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Critical period for dopaminergic neuroprotection by hormonal replacement in menopausal rats

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ABSTRACT

The neuroprotective effects of menopausal hormonal therapy in Parkinson's disease have not yet been clarified, and it is not known whether there is a critical period. Estrogen induced significant protection against 6-hydroxydopamine-induced dopaminergic degeneration when administered immediately or 6 weeks, but not 20 weeks after ovariectomy. In the substantia nigra, ovariectomy induced a decrease in levels of estrogen receptor-α and increased angiotensin activity, NADPH-oxidase activity, and expression of neuroinflammatory markers, which were regulated by estrogen administered immediately or 6 weeks but not 20 weeks after ovariectomy. Interestingly, treatment with angiotensin receptor antagonists after the critical period induced a significant level of neuroprotection. In cultures, treatment with 1-methyl-4-phenylpyridinium induced an increase in astrocyte-derived angiotensinogen and dopaminergic neuron death, which were inhibited by estrogen receptor α agonists. In microglial cells, estrogen receptor β agonists inhibited the angiotensin-induced increase in inflammatory markers. The results suggest that there is a critical period for the neuroprotective effect of estrogen against dopaminergic cell death, and local estrogen receptor α and renin-angiotensin system play a major role.

1. Introduction

Numerous studies have demonstrated that estradiol (E2) exerts trophic actions on neurons and glial cells, promotes neuron survival, and has a neuroprotective role in several models of neurologic diseases (Azcoitia et al., 2011; Smith and Dahodwala, 2014). Nonetheless, the effects of the loss of ovarian function and hormonal replacement therapy (estrogen replacement therapy, ERT) in humans are controversial. The conflicting data have been explained by the window of opportunity or critical period hypothesis, which suggests that the neuroprotective effects of estrogen depend on age at the time of administration, and that ERT must be initiated soon after loss of endogenous estrogen to be beneficial (for reviews see Daniel, 2013; Scott et al., 2012). However, the hypothesis is still controversial (Scott et al., 2014; de Villiers et al., 2013), and the mechanisms underlying the altered responsiveness to estrogen treatment following long-term estrogen deprivation (LTED) are still unclear. Recent studies suggest the loss of estrogen receptor alpha (ER-α) after LTED as a possible basis for the existence of the critical period (Daniel, 2013), whereas the role of ER-β is less clear (Dubal et al., 2006). The cellular target of estrogen-mediated neuroprotection is also unclear (i.e., ER-α or ER-β on astrocytes, microglia, or neurons) (Baker et al., 2004; Spence et al., 2013). The anti-inflammatory effects of estrogen appear to play a major role in the neuroprotective effects (Morale et al., 2006; Vegeto et al., 2008), although direct anti-apoptotic (Brendel et al., 2013) and trophic (Campos et al., 2012; Lopez-Martin et al., 1999) effects on neurons have also been suggested.

The findings of studies with animal models (Dluzen, 1997; Murray et al., 2003) and epidemiological and clinical evidence (Liu and Dluzen, 2007; Ragonese et al., 2006; Smith and Dahodwala, 2014) together suggest that sex hormones have a beneficial influence on the risk, onset, and severity of Parkinson's disease (PD). However, conflicting findings have also been reported from some clinical trials, and there is still a lack of consensus about the effects of the type of menopause (i.e., surgical or natural) and of ERT on PD (Miller et al., 2009; Ragonese et al., 2006). Interestingly, the renin-angiotensin system (RAS) mediates inflammation and oxidative stress, as well as effects of estrogen in several tissues (Dean et al., 2004; Nickenig et al., 1998). In recent studies in rat models of PD

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(Rodriguez-Perez et al., 2010, 2012), we have observed that the loss of estrogen following ovariectomy (surgical menopause) increases RAS activity and the susceptibility to neuroinflammation, oxidative damage, and dopaminergic neuron neurodegeneration, and that ERT induces pronounced RAS inhibition and neuroprotection when administered shortly after ovariectomy (i.e., short-term estrogen deprivation, STED). However, it is not known either whether there is a window of opportunity or critical period for the neuroprotective effects of estrogen in PD models, or whether the RAS is involved and administration of angiotensin antagonists may be an effective alternative therapy after the critical period. In the present study, we studied these questions as well as the estrogen receptors and cells involved in the observed effects.

2. Methods

2.1. Experimental design

In vivo and in vitro experiments were carried out to investigate the previously mentioned questions (Table 1). Adult female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN, USA; 10 weeks old at the beginning of the experiments and similar body weight) were used for in vivo studies. Rats were fed with 2014S Teklad Rodent Maintenance Diet (Harlan Laboratories) to minimize the presence of natural phytoestrogens. All experiments were carried out in accordance with Directive 2010/63/EU and Directive 86/609/CEE and were approved by the corresponding committee at the University of Santiago de Compostela. All surgery was performed under ketamine/xylazine anesthesia. The rats were divided into 6 groups. Rats in group A were ovariectomized (ovx; i.e., menopause was induced surgically; n = 36) and fitted with empty implants and killed at times post ovx similar to rats in groups B–F (i.e., 3 subgroups), to compare treated and not treated ovx rats of the same age and body weight. Rats in group B were ovariectomized and simultaneously received implants containing 17β-estradiol (E2; ovx + 0 weeks + E2; n = 12). Rats in group C were treated as group-B rats except that the E2 implants were inserted 6 weeks after ovariectomy (ovx + 6 weeks + E2; n = 12). Rats in group D were treated as group-B rats except that E2 implants were inserted 20 weeks after ovariectomy (ovx + 20 weeks + E2; n = 12). Rats in group E were treated as group-D rats but, instead of E2 implants, were administered with the angiotensin type 1 (AT1) receptor antagonist candesartan 20 weeks after ovariectomy (ovx + 20 weeks + candesartan; n = 6) or vehicle (n = 6). It has been shown that candesartan is the most effective AT1 antagonist in crossing the blood-brain barrier and that low doses have little effect on blood pressure and block brain angiotensin (AII) effects (Unger, 2003). Rats in group F comprised a control group of sham-operated (not ovariectomized; n = 12) rats that were sacrificed in proestrus (i.e., period with high levels of E2).

Table 1
Experimental design

Rats						
Series	Groups	OVX	Treatment	6-OHDA	Sacrifice	Methodology
1	A1	+	–	W 1,7,21	W3,9,23	Immunolabeling (TH, GFAP, OX-42, ERα, ERβ) and HPLC (E2 levels)
	B1	+	E2, W0	W1	W3	
	C1	+	E2, W6	W7	W9	
	D1	+	E2, W20	W21	W23	
	E	+	CAND, W20	W21	W23	
	F1	–	–	–	W0	
2	A2	+	–	–	W3,8,23	PCR, WB (AT1, AT2, AGT, p47, RhoA, ROCK II, IL1β, ERα, ERβ), ACE activity, HPLC (E2 levels)
	B2	+	E2, W0	–	W3	
	C2	+	E2, W6	–	W9	
	D2	+	E2, W20	–	W23	
	F2	–	–	–	W0	

Cell cultures		
Cell type	Treatments	Methodology
Neuron glia (primary)	No treatment	Immunolabeling (TH, GFAP, OX-42, ERα, ERβ), PCR, and WB (AT1, AT2, AGT, ROCK II, IL1β, TH)
Astrocytes (primary)	or MPP^+ and PPT/DPN	
Dopaminergic neuron (MES 23.5)		
Microglia (N9)	AII and PPT/DPN	

Key: 6-OHDA, 6-hydroxydopamine; ACE, angiotensin converting enzyme; AGT, angiotensinogen; AII, angiotensin II; AT1, angiotensin receptor type 1; AT2, angiotensin receptor type 2; CAND, candesartan; DPN, agonist of ER-β, 2,3-Bis(4-hydroxyphenyl)-propionitrile; E2, estradiol; ER-α, estrogen receptor alpha; ER-β, estrogen receptor beta; GFAP, glial fibrillary acidic protein; HPLC, high performance liquid chromatography; IL1-β, interleukin-1β; MPP+, 1-methyl-4-phenylpyridinium; OVX, ovariectomized; OX-42, anti-complement receptor-3, clone MRC; p47, NADPH oxidase subunit $p47^{phox}$; PCR, polymerase chain reaction; PPT, agonist of ER-α, 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; ROCK II, Rho kinase; TH, tyrosine hydroxylase; W, week; WB, Western blot.

In a first series of experiments, ovariectomized rats were used to determine the neuroprotective effect of ERT administered at different times (0, 6, and 20 weeks) after the loss of estrogen (to investigate the existence of a critical period in PD models). One week after initiation of ERT, some rats (n = 6 by group/experiment) from groups A (ovx), B (ovx + 0 weeks + E2), C (ovx + 6 weeks + E2), and D (ovx + 20 weeks + E2) were lesioned with the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) and killed 2 weeks after lesion to investigate 6-OHDA-induced loss of dopaminergic neurons. In addition, rats in group E (ovx + 20 weeks + candesartan or vehicle) were lesioned with 6-OHDA to investigate the neuroprotective effect of inhibition of RAS activity after LTED. Furthermore, location of ER-α and ER-β in the substantia nigra compacta (SNc; dopaminergic neurons, astrocytes, and microglia) was studied by immunolabeling and laser confocal microscopy. Rats were perfused first with 0.9% saline and then with cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and subsequently washed and cryoprotected in the same buffer containing 30% sucrose, and finally cut on a freezing microtome (see Section 2.7).

In a second series of experiment rats in groups A–D and F (i.e., rats with STED and LTED) were used to study the effects of ERT on RAS activity, including levels of angiotensinogen, AII type 1 (AT1) and type 2 (AT2) and angiotensin converting enzyme (ACE), markers of NADPH-oxidase activation ($p47^{phox}$ subunit) and neuroinflammation (interleukin-1β, IL-1β; RhoA/Rho kinase, ROCK), and levels of estrogen receptors (ER-α and ER-β) in the nigral area. The rats were killed by decapitation 3 weeks after treatment with E2 or empty implants. The brains were rapidly removed, and the mesencephalon was sliced coronally (1 mm) with a tissue chopper set. To isolate the nigral region (SNc), the individual 1 mm tissue slides were dissected on a precooled glass plate under a stereoscopic microscope (Leica M220, Heidelberg, Germany). The SNc was dissected according to Paxinos and Watson (2007), frozen on dry ice, and stored at −80 °C until processed for Western blot (WB), ACE activity, and real time quantitative polymerase chain reaction studies (see the following sections).

The in vitro experiments were carried out with primary (i.e., neuron glia) mesencephalic cultures and cultures of primary mesencephalic astrocytes, MES 23.5 dopaminergic neuron cell line, and N9 microglial cell line. Laser confocal microscopy was used to study location of ER-α and ER-β in different cell types. In addition, cultures were treated with the dopaminergic neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) and/or AII and agonists of ER-α or ER-β to study the role of different cells (dopaminergic neurons, astrocytes, and microglial cells) in the interaction of estrogen receptors and RAS activity for neuroprotection.

2.2. Estrogen and candesartan administration and intrastriatal injection of 6-OHDA

Rats were bilaterally ovariectomized through a dorsal incision and simultaneously (group B) or 6 weeks (group C) or 20 weeks