Neuroprotection by a selective estrogen receptor β agonist in a mouse model of global ischemia

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Carswell, H. V. O., I. M. Macrae, L. Gallagher, E. Harrop, and K. J. Horsburgh. Neuroprotection by a selective estrogen receptor β agonist in a mouse model of global ischemia. Am J Physiol Heart Circ Physiol 287: H1501–H1504, 2004.—The present study employs selective estrogen receptor (ER) agonists to determine whether 17β-estradiol-induced neuroprotection in global ischemia is receptor mediated and, if so, which subtype of receptor (ERα or ERβ) is predominantly responsible. Halothane-anesthetized female C57Bl/6J mice were ovariectomized, and osmotic minipumps containing ERβ agonist diarylpropionitrile (DPN) (8 mg·kg–1·day–1, n = 12) or vehicle (50% DMSO in 0.9% saline, n = 9) or ERα agonist propyl pyrazole triol (PPT) (2 mg·kg–1·day–1, n = 13) or vehicle (50% DMSO in 0.9% saline, n = 10) were implanted subcutaneously. One week later transient global ischemia was induced by bilateral carotid artery occlusion under halothane anesthesia, and the mice were perfusion fixed 72 h later. ERβ agonist DPN significantly reduced ischemic damage by 70% in the caudate nucleus and 55% in the CA1 region compared with controls (P < 0.05, Mann-Whitney U-statistic). In contrast, pretreatment with the ERα agonist PPT had no effect on the extent of neuronal damage compared with controls. The data indicate a significant estrogen receptor-mediated neuroprotection in a global cerebral ischemia model involving ERβ.

estrogen receptor subtypes; neuroprotection; diarylpropionitrile; propyl pyrazole triol

THE HORMONE 17β-estradiol has neuroprotective effects in animal models of brain injury, including global ischemia (12, 26), focal ischemia (5, 18, 21), and hippocampal neurotoxic lesions (2).

Estrogen has pleiotropic effects in the brain that may be explained by classic estrogen signaling via nuclear receptors that act as ligand-activated transcription factors. Two subtypes of estrogen receptors (ER; α and β) are known to exist. Estrogen receptors have been implicated in estrogen-mediated neuroprotection since ICI-182,780, a nonselective estrogen receptor antagonist, was shown to exacerbate ischemic damage in a mouse middle cerebral artery occlusion (MCAO) model (20). In terms of the subtype involved, conflicting data exist. For example, one group (8) demonstrated that IC182,780 significantly exacerbates ischemic damage in the CA1 region. However, another group (20) demonstrated that treatment of the mice with IC182,780 has no effect on ischemic damage in the CA1 region. These conflicting results may be due to the different strains of mice used in the two studies. It is possible that the difference in the results is due to the fact that the two strains of mice used in the two studies have different levels of estrogen receptor expression. This is supported by the fact that the two strains of mice used in the two studies have different levels of estrogen receptor expression.

Knowing which ER subtype is responsible for neuroprotection may lead to future drug treatments. In the present study we employed two recently developed selective ERα and ERβ agonists to directly determine the functional role of estrogen receptors in cerebral ischemia. Propyl pyrazole triol (PPT) is the first selective agonist for the ERα subtype to be developed with 410-fold binding selectivity over ERβ (22). Diarylpropionitrile (DPN) acts as an ERβ agonist with a 70-fold higher relative binding affinity over ERα (17). The present study was designed to reveal first whether estrogen receptor activation mediates estrogen neuroprotection in a mouse model of global ischemia and second which estrogen receptor subtype(s) is responsible.

METHODS

All procedures were carried out under license from the Home Office with the approval of the University of Edinburgh Ethical Review Panel and were subject to the Animals (Scientific Procedures) Act 1986. Adult female C57Bl/6J mice were obtained from Charles River, UK. In the first study, 3-mo-old mice were anesthetized with halothane (3% for induction and 1.5% for maintenance) in 70% N2O-30% O2 via a face mask and underwent a bilateral ovariectomy via a dorsal incision. A miniosmotic pump containing ERα agonist PPT (Tocris Cookson; Bristol, UK) (2 mg·kg–1·day–1 in 50% DMSO in 0.9% saline; n = 13) or vehicle (n = 10) was implanted subcutaneously at the nape of the neck. The animals recovered for 1 wk and were then anesthetized and exposed to a period of global ischemia as described below. The second study followed the same protocol as the first study, but this time miniosmotic pumps contained ERβ agonist DPN (Tocris Cookson) (8 mg·kg–1·day–1 in 50% DMSO in 0.9% saline; n = 12) or vehicle (n = 9). ALZET osmotic pumps were used (Charles River), and each batch of pumps was tested at DURECT to determine the exact pumping rate and reservoir volume and to ensure accurate compound delivery. The pumps had a reservoir volume of 100 μl and were validated to deliver at a constant rate of 0.25 μl/h.

To induce global ischemia, both common carotid arteries were isolated and then occluded by using microaneurysms clips applied bilaterally for 15 min. The clips were then removed, and blood flow through the arteries was confirmed before the wound was sutured. The body temperature was strictly controlled at 37°C during the surgical procedure and maintained thereafter by an incubator (2 h) until the animals fully recovered from anesthesia. Animals were perfusion fixed 72 h later with 4% paraformaldehyde in 50 mM phosphate buffer following perfusion with heparinized physiological saline under deep halothane anesthesia. The operator was blind to the drug treatment of the mice. The brains were removed, postfixied in 4% paraformaldehyde for 2 h, and then processed for paraffin embedding. Sections (6 μm) at the levels of the caudate nucleus and dorsal hippocampus were cut and stained with hematoxylin and eosin. The

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numbers of morphologically normal neurons and neurons showing the features of ischemic cell change (9) were counted in five different fields in the caudate nucleus and CA1 pyramidal cell layer in each hemisphere using a 10 × 10 mm grid (attached to the microscope optics) at ×400 magnification. Two sections were analyzed per animal, and, in each section, the grids were positioned throughout the region being analyzed such that data were collected as representatively as possible. In the caudate nucleus the five grids used were positioned such that data from the medial, dorsal, ventral, and lateral aspects of the nucleus were sampled. In the CA1 regions, grids were positioned equidistantly along the extent of the pyramidal layer. Great care was taken to ensure that the grids were positioned in the same manner in each animal to avoid bias. Morphologically viable neurons are identified in hematoxylin plus eosin-stained sections by a large round perikaryon with a thin cytoplasm within a compact and regular neuropil, whereas nonviable ischemic neurons have shrunken perikaryon, which are triangular in shape with vacuolation of the neuropil. The analyzer was blind to the drug treatment in each animal. The percentage of ischemic neurons in vehicle-treated mice was compared with ER-agonist-treated mice using the Mann-Whitney U-statistic.

RESULTS

An episode of transient global ischemia of 15 min duration is associated with moderate levels of ischemic neuronal damage in selectively vulnerable neuroanatomic regions such as the caudate nucleus and CA1 pyramidal cell layer of the hippocampus. The extent of neuronal damage was quantified in these regions and compared between vehicle- and ER agonist-treated mice. DPN-treated mice (n = 12) exhibited a significant reduction in neuronal damage compared with vehicle-treated mice (n = 9) in the caudate nucleus (18 ± 7% vs. 61 ± 18% ischemic neurons, means ± SE, P = 0.028, Fig. 1). Similarly, in the CA1 pyramidal layer, there was a significant reduction in the percentage of ischemic neuronal damage with DPN treatment compared with vehicle (17 ± 7% vs. 38 ± 12% ischemic neurons, P = 0.025). However, after PPT treatment (n = 13), there was no significant difference compared with vehicle (n = 10) in the caudate nucleus (34 ± 9% vs. 33 ± 9%, P = 0.78, Fig. 2) or in the CA1 pyramidal cell layer (20 ± 7% vs. 23 ± 8%, P = 0.72).

DISCUSSION

The key findings from the present study are that 1) estrogen neuroprotection against global ischemia in C57Bl/6 mice is mediated at least in part by estrogen receptor activation and 2) that ERβ is predominantly responsible. These results give direct evidence that ERβ activation is neuroprotective, providing essential information on the mechanisms by which estrogen can have a positive influence on ischemic damage. Physiological variables were not measured in the present study; however, studies in our own laboratory and others showed that 17β-estradiol does not influence variables such as blood pressure in normotensive rats (4, 18).

The present study is the first to report the effects of selective estrogen receptor agonists in cerebral ischemia and to show central effects of DPN in vivo. DPN acts as an agonist on both ER subtypes but has a 70-fold higher relative binding affinity and 170-fold higher relative estrogenic potency in transcription assays with ERβ than with ERα (17). DPN reduced ischemic damage by 55–70% in the present study. The dose of DPN (8 mg·kg⁻¹·day⁻¹) closely correlates with effective doses used in other in vivo studies. For example, Frasor et al. (8) showed decreased progesterone receptor mRNA in the uterus by using a similar dose of DPN.

In contrast to DPN, pretreatment with PPT, a selective ERα agonist, did not confer neuroprotection in hippocampus or caudate nucleus. PPT was the first selective agonist for the ERα subtype to be developed and is the best compound for ERα selectivity out of a series of tetrasubstituted pyrazole analogs (22). PPT binds to ERα with high affinity, displaying 410-fold binding selectivity over ERβ (22). The reasons for the lack of efficacy of PPT in the present study should be considered and include possible deterioration of the drug in the pump. The dose of PPT used in the present study was based on optimum activation of ERα as shown by Harris et al. (10). Systemically administered PPT can cross the blood-brain barrier and exert central effects such as increasing cerebral progesterone receptor mRNA (10). Also worthy of note is the evidence for ERα expression in the hippocampus (24) and caudate (14). The DPN result in the present study indicates that our group sizes were sufficient to pick up a 55% reduction in neuronal damage as statistically significant.
Chemical estrogen neuroprotection is mediated in part via ERα antagonist, ICI-182,780 (nonselective ER agonist). In another study, the nonselective estrogen receptor activation but provide no evidence that ER activation is protective. In another study, the nonselective estrogen receptor antagonist, ICI-182,780 had no effect on cerebral blood flow despite exacerbating striatal neuronal damage (20).

Fig. 2. Quantification of ischemic neuronal damage in the caudate nucleus (A) and CA1 pyramidal cell layer (B) after transient global ischemia for individual female ovariectomized C57B/6J mice receiving propyl pyrazole triol (PPT, n = 13) or vehicle (n = 10) delivered subcutaneously. The percentages of ischemic neurons in the PPT-treated and the vehicle-treated group were compared using the Mann-Whitney U-statistic. The median of each group is indicated. Ischemic neuronal damage was not significantly different in the PPT compared with vehicle group in either the caudate nucleus or the CA1 pyramidal cell layer.

Our results provide convincing evidence that in global ischemia estrogen neuroprotection is mediated in part via ERβ activation but provide no evidence that ERα activation is protective. In another study, the nonselective estrogen receptor antagonist, ICI-182,780 (nonselective ERα and β antagonist) increased caudate infarct volume after transient MCAO in female mice (20), also pointing to a receptor-mediated effect of estrogen, but it was not possible from that study to determine which receptor subtype(s) was involved. In agreement with our finding that ERα activation does not induce neuroprotection, Sampei et al. (19) found that ERα knockout mice do not display increased neuronal damage following MCAO compared with wild-type mice. In contrast, Dubal et al. (7) found estrogen-induced neuroprotection was retained in ERβ knockout mice but lost in ERα knockout mice, indicating a role of ERα in estrogen-induced neuroprotection. The disparity between the results of Dubal et al. and our own results may be explained by the experimental models employed. Focal ischemia (as used by Dubal et al.) has a quite distinct pathophysiology compared with global ischemia as used in the present study. Focal ischemia is associated with a spectrum of hypo-perfusion leading to infarction that is fast evolving. Global ischemia produces intense forebrain ischemia that leads to selective and slowly evolving neuronal damage in selectively vulnerable regions such as the caudate nucleus and CA1 region of the hippocampus. It is increasingly apparent that the actions of estrogen in cerebral ischemia are complex and may be dependent on the type and severity of ischemia and dosing regimen of estrogen.

ERβ receptors are present on neurons, astrocytes (24), and on blood vessels (15). Thus DPN may protect neurons by direct (neuronal) or indirect (astrocytes or blood vessels) means. An involvement of estrogen receptors located on astrocytes in estrogen-mediated neuroprotection has been proposed (1). However, the involvement of estrogen receptors located on blood vessels to increase cerebral blood flow is less likely to mediate estrogen neuroprotection because the nonselective estrogen agonist ICI-182,780 had no effect on cerebral blood flow despite exacerbating striatal neuronal damage (20). These results agree with previous evidence that estrogen-mediated neuroprotection is not associated with significant blood flow enhancement (3, 18). Future work will need to define whether the specific estrogen agonists used in the present study have an effect on cerebral blood flow that could account for the differences in conferring neuroprotection.

Both DPN and PPT cause estrogen receptor-mediated transcrip-tion (17, 22). Thus in the present study DPN is probably inducing neuroprotection, at least in part, by enhancing expression of genes containing estrogen response elements. Candidate estrogen-regulated genes include apoE (16), a lipid transport protein shown to be neuroprotective in global ischemia (13). We have recently shown that 17β-estradiol-induced neuroprotection in this model of transient global ischemia is apoE dependent (12). Indeed, 17β-estradiol has been shown to upregulated central nervous system apoE levels in vitro and in vivo (23). In addition, the antiapoptotic gene bcl-2 has also been shown to be upregulated by 17β-estradiol after cerebral ischemia (6).

Mechanistic insight into the actions of estrogen are important, especially because recent clinical (25) and animal studies (4, 11) have also revealed a detrimental influence of estrogen on cerebral ischemic damage. The overall aim of understanding the mechanisms of action of estrogen in brain injury is to develop compounds that mediate the beneficial effects but not the detrimental effects of estrogen. In summary: this is the first study to demonstrate the effects of selective estrogen receptor agonists on cerebral ischemic damage. The results provide clear evidence that ERβ activation is neuroprotective against global ischemia in vivo. Thus neuroprotection induced by 17β-estradiol is mediated, at least in part, by ERβ receptor activation.

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GRANTS

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