3 laser-Doppler single fiber probes – two stereotactically inserted into the striatum and hippocampus, and one overlying the surface of the sensorimotor cortex (see ref. 78). In all 3 groups (n = 5–6 for each), the eNOS-dependent portion of the response was estimated by comparing results in the absence and presence of the non-specific NOS inhibitor, N^G-nitro-L-arginine (L-NNA) (10 mg/kg, i.v.). The nNOS-independence of the oxotremorine response was demonstrated by the lack of any effect of the nNOS blocker, ARR 17477 (1 mg/kg, i.v.) (129). In cortex and striatum, but not hippocampus, there is an eNOS-dependent vasodilation that disappears in the absence of estrogen, to be recovered upon estrogen replacement. These observations reveal that estrogen can regulate the functional activity of brain eNOS, although regional differences exist.

Fig. 2. Endothelial NOS (eNOS) protein expression [via Western immunoblotting, using a monoclonal antibody from Transduction Laboratories (80)] in the cortex, hippocampus and striatum of intact, ovariectomized (OVX) and OVX + E₂-treated females (see refs. 78, 80, 177, 178). In the lower panel, the levels of eNOS protein are expressed in relation to a HUVEC standard (control) run with each gel (not shown). The data for each group was taken from 4–5 brains. In the striatum and, to a lesser extent, the cortex, but not hippocampus, expression of eNOS appears to correlate with the presence of estrogen.

changes in eNOS function tracking eNOS protein expression may relate to a capacity for estrogen to modulate eNOS function via interactions with other endogenous eNOS-regulating substances. This is discussed further below.

Another conundrum revealed in these experiments is that the pattern of regional changes in eNOS abundance and functional activity did not correlate with the known regional patterns of classical ER expression. In fact, reports to date strongly indicate an absence of such receptors in the striatum (83, 84). On the other hand, based upon evaluations of labeled estrogen binding or mRNA analyses, ERs, especially the β -isoform, appear to be widely distributed in the hippocampus and cortex (83–85). One rather intriguing implication of these findings is that estrogen-associated genomic regulation may operate via receptors and/or pathways that do not include the ER- α or ER- β isoforms. Recent findings, in fact, provide some support for the presence of “alternative” ERs in the brain—receptors that are insensitive to established ER inhibitors or ER- α or - β -selective ligands (5, 86). Whether such apparently novel receptors play a role in regulation of eNOS expression and function remains to be determined.

3.2.1.3. Estrogen regulation of eNOS activity by indirect mechanisms

Caveolin: It seems likely that the influence of chronic estrogen exposure on eNOS functional activity in the brain goes beyond simply modulating eNOS protein expression. Recent publications indicate that eNOS activity is regulated by a number of endogenous proteins. The best described among these is the plasma membrane protein, caveolin-1 (CAV-1), which binds to and acts as a potent negative regulator of eNOS activity in vascular endothelial cells (87, 88). Stimulation of eNOS occurs when Ca²⁺-activated calmodulin displaces CAV-1 from its binding site on the eNOS molecule. Caveolin-1, a 22–24-kDa protein, is the major structural protein of endothelial cell plasma membrane invaginations called caveolae. Optimal sensitivity of eNOS to its endogenous activators requires its localization to caveolae. The importance of caveolar localization for efficient eNOS regulation may relate to the observation that most of the elements needed for activation of eNOS are concentrated in the microdomains of these structures. These include cofactors; L-arginine transporters (89); proteins regulating Ca²⁺ influx, extrusion and intracellular release (90); and even muscarinic receptors (91). In a recent publication, Feron et al. (92) found that upregulation of

CAV-1 expression in non-cerebral endothelium (accompanying chronic elevations in free and LDL-cholesterol) was associated with an impairment in the ability to stimulate NO generation via eNOS. Thus, a precedent exists for a reduction in eNOS function in the absence of any changes in eNOS expression.

In pial vessels, we found that chronic estrogen depletion (ovariectomy) was associated with increased cerebral endothelial CAV-1 expression (80) (Fig. 3). That upregulation of CAV-1 could be prevented with prolonged E₂ supplementation (Table 1). Preliminary findings from our laboratory (93) indicated that this estrogen effect on CAV-1 could also be extended to include parenchymal microvessels (Fig. 3). The specific mechanisms through which E₂ acts to reduce CAV-1 expression have not as yet been identified. Nevertheless, some interesting possibilities can be taken from the aforementioned studies on cholesterol and its influence on CAV-1 gene transcription in peripheral cells. Thus, via preventing transcription-repressing binding protein interactions with sterol response elements (SREs) on the CAV-1 promoter, free cholesterol can increase the formation of CAV-1 mRNA (94). On the other hand, oxysterols have been reported to potentiate binding protein interactions with SREs, thereby reducing CAV-1 gene transcription (95). Whether estrogen acts in brain in a manner similar to that of oxysterols is unknown. Another rather straightforward consideration is that estrogen influences CAV-1 expression in brain endothelium simply through its well-known cholesterol-lowering effect (see, for example, ref. 96). Those two possibilities should make interesting subjects for future study.

Heat shock protein 90 (hsp90): Although direct evidence is lacking, the results of a number of studies, showing separately that hsp90 can interact with both eNOS and estrogen receptors, hint at the possibility of hsp90 participating in estrogen regulation of eNOS function. For example, results from one report showed that the putative hsp90 inhibitor, geldanamycin, can interfere with ER function (97). This could impact on eNOS function, since ERs appear to be involved in both acute (40) and chronic (98) effects of estrogen on eNOS in cerebral vessels. Of additional relevance to the cerebral circulation, results from another study demonstrated that hsp90 inhibition elicited a substantial reduction in NOS-dependent vasodilation in isolated rat middle cerebral arteries (99). Nevertheless, whether such findings can truly be interpreted as estrogen modulating eNOS function in cerebral vessels via influences on hsp90 remains to be established experimentally.

Akt: The serine/threonine kinase, Akt (also called protein kinase B [PKB]), has been linked to eNOS activation (100–102). The Akt kinase phosphorylates eNOS on serine 1177, rendering eNOS far more sensitive to calmodulin-induced activation (102). The major upstream activator of

Fig. 3. Caveolin-1 (CAV-1) expression (Western immunoblots) in cerebral cortical tissue (upper panel) and in pial vessels or cerebral parenchymal microvessels (lower panel) harvested from intact and ovariectomized (OVX) female rats. The cortical immunoblots were taken from single intact and OVX animals, while for the pial and microvessel immunoblots, 3 rats were pooled in each group. The microvessels were prepared according to methods described by McNeill et al. (82). With the pial vessel analysis, the entire pial layer overlying the hemispheres was obtained. The linearity of the analysis is demonstrated in the upper panel, where protein loading was done at 10, 15 and 20 µg/lane for each group. For the pial vessels and parenchymal microvessels, 10 µg protein/lane was loaded. A rabbit polyclonal antibody (from Transduction Laboratories) was used for this assay (80). In each case, the selectivity of the antibody was confirmed by the fact that only a single band (at 22–24 kDa) was visible. Pilot studies showed that the polyclonal antibody produced a far more robust reactivity than any of the monoclonal antibodies tested (e.g., m2297 or m2234 from Transduction Laboratories), indicating a higher affinity of the polyclonal vs the monoclonal forms, at least in tissue lysates, as the literature suggests (192). The major point emphasized in this figure is that depletion of estrogen induces the up-regulation of CAV-1 in total brain tissue as well as in vascular fractions isolated from pial membranes and the brain parenchyma. This evidence suggests that, in normal rats, the cerebrovascular expression of CAV-1 may be tonically repressed by chronic exposure to estrogen.

Akt appears to be phosphatidylinositol 3-kinase (PI3-K), which is capable of phosphorylating Akt at two serine/threonine sites (100). One mechanism for activation of PI3-K involves tyrosine kinase receptor-linked growth/trophic factors (100). That mechanism could be linked to estrogen by virtue of evidence provided in multiple studies showing that brain neurotrophin and tyrosine kinase receptor expression appear to vary directly with long-term estrogen changes (103–106). Furthermore, E₂ was recently

found to promote an ER-dependent, rapid, and sustained phosphorylation of Akt in neurons via a PI3-K-mediated process (101). While these particular mechanisms have not been confirmed in endothelial cells, it does suggest the possibility of yet another estrogen-associated influence on eNOS.

3.2.2. Regulation of endothelial prostanoid release

3.2.2.1. Non-cerebral tissues

Although the number of publications devoted to long-term estrogen influences on vasodilator prostanoid synthesizing capacity, compared to NO, are far fewer in number, some data are available. In peripheral vascular tissues, a majority of relevant publications point to a capacity for estrogen, over the long term, to enhance prostacyclin production (107–110). The mechanism behind this E_2 action may involve upregulation of vascular endothelial cyclooxygenase-1 (COX-1) expression (108, 109) and/or prostacyclin synthase (111). However, this action of estrogen may vary according to the vascular bed being studied. For example, increases in COX-1 expression or prostacyclin synthesis were seen in uterine (107, 111), aortic (110), pulmonary (109) and umbilical (108) tissue, but not omental (107) and vascular tissue. In mesenteric vessels, one study reported E_2 to be associated with a diminished prostanoid-dependent vasodilating function (112), while another provided evidence for an E_2 -related capacity to repress prostanoid-associated vasoconstrictor function (113).

3.2.2.2. Cerebral tissue

In the brain, reports on the effects of long-term estrogen depletion or supplementation are virtually absent from the literature. There is some evidence implying an estrogen-linked increase in brain prostaglandin E_2 production or expression of the prostanoid receptor (114, 115). Also, findings from a recent study suggested that a capacity for estrogen to promote prostanoid-dependent vasodilation in cerebral arteries could only be revealed when NO production was inhibited (116). Clearly more work is needed before any conclusions can be made regarding long-term estrogen influence on prostanoid-dependent cerebral vasodilating function.

3.2.3. Regulation of endothelium-derived hyperpolarizing factor in peripheral and cerebral tissue

With respect to estrogen-related influences, of the three major endothelium-derived vasodilating factors, the endothelium-derived hyperpolarizing factor (EDHF) has received the least amount of attention. Results from two published reports indicated that the chronic estrogen elevations accompanying pregnancy were associated with increased release of a non-NO/non-prostanoid hyperpolarizing factor in mesenteric arteries (117) and aorta (118) when ACh was applied. On the other hand, in cerebral arteries, a seemingly

opposite estrogen-related effect was observed. Thus, the contributions from a putative EDHF to ATP-induced dilations in the isolated rat middle cerebral artery were increased in ovariectomized rats relative to intact female rats (119). The possibility that the results of these studies reflect an organ-specific effect of estrogen on EDHF-mediated vasodilatory function cannot be established at this time, due to the limited nature of these data. Furthermore, the specific EDHF-related enzymes modulated by chronic estrogen changes are uncertain. In the aorta, circumstantial evidence points to an endothelial P450 epoxygenase product being involved (118). On the other hand, in the brain, the epoxygenases participating in the regulation of cerebrovascular tone (120) may be localized to astrocytes rather than endothelium (121). Thus, at least in the brain, the “endothelium-derived” label for a paracrine factor that elicits smooth muscle hyperpolarization may be too limiting. One implication of this is that, if estrogen indeed modulates expression of epoxygenases in the brain, the manifestation of that action may not be revealed in an isolated vessel. Moreover, the consistent and strikingly different effects of chronic estrogen changes in isolated cerebral vessels versus peripheral vessels may be suggestive of an estrogen influence on a non-epoxygenase, EDHF in cerebral arteries. Such issues need to be addressed in future studies.

3.2.4. Endothelium-independent regulation of cerebral vasodilation

3.2.4.1. Role of the neuronal NOS

The likelihood that estrogen may potentiate cerebral vasodilating function through regulation of protein expression in non-endothelial cells warrants some consideration. One particularly intriguing candidate is the other Ca^{2+} -dependent NOS isoform found in the brain, the type I or neuronal NOS (nNOS). This isoform has received far less attention than eNOS, when considering estrogen-associated regulation. In the periphery, direct variations in nNOS mRNA and/or protein associated with long-term E_2 treatment or depletion have been shown to occur in skeletal muscle (69), uterus (122, 123) and vaginal tissues (124). Significant increase in cerebellar NOS activity during pregnancy or following chronic E_2 treatment have been observed in the guinea pig (69). However, the estrogen-associated enhancement of cerebellar nNOS activity may not necessarily relate to increased nNOS expression, but rather to an increase in calmodulin levels (125). Okamura et al. (126) showed a clear and positive effect of chronic E_2 administration on NADPH diaphorase immunoreactivity (an indicator of nNOS) in rat hypothalamic neurons. Similarly, Rachman et al. (127) found an increase in hypothalamic nNOS mRNA following E_2 treatment. In the developing brain, the rise in brain NOS activity follows the same time course as the appearance of brain ERs (128). In that study and in

the study of Weiner et al. (69), blockade of ERs with tamoxifen reversed the NOS upregulation. This suggests that the E₂ effects on brain nNOS are mediated by ERs. Results from our laboratory also support a connection between chronic estrogen status and brain nNOS activity. We found that Ca²⁺-dependent NOS activity in the soluble fractions of cerebral cortical homogenates (a reflection of nNOS) varied according to the circulating E₂ level, with activities in ovariectomized rats approx. 50% lower than those in intact or E₂-treated females (129). On the other hand, estrogen treatment has been associated with a down-regulation of nNOS mRNA and protein in pituitary tissue – an effect that may be specific to a particular splice variant of nNOS (130). However, these findings could also be explained on the basis of a regional selectivity in estrogen regulation of nNOS, although that possibility has yet to be confirmed.

3.2.4.2. Role of the cholinergic system

Changes in the levels of estrogen have also been linked to variations in the expression of enzymes responsible for the synthesis of vasodilating neurotransmitters, as well as the expression of receptors participating in vasodilating responses. One of the best-studied of these neurotransmitter pathways is the muscarinic cholinergic system. Certainly, the connection between this neurotransmitter system and endothelium- and eNOS-dependent vasodilation is well-established. However, in the present context, we will expand this to include endothelium-independent mechanisms like muscarinic receptor-mediated neuronal activation. One of the most important clinical implications arising from a potentiating influence of estrogen on this system is a reduction in the incidence and severity of Alzheimer's disease symptoms (e.g., ref. 131). There may be several sites for estrogen control of cholinergic function. The first relates to the rate-limiting enzyme in the ACh biosynthetic pathway – choline acetyltransferase (ChAT). Thus, the expression ChAT message and protein, especially in neurons of the basal forebrain, have been found to vary directly with estrogen status (132 – 134). Estrogen has also been reported to be associated with increased muscarinic receptor sensitivity to agonist-induced activation (135) and diminished potency of muscarinic receptor antagonists (136). The hippocampus appears to be particularly sensitive to these effects (136). Other investigators have reported an estrogen-related increase in the number of muscarinic receptors (137, 138), although such changes may be confined to the hypothalamus (137) and, therefore, may have little relevance to overall circulatory control. An absence of estrogen-associated changes in muscarinic receptor abundance was supported by preliminary findings from our laboratory (unpublished) indicating no differences in cerebral cortical, striatal or hippocampal arterial/arteriolar muscarinic receptor expression in intact versus ovariectomized rats. Never-

theless, even in the absence of changes in receptor numbers, one might consider the possibility that the loss and restoration of muscarinic agonist-induced cerebral vasodilation accompanying chronic estrogen depletion and repletion, respectively (discussed earlier), may be more than just a simple function of changes in eNOS and CAV-1 expression.

3.2.4.3. Role of synaptic remodelling

Another documented phenomenon associated with prolonged (i.e., ≥24 h) estrogen exposure is increased brain synaptic density (see, for example, refs. 139, 140). Since the extent of synapse density and neuronal connectivity has been linked to the level of brain perfusion (e.g., ref. 141), it is tempting to suggest that estrogen-associated synaptogenesis may provide an additional mechanism for improving cerebral vasodilating function. From findings obtained in rats, two brain regions, the CA1 sector of the hippocampus and the ventromedial hypothalamus (VMH), appear to be particularly sensitive to estrogen-related increases in synapse density, as evidenced by increased formation of dendritic spines. In the hippocampus, the density of dendritic spines on *N*-methyl-D-aspartate (NMDA) receptor-expressing synapses have been found to vary with changes in estrogen levels (139). In the VMH, circumstantial evidence implies similar changes, but direct confirmation is lacking (140). Whether synaptogenesis-associated expression of other neurotransmitter receptors, with links to vasodilation, are similarly increased by estrogen, remains to be established. Cyr et al. (142) recently reported that estrogen exhibits a regional heterogeneity in its influence on NMDA receptor density, with increases seen for the hippocampus (CA1 and dentate gyrus), but reductions observed in the striatum and frontal cortex. The link between activation of NMDA receptors and cerebral vasodilation, via a process that is largely dependent upon nNOS-derived NO, is well-established in many brain regions, including the hippocampus (143). Nevertheless, although estrogen may increase NMDA receptor numbers in some brain regions, estrogen has also been associated with diminished NMDA receptor activity (144). This may limit the overall impact of estrogen-induced increases in NMDA receptor expression on cerebral vasodilating function.

3.2.4.4. Role of noradrenaline and adenosine

Independent from factors related to synaptic remodeling, other vasodilating neurotransmitter/neuromodulator candidates, that merit consideration as potential targets of estrogen influence, are norepinephrine (NE) and adenosine. Compared to the body of information related to cholinergic mechanisms, the literature regarding long-term estrogen influence on cerebral adrenergic or adenosinergic systems is sparse. There is some evidence of a positive estrogen effect on the expression of tyrosine hydroxylase (the rate-limiting enzyme in the synthesis of NE) in cortical neurons

(145). The results of another study indicated that estrogen can potentiate NE responses (cAMP accumulation) in brain *neurons*, with α_2 , rather than β -adrenoceptors, being affected (146). Activation of α_2 -adrenoceptors has been shown to elicit an endothelium-dependent cerebral vasodilation, via receptors present on the vessels themselves (147), but there is no information as to whether estrogen influences vascular adrenoceptors. With respect to adenosine, estrogen has been linked to increased cerebral activity of the enzyme responsible for adenosine synthesis – 5'-nucleotidase (148). However, there is no currently available data as to whether estrogen influences the expression or activity of adenosine synthesis, degradation or receptor proteins in vascular cells.

4. Estrogen and cerebrovascular inflammation

The discussion up to this point has dealt with estrogen control of vascular function within normal physiologic boundaries. In this section, the focus will be on how estrogen can modulate the changes in vascular function occurring under pathologic conditions. The inflammatory reactions accompanying a number of pathologic states can lead to the localized upregulation of proteins mediating the production of potent vasodilating substances as well as locally-damaging reactive oxygen species. There is a growing body of information linking inflammation and inflammatory proteins to the brain cell damage associated with neurodegenerative brain diseases, brain and spinal cord trauma, and stroke (for a review, see ref. 149). The number of inflammatory proteins that might participate in neuropathologic changes is extensive. For the purpose of simplification, the present discussion will be limited to only a few proteins, where some connection to estrogen has been identified. Examples include cytokines, adhesion molecules, iNOS and cyclooxygenase-2 (COX-2).

4.1. Inflammatory events in endothelial cells

As a generalization, the process of inflammation is initiated principally in post-capillary venules through enhanced interactions between receptor-ligand pairs on both leukocytes and vascular endothelial cells. Leukocyte capture and rolling comprise the first phase of this process (150). This stage is mediated primarily by the selectin family of adhesion molecules. The principal member of this family is the endothelial P-selectin, which facilitates rolling through interactions with its counter-ligand, P-selectin glycoprotein ligand-1 (PSGL-1), on leukocytes (151). Other selectins, such as the endothelial E-selectin, may play a role in further slowing leukocyte rolling velocity, thereby increasing the interaction between leukocytes and locally-produced inflammatory mediators (e.g., cytokines, chemokines – see below). Subsequent firm adhesion and sticking is mediated

by the interaction between leukocyte β -integrins *and* intercellular and vascular cell adhesion molecules (ICAM-1, ICAM-2, VCAM-1) on the endothelial surface. This process is facilitated by the presence of cytokines, like interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF- α), and local chemoattractants (149). The last step in the adhesion cascade is leukocyte transendothelial migration and involves, among other factors, the participation of an adhesion molecule present in both endothelium and leukocytes (i.e., PECAM-1).

4.2. Effect of estrogen on inflammation in non-cerebral endothelium

In peripheral tissues, with few exceptions (see, for example, ref. 152), most of the data obtained from human subjects and experimental animals point to an estrogen-related limitation in the expression of adhesion molecules under basal conditions and following pro-inflammatory stimuli. Several studies have indicated counter-inflammatory actions accompanying *short-term* (15 minutes to several hours) estrogen exposure, as evidenced by reduced post-ischemic leukocyte accumulation in cardiac and mesenteric tissue (153). The relatively short time frame of estrogen exposure, especially over periods of less than one hour, implies the participation of mechanisms apart from direct estrogen modulation of adhesion molecule transcriptional activity. One possibility is a restriction in the mobilization of constitutively-expressed adhesion molecules from intracellular reservoirs to their sites of action on the cell surface. A potential candidate is P-selectin, which under resting conditions, is present in intracellular storage granules (Weibel-Palady bodies) (154). The secretion of Weibel-Palady bodies has been reported to be inhibited by NO (e.g., ref. 155). Therefore, one might consider that estrogen's acute counter-inflammatory effects relate, as described earlier, to an increase in NO generation via stimulation of a constitutive NOS (156). However, an increase in the levels of NO, when extended over periods of several hours, may also limit adhesion molecule *expression* (157).

In conjunction with *long-term* (≥ 1 day) estrogen exposure, cultured human umbilical vein, saphenous vein or rabbit aortic endothelial cells displayed a decreased expression of E-selectin, ICAM-1 or VCAM-1 following cytokine, LPS or lysophosphatidylcholine treatment (158 – 160). In human subjects, circulating levels of adhesion molecules were found to be lower in postmenopausal females receiving estrogen replacement therapy, compared to their untreated counterparts (161). These results support a genomic action of estrogen, perhaps via blocking the transcription of adhesion molecules (158).

Because of the evidence that estrogen up-regulates the expression and/or the activity of constitutive NOS's and the evidence that NO has anti-adhesive properties, it is

tempting to ascribe the anti-inflammatory effects of estrogen to an enhancement of NO generation within the sub-micromolar ranges attributable to eNOS and nNOS. Changes in leukocyte adhesion and adhesion molecule expression accompanying administration of NOS inhibitors or NO donors have been widely observed, both under basal conditions and in association with ischemia/reperfusion (162 – 167). Furthermore, an increase in mesenteric leukocyte adhesion was recently observed in both eNOS and nNOS knock-out mice (168). These findings demonstrated the anti-adhesive capabilities not only of the NO produced at the endothelium, which is ideally situated to influence both endothelial and leukocyte adhesion molecule expression, but also of nNOS-derived NO. These studies suggest, therefore, that at least a portion of estrogen's capacity to reduce leukocyte adhesion may be related to stimulation of constitutive NOS activity, either eNOS or nNOS. Relevant to this discussion, Garcia-Duran et al. (169) showed that estrogen stimulates nNOS protein expression in human neutrophils. This was associated with a reduction in the expression of the CD18 integrin on the surface of E₂-incubated neutrophils, producing a decrease in adhesive capacity.

4.3. Counter-inflammatory effects of estrogen: role of NF κ B

At the molecular level, the purported ability of estrogen to interfere with the adhesion process may be due to blocking NF κ B activation (170). NF κ B is widely accepted as a central transcription factor in inflammation. Its activity is regulated by inhibitory proteins such as I κ B- α . Evidence to date indicates that modulation of I κ B degradation and/or synthesis is indeed a common mechanism in the regulation of NF κ B activity (170). The I κ B proteins bind to NF κ B dimers, thus stabilizing them in the cytoplasm of resting cells. When cells are exposed to inflammatory agents, like cytokines, I κ Bs are phosphorylated at serine residues by I κ B-kinases. That action targets these proteins for ubiquitination and degradation by proteasomes. This releases NF κ B and exposes a nuclear localization signal, which leads NF κ B to the nucleus, where it binds to sites in gene promoters, usually triggering transcription. Most of the genes encoding for cell adhesion molecules have κ B binding sites in their promoters. The fact that NF κ B is ubiquitous, responds to a wide variety of pathogenic signals and functions as a pleiotropic inducer of transcription supports the central role that this factor plays in inflammation. Estrogen influence on NF κ B may involve both NO-dependent and -independent actions. With respect to the former and estrogen's known capacity to enhance constitutive NO generation, there is a reasonable body of evidence indicating that NO can reduce NF κ B function in multiple cells, endothelial cells in particular, via influences on I κ B. The exact mechanism is unclear, but it may involve trans-

criptional induction of I κ B (170) and/or prevention of I κ B degradation (171). That action of NO may be a function of its ability to behave as an antioxidant (172), since oxidative stress is one of the major triggers for NF κ B activation (173). There is also evidence to indicate that estrogen (via interactions with its receptor [174]), without invoking any contributions from NO, may repress the transcriptional activation of adhesion molecules via preventing the degradation of I κ B- α and maintaining NF κ B in its inactive form (175). Also, estrogen, by virtue of its rather potent antioxidant properties (176), could contribute to the prevention of oxidative stress-induced NF κ B activation (173). Nevertheless, although estrogen-associated repression of inflammation appears to include actions toward NF κ B, current evidence does not permit one to dismiss the possibility that estrogen may act to prevent adhesion molecule transcription via mechanisms (receptor-dependent?) not involving NF κ B.

4.4. Effect of estrogen on inflammation in cerebral tissue

4.4.1. Effect on adhesion molecule expression

Studies addressing the effects of estrogen on cerebral inflammatory mechanisms are relatively few in number. Results from our own laboratory revealed that chronic estrogen depletion increased basal and PKC-stimulated leukocyte adhesion in pial venules, with those changes being reversible in the presence of chronic E₂ replacement (177). In another study, we observed that the increase in pial venular leukocyte adhesion, during the reperfusion period following forebrain ischemia, was influenced by chronic estrogen status (178). In that investigation, no differences in the extent of leukocyte adhesion, when comparing intact, ovariectomized, and E₂-treated ovariectomized females, were observed at 2 h of reperfusion. However, when the reperfusion time was extended to 4 and 6 h, the level of leukocyte adhesion in the intact and E₂-treated, but not untreated, animals declined substantially from the 2 h level. That reduction in the number of adherent leukocytes could not be attributed to de-adhesion and subsequent tissue infiltration, since no leukocytes were seen within the brain parenchyma at 6 h of reperfusion. Although the specific mechanisms are not known, these results suggest a process whereby estrogen promotes de-adhesion, allowing leukocytes to return to the circulation. The absence of any signs of leukocyte infiltration in this study was not completely unexpected, since, in forebrain ischemia, leukocyte infiltration does not occur until about 24 h of reperfusion. Furthermore, chronic estrogen depletion appears not only to potentiate, but also to accelerate the process of post-ischemic diapedesis. That is, preliminary findings from our laboratory (Fig. 4) indicated a substantial degree of leukocyte infiltration already at 12 h of reperfusion in ovariectomized rats, as opposed to a virtual absence of infiltrated leukocytes in intact females.

The mechanisms responsible for the estrogen-related repression in leukocyte adhesion in cerebral vessels has not as yet been clearly defined. It is tempting to ascribe these effects to the same mechanisms modulating inflammatory processes in peripheral vascular tissue. One possibility relates to the ability for estrogen to upregulate both eNOS and nNOS expression and activity in the brain, as discussed earlier. That NO may play an anti-adhesive role in the cerebral circulation was shown in a recent paper by Hudetz et al. (179). In that study, administration of an NOS inhibitor, L-NAME, just prior to reperfusion in rats subjected to forebrain ischemia, was associated with an increase in the level of leukocyte adhesion normally seen over 60 min of reperfusion. However, a recent preliminary study from our laboratory (180) revealed that the chronic absence of NO production in brain did not increase adhesion molecule expression in parenchymal microvessels, at least under resting (non-ischemia/trauma-associated) conditions. This result runs contrary to evidence in peripheral circulation where chronic inhibition of basal release of NO induces adhesion molecule expression (181), indicative of a tonic anti-inflammatory role of NO. One might speculate that the different findings in cerebral parenchymal (180), compared to peripheral (181), vascular tissue are due to the existence of NO-independent, yet estrogen-sensitive, counter-inflammatory processes in the former, at least with respect to resting, non-pathologic states. We do not, however, view our findings in parenchymal vessels as contradictory to the

results of Hudetz et al. (179) in pial vessels, since those authors monitored leukocyte adhesion following an ischemic insult.

4.4.2. Effect on the inducible nitric oxide synthase and other inflammatory proteins

Data obtained to date in studies on peripheral vascular tissues would suggest that estrogen is capable of restricting the upregulation of iNOS elicited by inflammatory stimuli (68, 182). On the other hand, simple E₂ exposure (1 – 2 days) was found to increase the expression of iNOS in rat aorta (183) and in cultured endothelial cells (184). However, since these latter data were obtained under conditions not associated with inflammation, their relevance to the present discussion is limited.

In the brain, preliminary findings from our laboratory appear to support a mechanism whereby estrogen acts to prevent iNOS upregulation under conditions of increased inflammatory activity. That is, detectable iNOS immunoreactivity, at 24 – 72 h following forebrain ischemia, was observed only in ovariectomized females and not in E₂-treated or intact females (Fig. 5). Multiple cell types were found to express iNOS at 72 h. The major iNOS-expressing cells appeared to be intraparenchymal polymorphonuclear leukocytes and monocytes/macrophages. Some iNOS expression was also observed in endothelium and in cells resembling activated microglia (not shown). The observation that such expression was minimal in post-ischemic

Fig. 4. Actual frames captured from video recordings of leukocyte adhesion at 6 h and 10 – 12 h reperfusion following 30 min of transient forebrain ischemia in an ovariectomized (OVX) vs an intact female rat. Adherent rhodamine-6G-labeled leukocytes (white and gray “spots”) were viewed through a closed cranial window, using intravital fluorescence microscopy, according to procedures described in earlier publications from our laboratory (177, 178). Note, in images captured during the experiments reported in ref. 178, the far greater magnitude of leukocyte adhesion in the OVX vs intact rat at 6 h reperfusion. In several rats (data not previously reported), the period of observation was extended out to 10 – 12 h. Note the extensive leukocyte infiltration into the parenchyma at 10 – 12 h in the OVX rat and the absence of infiltration in the intact rat. Also shown – pial venules under standard light. Note that vertically-oriented arteriole in the OVX rat is devoid of adherent leukocytes.

brains taken from intact and E_2 -treated ovariectomized females certainly adds to the body of evidence favoring a counter-inflammatory estrogen action in the brain.

The iNOS upregulation is likely to be accompanied by prolonged localized increases of NO in micromolar quantities. Nevertheless, by itself, NO is not likely to cause any substantial cellular damage. The cytotoxic actions of iNOS-derived NO probably derive from the rapid, diffusion-limited reaction between NO and superoxide radical to form the potent pro-oxidant, peroxynitrite (185). We have obtained evidence of extensive peroxynitrite generation (as indicated by measurements of nitrotyrosine expression) in brains obtained from ovariectomized females at 72 h following forebrain ischemia (Fig. 6). That apparent increase in post-ischemic peroxynitrite generation requires both a source for NO (seemingly provided by iNOS) and for superoxide radical. One likely superoxide source is the NADPH oxidase present in activated leukocytes and microglia. Another possible source is cyclooxygenase, the inducible COX-2 isoform in particular. Like iNOS, COX-2 can also be labeled as an inflammatory protein (149), and it is known to be up-regulated following forebrain ischemia (186). Although no studies have addressed whether estrogen influences cerebral COX-2 expression, estrogen has been associated with reduced COX-2 expression in peripheral tissues (187). Furthermore, COX-2, as well as iNOS, induction in the brain appear to involve NF κ B activation (188). Since estrogen has been linked to repression of NF κ B activity (see earlier), it would not be altogether surprising to find that post-ischemic expression of COX-2, like iNOS, will vary inversely with chronic estrogen status.

In our experiments, only limited signs of peroxynitrite formation were seen in estrogen-repleted or intact females (Fig. 6). In the same rats displaying enhanced iNOS and nitrotyrosine expression, there was evidence of substantial lipid peroxidation – an indicator of oxidative damage. Moreover, much of the lipid peroxidation was concentrated in vascular endothelial cells (Fig. 7). No signs of lipid peroxidation were observed in intact or E_2 -treated rats. Thus, one manifestation of estrogen's counter-inflammatory effects in the brain appears to be vascular endothelial protection. In support of this, recent findings have shown that chronic estrogen exposure can block the cerebral vascular damage induced by amyloid β -protein (189).

A composite illustration of the suggested mechanisms involved in estrogen's counter-inflammatory actions in cerebral endothelial cells is presented in Fig. 8. The figure represents a speculative synthesis of experimental evidence obtained in both cerebral and non-cerebral vascular tissue (see earlier). The principal emphasis is on estrogen's ability to disrupt the inflammatory cascade unleashed following a cerebral insult like ischemia and reperfusion. Figure 8 also considers NF κ B as a major target of estrogen actions and

Fig. 5. Inducible NOS (iNOS) immunofluorescence at 72 h post-ischemia in ovariectomized (OVX) females in cells within the brain parenchyma of the striatum (A) and hippocampus (B), in endothelium (C) and in cells embedded in endothelium (D). Few or no iNOS immunoreactive cells were observed in intact or E_2 -treated OVX rats (not shown). For this analysis, we used 7- μ m coronal sections of paraformaldehyde-fixed and paraffin-embedded rat brains, obtained in an earlier investigation (78). A rabbit polyclonal anti-iNOS antibody (from Transduction Laboratories) was employed. FITC was used as the fluorophore (see ref. 80). The scale marker, shown in panel A, applies to panels A and B. The scale marker presented in panel C applies to panels C and D.

Fig. 6. 3-Nitrotyrosine immunofluorescence (a measure of ONOO⁻ generation) at 72 h post-ischemia in the hippocampus (upper panels) and striatum (lower panels) of intact (left panels) vs ovariectomized (OVX) (right panels) female rats. Seven-micrometer coronal sections were prepared from paraformaldehyde-fixed and paraffin-embedded rat brains obtained in an earlier investigation (78). A rabbit polyclonal anti-3-NT antibody (from Upstate Biotechnology) was employed. Cy³ was used as the fluorophore (see ref. 80). Note the far greater intensity of 3-NT expression in the OVX rats.

also places some emphasis on estrogen's purported capacity to upregulate eNOS and to restrict oxidative activity.

Fig. 7. Malondialdehyde (MDA)-modified protein immunofluorescence (a measure of lipid peroxidation) at 72 h post-ischemia in the hippocampus, striatum and cortex of E₂-treated vs untreated ovariectomized (OVX) female rats. Seven-micrometer coronal sections were obtained from paraformaldehyde-fixed and paraffin-embedded rat brains, as previously described (80). A polyclonal (rabbit) antibody (see ref. 193) was used for this analysis, with FITC as the fluorophore. A) Low magnification. B) Higher magnification showing expression concentrated in endothelial cells (examples indicated by arrowheads).

5. Concluding remarks

The broad spectrum of actions linked to estrogen are probably a reflection of multiple sites and pathways of E₂ influence. That wide range of estrogen effects, on the one hand, may be a principle reason for its vasculoprotective efficacy. On the other hand, the complex pattern of estrogen's influence may also contribute to the rather substantial disparity in published findings with respect to specific mechanisms of estrogen action. One factor not covered in this review is the influence of progesterone—the other major female sex steroid. Multiple studies have been published indicating that progesterone can reverse or prevent many of the actions of estrogen described in this review. Other reports, only touched upon in the present paper, have hinted at the possibility that the vascular influence of estrogen treatment may be less pronounced in males. Thus, the manner in which a vessel or vascular cell responds to estrogen may depend, among other things, upon the presence of progesterone, gender, the duration of estrogen exposure, the dose, the vascular bed, vessel size or segment, whether that tissue is studied in vivo or in vitro, or species. Despite these “complications”, our understanding of how estrogen influences vascular cells continues to expand. However, our quest to gain insight into the mechanisms involved in estrogen's effects on cerebral vessels is still in the early

stages. Obviously, more research in this area is needed.

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Fig. 8. Multiple pathways may participate in mediating the reported capability of E_2 to suppress leukocyte-endothelial interactions and limit tissue inflammation. Upper panel: E_2 may act via its receptor (ER, pathway 1), forming a complex (E_2 -ER) that interacts with the transcriptional machinery in the nucleus (pathway 2 and lower panel), leading to increased expression and activity of eNOS and reduced expression of adhesion molecules (e.g., ICAM, VCAM and E-selectin [see ref. 175]). E_2 may also behave as an antioxidant (176) (pathway 7), diminishing the impact of the increase in reactive oxygen intermediates (ROI) associated with pathologic conditions like ischemia and reperfusion (pathway 10). Limiting increases in ROI levels will act to limit phosphorylation of the $NF\kappa B$ inhibitory protein, $I\kappa B-\alpha$ [pathway 8 (173)], and its subsequent degradation, resulting in increased $I\kappa B-\alpha$ levels (pathway 9). In turn, the higher $I\kappa B-\alpha$ levels will diminish the amount of active $NF\kappa B$ that is present (pathway 5). Lesser $NF\kappa B$ abundance will limit the cell's capacity to increase the synthesis of adhesion molecules, iNOS or COX-2 (pathway 2 and lower panel). Increased generation of eNOS-derived NO has been linked to prevention of mobilization of endothelial adhesion molecules to the cell surface [pathway 3 (155)], increased de novo synthesis of $I\kappa B-\alpha$ [pathway 4 (170)], antioxidant actions (pathway 11) and diminished β -integrin expression [pathway 12 (169)]. The ability to prevent upregulation of iNOS and COX-2 will act to minimize ROI-related actions (pathway 6), which could also contribute to increased $I\kappa B-\alpha$ activity. Lower panel: This panel depicts the possible transcriptional mechanisms influenced by E_2 -ER actions within the nucleus, as well the transcriptional effects of a diminished $NF\kappa B$ activity. A reduction in $NF\kappa B$ activity is likely to limit or prevent upregulation of iNOS, COX-2, or adhesion molecules [pathways 1 and 2 (149, 188)]. Estrogen, via the complex formed with its receptor, has also been associated with reduced transcription of adhesion molecules [pathway 3 (173, 174)]. The E_2 -ER complex has been linked to enhanced eNOS transcription (pathway 4). Recent findings have suggested an E_2 -associated reduction in CAV-1 expression [pathway 5 (80)], although it is not known whether that mechanism involves an E_2 -ER interaction with the CAV-1 gene. The combination of increased eNOS and diminished CAV-1 expression will greatly enhance eNOS-derived NO generation.

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