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Inhibition of Rho kinase mediates the neuroprotective effects of estrogen in the MPTP model of Parkinson's disease

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ABSTRACT

The mechanism by which estrogen protects dopaminergic neurons has not yet been clarified. It is not known if changes in RhoA/Rho kinase activity are involved in the enhanced vulnerability of dopaminergic neurons observed after estrogen depletion. The present study shows that the MPTP-induced loss of dopaminergic neurons is increased by estrogen depletion and inhibited by estrogen replacement, the Rho kinase inhibitor Y27632 and deletion of the angiotensin type-1 receptor. In ovariectomized mice, treatment with MPTP induced a marked increase in Rho kinase activity, and RhoA and RhocK II mRNA and protein expression, which were significantly higher than in ovariectomized mice treated with MPTP and estrogen replacement or type-1 receptor deletion. Estrogen depletion increased Rho kinase activity, via enhancement of the angiotensin type-1 receptor pathway, and Rho kinase activation increased type-1 receptor expression suggesting a vicious cycle in which Rho kinase and type-1 receptor activate each other and promote the degenerative process. The results suggest that type-1 receptor antagonists and Rho kinase inhibitors may provide a new neuroprotective strategy, which may circumvent the potential risks of estrogen replacement therapy and be particularly useful in elderly women or women affected by long-term lack of estrogen.

Introduction

The results of studies with animal models (Dluzen, 1997; Murray et al., 2003) and clinical evidence (Liu and Dluzen, 2007; Ragonese et al., 2006a,b; Shulman, 2002) suggest that the ovarian steroid hormone 17β-estradiol (E2) exerts a neuroprotective effect against Parkinson's disease (PD). Furthermore, a number of epidemiological studies have reported that the incidence and prevalence of PD are higher in men (Baldereschi et al., 2000; Mayeux et al., 1995; Van Den Eeden et al., 2003) and postmenopausal women (Currie et al., 2004; Ragonese et al., 2006a,b) than in premenopausal women of similar age. Controversial effects of estrogen replacement therapy (ERT) have been also reported (Popat et al., 2005; Shulman, 2002). However, the effects of the timing of postmenopausal ERT and the age of the women receiving the treatment may be major factors in these discrepancies (Ragonese et al., 2006a,b; Rodriguez-Perez et al., 2012). The mechanism by which E2 protects dopaminergic (DA) neurons has not been clarified. Recent studies have suggested that modulation of the glial neuroinflammatory response by E2 is involved in the neuroprotective effects (Morale et al., 2006; Suzuki et al., 2007; Tripanichkul et al., 2006; Vegeto et al., 2008), and it is known that neuroinflammation and microglial activation play a major role in the progression of PD (Gerhard et al., 2006; Ouchi et al., 2005; Rodriguez-Pallares et al., 2007). It is known that the peptide angiotensin (AII), via type-1 (AT1) receptors, is one of the most important inducers of inflammation and oxidative stress in several tissues (Lühder et al., 2009; Ruiz-Ortega et al., 2001; Stegbauer et al., 2009). In several studies with male and female rats treated with 6-hydroxydopamine (i.e., 6-OHDA model of PD), we have shown that E2 inhibits the 6-OHDA induced neuroinflammatory response and dopaminergic cell death, and that inhibition of nigral renin–angiotensin (RAS) activity plays a major role in the anti-inflammatory and neuroprotective effects of E2 (Rodriguez-Perez et al., 2010, 2011, 2012).

We have shown for the first time that activation of the RhoA-ROCK II pathway is involved in the dopaminergic cell degeneration induced by MPTP and possibly in PD, and that ROCK inhibitors decrease MPTP-induced dopaminergic cell death (Villar-Cheda et al., 2012), which was recently confirmed by Tönges et al. (2012). The small

Abbreviations: 6-OHDA, 6-hydroxydopamine; AII, Angiotensin II; AT1, Angiotensin type-1 receptor; AT2, Angiotensin type-2 receptor; DA, Dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; E2, 17β-estradiol; ERT, estrogen replacement therapy; HPLC, High performance liquid chromatography; Ovx, Ovariectomized; PD, Parkinson's disease; RAS, Renin–angiotensin system system; ROCK, Rho-associated kinase; RT-PCR, Real time polymerase chain reaction; SNc, Substantia nigra compacta; TH, Tyrosine hydroxylase; TH-ir, TH-immunoreactivity; WB, Western blot.

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GTP-binding protein Rho plays an important role in several cellular functions and upregulates different molecules that accelerate inflammation and oxidative stress (Hiroki et al., 2004; Schmandke et al., 2007). RhoA, a member of the Rho family initiates cellular processes by acting on Rho-associated Kinase (ROCK), a direct downstream effector of RhoA (Katoh et al., 1998; Zhang et al., 2007). Abnormal activation of the RhoA/ROCK pathway has been observed in animal models of several brain diseases in which neuroinflammation may play a major role (Mueller et al., 2005; Toshima et al., 2000). However, it is not known if changes in RhoA/ROCK activity are involved in the enhanced vulnerability of dopaminergic neurons and enhanced neuroinflammatory response observed after E2 depletion, or in the beneficial effects induced by ERT. Furthermore, it is not known whether the increase in nigral RAS activity (i.e., AT1 receptor upregulation) induced by E2 depletion leads to increased RhoA/ROCK activity, which may mediate neuroinflammation and progression of dopaminergic neuron death. In the present study, we investigated these questions in the MPTP model of PD, using female (wild type or AT1-null) mice subjected to E2 depletion by ovariectomy and treated or not treated with E2.

Methods

Experimental design

C57BL-6 female mice (8 weeks old at the beginning of the experiments) were used in the present study. Mice were wild type (Charles River, France) or (group B5, see below) homozygous mice deficient for AT1a (the major mouse AT1 isoform and closest murine homolog to the single human AT1; Jackson Laboratory, Bar Harbor, ME, USA; Sugaya et al., 1995). The mice were fed with 2019S 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 approved by the corresponding committee at the University of Santiago de Compostela. All mice were anesthetized with ketamine/xylazine prior to surgery. Mice (excepting group A5) were subjected to E2 depletion by ovariectomy (ovx). Intact female mice have a 4–5-day estrous cycle (Byers et al., 2012), with a very short proestrus period (i.e. only 12 h with high levels of E2). Most intact mice are therefore expected to have levels of E2 similar to ovx mice when euthanized. Furthermore, intact mice have low levels of E2 during most of the MPTP lesion period and only several short (12 h) periods with high levels of E2, and they are thus not suitable for comparison with E2 treated and untreated models. In addition, ovx also removes other sex steroids (in addition to E2). However, only E2 was reintroduced in the ovx + E2 groups to confirm that the observed effects were due to E2 and not to other sex steroids. Three series of experiments were then carried out with different groups of mice (Table 1). In the first series of experiments (A; n = 6 per group), ovariectomized (ovx) mice received empty implants and vehicle treatment (group A1; controls), or implants containing 17β-estradiol and vehicle injections (group A2), or empty implants + treatment with the ROCK inhibitor Y-27632 (group A3), or empty implants + treatment with the AT1 receptor antagonist candesartan (group A4). In addition, a control group (A5) of intact mice in proestrus (i.e., levels of E2 similar to group A2; n = 8) was selected by vaginal cytology (see Byers et al., 2012; McLean et al., 2012) and immediately euthanized. In the second series of experiments (B; n = 12 per group), ovariectomized mice received empty implants and vehicle treatment (group B1; controls), or empty implants and treatment with MPTP (group B2), or implants containing 17β-estradiol and treatment with MPTP (group B3), or empty implants + MPTP + treatment with the ROCK inhibitor Y-27632 (group B4), or empty implants + treatment with MPTP + AT1 deletion (i.e., AT1-null mice ovariectomized and treated with MPTP; group B5). The motor behavior of mice was tested in the rotarod before lesion and 1 week after treatment with MPTP alone or MPTP and the different treatments.

Table 1
Experimental design.

Series	Group	n	Treatment	Methodology
A	A1	6	OVX + vehicle	Rho Kinase activity
	A2	6	OVX + E2	HPLC (E2 levels)
	A3	6	OVX + Y-27632	WB (AT1)
	A4	6	OVX + CAND	Immunohistochemistry (A5)
	A5	8	CONTROL (proestrus)	
B	B1	12	OVX + vehicle	Immunohistochemistry, Nissl
	B2	12	MPTP(×5) + OVX	HPLC (dopamine and
	B3	12	MPTP(×5) + OVX + E2	metabolites; E2 levels)
	B4	12	MPTP(×5) + OVX + Y-27632	Rho Kinase Activity
	B5	12	MPTP(×5) + OVX + AT1-null	RT-PCR (RhoA, Rock II)
				WB (RhoA, Rock II)
				Rotarod
C	C1	5	OVX + vehicle	Mass spectrometry
	C2	5	MPTP(×1) + OVX	(MPP + levels)
	C3	5	MPTP(×1) + OVX + E2	
	C4	5	MPTP(×1) + OVX + Y-27632	
	C5	5	MPTP(×1) + OVX + AT1-null	

OVX = ovariectomized; E2 = estrogen; CAND = candesartan; WB = western blot; HPLC = high performance liquid chromatography; RT-PCR = real time polymerase chain reaction.

Two weeks after ovariectomy (6 h after the last MPTP or vehicle injection) some mice in different groups were euthanized by decapitation to study RhoA and ROCK II expression and ROCK activity in the ventral midbrain including the substantia nigra compacta (SNc). In a previous study (Villar-Cheda et al., 2012), we observed a high level of ROCK activity in the SNc as soon as 1 h after the last MPTP injection, although a smaller increase in ROCK activity was also observed after 24 h after the first MPTP injection and 7 days after the last MPTP injection. Two isoforms encoded by two different ROCK genes have been described: ROCK I and ROCK II (Nakagawa et al., 1996). ROCK II (which is also known as ROCKα) is preferentially expressed in the brain. The brains were rapidly removed and the mesencephalon sliced coronally using a tissue chopper set to 1 mm. To obtain SNc, the individual 1 mm tissue slides were dissected on a pre-cooled glass plate under a stereoscopic microscope (Leica M220). The SNc was dissected according to Paxinos and Watson (1986), frozen on dry ice, and stored at −80 °C until processed for western blot (WB), real-time quantitative RT-PCR and enzyme immunoassay studies (see below). In addition, three weeks after ovariectomy (1 week after MPTP or vehicle treatment) some mice in the different groups (i.e. those used to study neuroprotective effects of treatments) were tested in the rotarod and then euthanized for histological (tyrosine hydroxylase immunohistochemistry and cresyl violet staining) and HPLC (dopamine and metabolites) studies, as described below.

In a third series of experiments (C; n = 5 per group), brains from mice treated with a single dose of MPTP (30 mg/kg) or MPTP and the different treatments were euthanized 90 min after the MPTP injection to quantify striatal levels of MPP^+ (Hows et al., 2004; Przedborski et al., 1996; see below). In order to confirm the efficiency of ovariectomies and implants, blood samples were obtained from mice treated with empty implants (ovx) or implants with E2 (ovx + E2), by cardiac puncture just immediately before the animals were euthanized. Blood was collected on ice and serum samples were immediately frozen at −80° until analysis for 17β-estradiol (see below).

Estrogen depletion by ovariectomy and replacement therapy

Female mice were bilaterally ovariectomized through a dorsal incision and received Silastic implants placed subcutaneously in the midscapular region (Dziuk and Cook, 1966; Febo et al., 2005). Silastic implants were prepared with Silastic® tubing (1.47 mm ID × 1.95 mm OD, Dow Corning 508-006; VWR Scientific, Bridgeport, NJ) as described by Febo et al. (2005). Briefly, 5-mm-long sections of tubing were sealed