Dopaminergic neuroprotection of hormonal replacement therapy in young and aged menopausal rats: role of the brain angiotensin system

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There is a lack of consensus about the effects of the type of menopause (surgical or natural) and of oestrogen replacement therapy on Parkinson’s disease. The effects of the timing of replacement therapy and the female’s age may explain the observed differences in such effects. However, the mechanisms involved are poorly understood. The renin-angiotensin system mediates the beneficial effects of oestrogen in several tissues, and we have previously shown that dopaminergic cell loss is enhanced by angiotensin via type 1 receptors, which is activated by ageing. In rats, we compared the effects of oestrogen replacement therapy on 6-hydroxydopamine-induced dopaminergic degeneration, nigral renin-angiotensin system activity, activation of the nicotinamide adenine dinucleotide phosphate oxidase complex and levels of the proinflammatory cytokine interleukin-1β in young (surgical) menopausal rats and aged menopausal rats. In young surgically menopausal rats, the renin-angiotensin system activity was higher (i.e. higher angiotensin converting enzyme activity, higher angiotensin type-1 receptor expression and lower angiotensin type-2 receptor expression) than in surgically menopausal rats treated with oestrogen; the nicotinamide adenine dinucleotide phosphate oxidase activity and interleukin-1β expression were also higher in the first group than in the second group. In aged menopausal rats, the levels of nigral renin-angiotensin and nicotinamide adenine dinucleotide phosphate oxidase activity were similar to those observed in surgically menopausal rats. However, oestrogen replacement therapy significantly reduced 6-hydroxydopamine-induced dopaminergic cell loss in young menopausal rats but not in aged rats. Treatment with oestrogen also led to a more marked reduction in nigral renin-angiotensin and nicotinamide adenine dinucleotide phosphate oxidase activity in young surgically menopausal rats (treated either immediately or after a period of hypo-oestrogenicity) than in aged menopausal rats. Interestingly, treatment with the angiotensin type-1 receptor antagonist candesartan led to remarkable reduction in renin-angiotensin system activity and dopaminergic neuron loss in both groups of menopausal rats. This suggests that manipulation of the brain renin-angiotensin system may be an efficient approach for the prevention or treatment of Parkinson’s disease in oestrogen-deficient females, together with or instead of oestrogen replacement therapy.

Keywords: ageing; angiotensin; oestrogen; menopause; Parkinson’s disease
Introduction

Sex steroids have been shown to have a neuroprotective role in several models of neurological diseases, although the effects of the loss of ovarian function and hormonal replacement therapy in humans are controversial. Numerous observational studies have supported the concept that oestrogen therapy in postmenopausal females protects against ageing-related diseases, including cardiovascular diseases, stroke and neurodegeneration. This was not confirmed in several randomized controlled trials that reported no or even detrimental effects (Rossouw et al., 2002; Chlebowski et al., 2010). Several possible explanations have been proposed for the discrepant results (Clarkson and Mehaffey, 2009). However, the age of the females receiving the treatment appears to be a major factor. The vast majority of females participating in these trials were on average ≥65 years, and had started oestrogen therapy 12 years after undergoing menopause (Turgeon et al., 2004, 2006); on the contrary, in observational studies that reported beneficial effects, most females had initiated oestrogen therapy in their perimenopausal period (Harman et al., 2005; Miller et al., 2005, 2009). The mechanisms underlying the differences in the effects of oestrogen therapy between young perimenopausal females (including early surgical menopause) and senescent females are still poorly understood (Sohrabi and Bake, 2006; Suzuki et al., 2007).

Experiments with animal models (Dluzen, 1997; Murray et al., 2003), epidemiological and clinical evidence (Shulman, 2002; Ragonese et al., 2006a, b; Liu and Dluzen, 2007) suggest that sex hormones have a beneficial influence on the risk, onset and severity of Parkinson’s disease, and that oestrogen modulates dopaminergic function. The anti-inflammatory effects of oestrogen appear to play a major role in the neuroprotective effects on Parkinson’s disease (Suzuki et al., 2007; Vegeto et al., 2008). However, conflicting findings have 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 versus natural) and of the oestrogen therapy on Parkinson’s disease (Ragonese et al., 2006a, b; Miller et al., 2009). As already mentioned for other diseases, the effects of the timing of postmenopausal oestrogen therapy and the age of the females receiving the treatment may be a major factor.

The renin-angiotensin system mediates the beneficial effects of oestrogen in several tissues (Nickenig et al., 1998; Dean et al., 2004; Chen et al., 2008), and interactions between oestrogen and angiotensin receptors have also been observed (Liu et al., 2002; Tasuda et al., 2005; Xue et al., 2007; Hoshi-Fukushima et al., 2008). In addition, the local renin-angiotensin system has been reported to be involved in age-related degenerative changes in several tissues (Heymes et al., 1998; Basso et al., 2005; Benigni et al., 2009). The peptide angiotensin, via type 1 (AT1) receptors, is one of the most important known inducers of inflammation and oxidative stress, and produces reactive oxygen species by activation of the NADPH oxidase complex (Seshiah et al., 2002; Touyz et al., 2002; Cai et al., 2003), which is the most important intracellular source of reactive oxygen species apart from mitochondria (Babior, 1999, 2004). The brain possesses a local renin-angiotensin system (Mckinley et al., 2003; Savedra et al., 2005), and we have previously shown that the dopaminergic cell loss is enhanced by angiotensin via AT1 receptors (Rey et al., 2007; Rodriguez-Pallares et al., 2008, Joglar et al., 2009). In addition, we have observed that oestrogen decreases and ageing increases the effect of the nigral renin-angiotensin system on enhancing dopaminergic neuron degeneration in young female rats and aged male rats, respectively (Rodriguez-Perez et al., 2010; Villar-Cheda et al., 2010b). It is therefore possible that ageing modifies the beneficial effects of oestrogen therapy in postmenopausal females, and that the nigral renin-angiotensin system plays a major role. In the present study, we compared the effects of oestrogen therapy on dopaminergic degeneration, nigral renin-angiotensin system activity, activation of the NADPH oxidase complex and levels of the pro-inflammatory cytokine interleukin-1β (IL-1β) in rat models of untreated and treated (either immediate or delayed oestrogen therapy) surgical (young ovariectomized rats) and natural (aged menopausal rats) menopause, and investigated if renin-angiotensin system manipulation may improve or replace the effects of oestrogen therapy.

Materials and methods

Experimental design

Young adult and aged acyclic female Sprague-Dawley rats (10 weeks and 24 months old at the beginning of the experiments, respectively) were used. All experiments were carried out in accordance with the ‘Principles of laboratory animal care’ (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the University of Santiago de Compostela. All surgery was performed under ketamine/xylazine anaesthesia, and rats were fed with 2019S Teklad Rodent Maintenance Diet (Harlan Laboratories) to minimize the occurrence of natural phytoestrogens. Five rat models corresponding to five major possibilities in clinical menopause were studied: untreated surgical menopause (young ovariectomized rats), surgical menopause immediately treated with oestrogen therapy, surgical menopause treated with oestrogen therapy after a period of hypo-oestrogenicity, untreated natural menopause (untreated aged rats), and natural menopause treated with oestrogen therapy. In addition, a group of aged rats were ovariectomized and treated with oestrogen to study the potential effects of the presence of ovaries on renin-angiotensin system activity in aged rats.

The rats were divided into six groups. Rats in Group A were young rats (10-weeks-old at the beginning of the experiments), which were ovariectomized (i.e. menopause was induced surgically) and given empty implants (n = 38). Rats in Group B were young rats that were ovariectomized and simultaneously received implants containing 17β-oestradiol (n = 31). Rats in Group C were treated as Group B rats but the oestrogen implants were given 3 weeks after ovariectomy. Rats in Group D were aged rats (24 months old; aged; n = 31) given empty implants (i.e. natural senescent menopause). Rats in Group E were aged rats given implants containing 17β-oestradiol (n = 25). Rats in Group F were aged rats that were ovariectomized and given implants containing
17β-oestradiol (n = 5). Two series of experiments were carried out with the different groups of rats (Groups A–F). In the first series of experiments, young ovariectomized or aged menopausal rats (n = 55) were used to determine the effect of oestrogen and candesartan (i.e. inhibition of renin-angiotensin system activity by this AT1 receptor antagonist) or treatment with oestrogen + candesartan on the dopaminergic degeneration induced by the neurotoxin 6-hydroxydopamine, relative to rats in the different groups treated with 6-hydroxydopamine alone and compared with the corresponding controls injected with vehicle. A second series of experiments was carried out to investigate the effect of treatment with oestrogen or candesartan on markers of NADPH oxidase activity/oxidative stress, inflammation and renin-angiotensin system activity in young ovariectomized and aged menopausal rats (n = 87). Rats in the first series of experiments were injected intrastrially with 6-hydroxydopamine or vehicle (controls) and treated with oestrogen and/or candesartan or vehicle (controls), then killed for immunohistochemical studies (i.e. quantification of dopaminergic cell death), as described below. Rats in the second series of experiments were killed by decapitation 3 weeks after ovariectomy and/or treatment with implants. The brains were rapidly removed and coronal slices of the mesencephalon were cut with a tissuecutter set to 1 mm. To obtain substantia nigra compacta, the individual 1-mm tissue slides were dissected on a pre-cooled glass plate under a stereoscopic microscope (Leica M220). The substantia nigra compacta was dissected according to Paxinos and Watson (1985), frozen on dry ice, and stored at −80°C until processed for investigation of expression of AT1 and AT2 receptors, expression of the NADPH oxidase cytosolic subunit p47phox and expression pro-inflammatory cytokine IL-1β by western blot and reverse transcriptase–polymerase chain reaction studies.

Expression of the NADPH oxidase cytosolic subunit p47phox is an indicator of the level of activation of the NADPH oxidase complex. The NADPH oxidase complex is composed of membrane-bound and cytosolic subunits such as p47phox, which is considered key for NADPH oxidase activation (Li and Shah, 2003). Translocation of cytosolic subunits to the membrane, which leads to reactive oxygen species generation, is a necessary step for NADPH oxidase activation. The level of the NADPH oxidase subunit p47phox is correlated with NADPH oxidase activity and NADPH-derived superoxide formation (Rueckschloss et al., 2002; Touyz et al., 2002). Finally, angiotensin converting enzyme activity was determined by lucigenin-enhanced chemiluminescence (see below).

**Oestrogen and candesartan administration**

Rats were bilaterally ovariectomized through a dorsal incision and simultaneously (Groups A, B and F) or 3 weeks later (Group C) received Silastic implants, placed subcutaneously in the midscapular region (Dziuk and Cook, 1966; Febo et al., 2005). Aged acyclic rats (Groups D and E) received similar implants without prior ovariectomy. Silastic implants were prepared with Silastic® tubing (1.47 mm inner diameter x 1.95 mm outer diameter, Dow Corning 508-006; VWR Scientific), as described by Febo et al. (2005). Briefly, 5-mm long sections of tubing were sealed at one end with Silastic silicone sealant (Dow Corning 732; VWR) and left to dry for 30 min. The implants were then either filled with crystalline 17β-oestradiol (17β-oestradiol benzoate; Sigma-Aldrich; Groups B, C, E and F) or were left empty (Groups A and D); the open end was then sealed in the same way as the other end. Implants were air-dried and incubated in sterile saline for at least 12–16 h to allow the initial surge of high oestradiol levels to be released before use. It has been observed that such implants achieve stable levels of plasma oestradiol over 30 days, with a release rate of 75–100 pg/ml per 24 h (Febo et al., 2005), as confirmed in our previous studies (Rodriguez-Perez et al., 2010). However, stable levels of oestrogen have also been found to persist for only 7–24 days (Mannino et al., 2005). Therefore, rats were killed 3 weeks after oestrogen implantation (i.e. 2 weeks after 6-hydroxydopamine injection).

Some rats in the different groups received candesartan in their drinking water (candesartan cilexetil, AstraZeneca; 3 mg/kg/day) from 7 days before the empty or oestrogen implants were fitted until they were killed. The oral bioavailability of candesartan cilexetil is 5–10% oral (Kondo et al., 1996; Zorad et al., 2006); the drug was diluted (0.025 mg/ml) following the protocol suggested by AstraZeneca and administered to the rats in their drinking water (25–30 ml/day/rat). It has been reported 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 effects (Unger, 2003).

**Intrastral injection of 6-hydroxydopamine**

One week after receiving empty or oestrogen implants, some rats in the different groups were injected intrastrially with 6-hydroxydopamine or vehicle. Thirty minutes prior to intrastriatal injection with 6-hydroxydopamine or vehicle, rats were treated with the selective inhibitor for the norepinephrine transporter desipramine (Sigma, 25 mg/kg i.p.) to prevent uptake of 6-hydroxydopamine by noradrenergic terminals. The rats were injected in the right striatum with 7 μg of 6-hydroxydopamine (in 3 μl of saline containing 0.2% ascorbic acid; Sigma). Stereotaxic coordinates were 0.8 mm anterior to bregma, 3.0 mm right of midline and 5.0 mm ventral to the dura; tooth bar at −3.3. Control animals were injected with 3 μl of vehicle alone. Rats were killed 2-week post-lesion (i.e. 3 weeks post-implant). Previous studies on the time course of 6-hydroxydopamine lesions have shown that the loss of tyrosine hydroxylase immunoreactive neurons is complete (Przedborski et al., 1995) or practically complete (Sauer and Oertel, 1994) 2 weeks after administration of intrastriatal injections. Although a few dopaminergic neurons may degenerate after the 2-week period, we considered it more important to kill the rats before any possible loss of oestrogen levels (i.e. 3 weeks after implantation and 2 weeks after 6-hydroxydopamine injection).
Radioimmunoassay
In order to confirm the efficiency of ovarioctomies and implants, blood samples were taken from the rats by cardiac puncture just before the animals were killed. Blood was collected on ice and serum samples immediately frozen at −70°C until analysis for 17β-oestradiol by radioimmunoassay, with the Diagnostic Products Corporation Coat-a-Count kit and protocol. The samples were counted for 1 min in a gamma counter, and assayed in triplicate.

RNA extraction and real-time quantitative reverse transcriptase–polymerase chain reaction
Total RNA from the nigral region was extracted with TRizol® (Invitrogen), according to the manufacturer’s instructions. Total RNA (2.5 µg) was reverse-transcribed to complementary DNA with deoxynucleotide triphosphates, random primers and Moloney murine leukaemia virus reverse transcriptase (M-MLV; 200 U; Invitrogen). Real-time polymerase chain reaction was used to examine relative levels of angiotensin receptors type 1 (AT1a) and type 2 (AT2) messenger RNA, p47 and IL-1β. Experiments were performed with a real-time iCycler™ polymerase chain reaction platform (Bio-Rad). β-Actin was used as housekeeping gene and was amplified in parallel with the genes of interest. The comparative Ct method was used to examine the relative messenger RNA expression. The expression of each gene was obtained as relative to the housekeeping transcripts. The data were then normalized to the values of the control group of the same batch (i.e. expressed as a percentage of the control values; 100%) to counteract any possible variability among batches. GAPDH and β-actin were simultaneously included as loading control in some experiments to exclude any potential effects of oestrogen on the internal control: no significant differences were detected. Finally, the results were expressed as means ± SEM.

Angiotensin converting enzyme activity in ventral mesencephalic tissue was assayed with hippuryl-l-histidyl-l-leucine (Hip-His-Leu; Sigma) as substrate, as described by Hemming et al. (2007). Fluorescence was assayed in 96-well plates in an Infinite M200 multi-well plate reader (TECAN; excitation, 355; emission, 535) and determined as nmol his-leu/mg protein/min. The data were then normalized to the values of the control group of the same batch (i.e. expressed as a percentage of the control values; 100%)

Immunohistochemistry and dopaminergic neuron quantification

The animals used for immunohistochemistry (i.e. those injected intrastriatally with 6-hydroxydopamine or vehicle) were first perfused 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 20% sucrose, and finally cut into 40-µm sections on a freezing microtome. The sections were incubated for 1 h in 10% normal swine serum with 0.25% Triton X-100 in 20 mM potassium phosphate-buffered saline containing 1% bovine serum albumin and then incubated overnight at 4°C with a mouse monoclonal anti-tyrosine hydroxylase (Sigma; 1:10 000), as dopaminergic marker. The sections were subsequently incubated, first for 60 min with the corresponding biotinylated secondary antibody, and then for 90 min with avidin–biotin–peroxidase complex (ABC, 1:100, Vector). Finally the labelling was revealed by treatment with 0.04% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine (DAB, Sigma). In all experiments the control sections, in which the primary antibody was omitted, were immunonegative for these markers.

The total number of tyrosine hydroxylase immunoreactive neurons in the substantia nigra compacta was estimated by an unbiased stereological method (the optical fractionator).
The stereological analysis was carried out with the Olympus CAST-Grid system (Computer Assisted Stereological Toolbox; Olympus). Uniform randomly chosen sections through the substantia nigra (every fourth section) were analysed for the total number of tyrosine hydroxylase immunoreactive cells by means of a stereological grid (fractionator), and the nigral volume was estimated according to Cavalieri’s method (Gundersen et al., 1988). To confirm that 6-hydroxydopamine induces cell death, series of sections through the entire substantia nigra of control rats and rats treated with 6-hydroxydopamine were counterstained with Cresyl violet, and the total number of neurons in the substantia nigra was estimated by the unbiased stereology method described above for tyrosine hydroxylase immunoreactive cells. Neurons were distinguished from glial cells on a morphological basis, and neurons with visible nuclei were counted as above for tyrosine hydroxylase immunoreactive neurons (see Rey et al., 2007 for details).

Statistical analysis

All data were obtained in triplicate (i.e. from each rat at least three samples were independently processed and the mean value for each rat was used for statistical analysis). Data were expressed as means ± SEM. Two group comparisons were analysed by a Student’s t-test and multiple comparisons were analysed by one-way ANOVA followed by a post hoc Bonferroni test. The normality of populations and homogeneity of variances were tested before each ANOVA. Differences were considered significant at \( P < 0.05 \). Statistical analyses were carried out with SigmaStat 3.0 from Jandel Scientific.

Results

Effect of the dopaminergic neurotoxin 6-hydroxydopamine on different groups of menopausal rats

Intrastratial injection of 6-hydroxydopamine induced a marked loss of dopaminergic neurons in the substantia nigra of aged rats and young ovariectomized rats, which was significantly higher than that induced by 6-hydroxydopamine in young ovariectomized rats immediately treated with oestrogen, or young ovariectomized rats treated with oestrogen 3 weeks after ovariectomy. The loss of dopaminergic neurons induced by the neurotoxin in aged rats treated with oestrogen tended to be lower than in untreated aged rats, but the difference was not significant. Interestingly, however, the loss of dopaminergic neurons was significantly lower in both groups of menopausal rats treated with the AT1 antagonist candesartan, and treatment with oestrogen + candesartan did not increase the neuroprotective effect induced by treatment with candesartan alone (Fig. 1A–E). Therefore, the present results indicate that either oestrogen or candesartan induced significant neuroprotection against 6-hydroxydopamine in young menopausal rats (surgically induced menopause). However, only candesartan induced significant neuroprotection in aged menopausal rats (natural menopause).

Renin-angiotensin system activity in young surgically menopausal and aged menopausal rats

Both groups of menopausal rats showed very low blood levels of oestrogen. Blood levels of oestrogen in young ovariectomized rats were 18.6 ± 2.4 pg/ml, and similar to those observed in aged menopausal rats (26.2 ± 4.5 pg/ml). Comparison between both groups of menopausal rats did not reveal any significant differences in the expression of AT2 and the NADPH subunit p47phox and IL-β (Fig. 2A and B). However, differences were observed between both groups of menopausal rats in AT1 receptor expression; AT1 messenger RNA expression was higher in aged rats. Since the present and previous studies have shown that oestrogen affects AT1 expression at post-transcriptional levels (see below), the increased messenger RNA expression in aged rats must be related to ageing-related factors other than low levels of oestrogen. Interestingly, AT1 protein expression was higher in young ovariectomized rats, which suggests that the lack of oestrogen affects the expression of AT1 receptors to a greater extent in young ovariectomized rats than in aged rats. Similar levels of AT2 messenger RNA and protein expression in young ovariectomized and aged rats also suggest that ageing-related factors other than low levels of oestrogen reduce the expression of AT2 receptors in aged rats, because the lack of oestrogen reduced AT2 receptor levels in young rats but did not significantly change AT2 receptor levels in aged rats (see below). Angiotensin converting enzyme activity was 0.0798 ± 0.00723 nmol his-leu/mg protein/min in ovariectomized rats and 0.0890 ± 0.0117 in aged rats.

Effect of oestrogen therapy in young surgically menopausal and aged menopausal rats compared with the corresponding untreated menopausal rats

In another set of experiments, we tested the effects of oestrogen therapy with respect to the corresponding untreated menopausal rats. Blood levels of oestrogen in young ovariectomized rats that received implants with oestrogen were 144.8 ± 16.7 pg/ml, which were similar to values observed in normal rats in proestrous (refer to ‘Discussion’ section). Plasma oestrogen concentrations in aged menopausal rats that received implants with oestrogen were not significantly different from those of young ovariectomized rats treated with oestrogen (151.1 ± 22.4 pg/ml immediately before sacrifice). However, the effects of oestrogen treatment on renin-angiotensin system activity were different. In young surgically ovariectomized rats, treatment with oestrogen at the time of ovariectomy induced a marked decrease in renin-angiotensin system activity as well as in the expression of the NADPH oxidase subunit and the pro-inflammatory cytokine IL-1β. The AT1 receptor protein decreased by ~80%. However, there were no
Figure 1  Dopaminergic (tyrosine hydroxylase immunoreactive, TH-ir) neurons (A–E) in the substantia nigra compacta (SNC) 2 weeks after intrastriatal injection of vehicle or 6-hydroxydopamine (6-OHDA) in young ovariectomized (ovx) rats or aged acyclic rats not treated or treated with 17-β-oestradiol, either immediately (E2) or 3 weeks later (+E2), and/or the AT1 antagonist candesartan (CAND). Representative photomicrographs of the substantia nigra compacta of different groups of aged rats are shown in B–E. The estimated total number of dopaminergic (tyrosine hydroxylase immunoreactive) neurons in the substantia nigra compacta of the different experimental groups is shown in A. Data are means ± SEM; n = 5. *P < 0.05 relative to the corresponding vehicle-treated group (ovariectomized or aged rats), +P < 0.05 relative to the group treated with ovariectomized + 6-hydroxydopamine alone, #P < 0.05 relative to the group aged + 6-hydroxydopamine alone, *P < 0.05 relative to the group ovariectomized + 6-hydroxydopamine + oestrogen and ovariectomized + t + 6-hydroxydopamine + E2 (one-way ANOVA and Bonferroni post hoc test). Scale bar = 500 µm.
significant changes in AT1 messenger RNA expression, which suggests that oestrogen regulates AT1 expression at a post-transcriptional level, possibly via cytosolic proteins that bind to cis elements in the 5’ leader sequence of the AT1 messenger RNA, interfere with ribosomal scanning and inhibit translational efficiency as observed in other cell types (Krishnamurthi et al., 1999; Wu et al., 2003b). In young menopausal rats, treatment with oestrogen induced a marked increase in AT2 messenger RNA and protein expression (~200%), and a significant decrease in angiotensin converting enzyme activity. Angiotensin converting enzyme activity was 0.0798 ± 0.00723 nmol his-leu/mg protein/min in ovariectomized rats and 0.0562 ± 0.00419 in rats with surgical menopause immediately treated with oestrogen therapy. In accordance, the results indicate that oestrogen induced a significant decrease in NADPH-derived reactive oxygen species and pro-inflammatory changes, as revealed by decreased p47phox expression and IL-1β expression, respectively (Fig. 3A and B).

The effect of treatment with oestrogen in aged acyclic rats was, however, different. Oestrogen induced a significant decrease in AT1 protein expression although much less than in young ovariectomized rats (i.e. 76.7% and 22.4% of the corresponding untreated menopausal rats, respectively), and no significant changes were observed in AT2 and p47phox messenger RNA or protein expression. Slight but significant decreases were observed in angiotensin converting enzyme activity (0.0890 ± 0.00117 and 0.0666 ± 0.00182) and IL-1β expression (~70% of untreated aged controls; Fig. 3C and D).

**Effect of delayed oestrogen therapy in young surgically menopausal rats and in aged ovariectomized rats**

As previously observed in young rats immediately subjected to oestrogen therapy, renin-angiotensin system activity and expression of the NADPH oxidase subunit and the pro-inflammatory cytokine IL-1β were lower in young ovariectomized rats treated with oestrogen 3 weeks after ovariectomy than in untreated ovariectomized rats. The AT1 receptor protein was ~80% lower, although there were no significant changes in AT1 messenger RNA expression (see above). In addition, AT2 messenger RNA and protein expression were significantly higher (~140%), and angiotensin converting enzyme activity (0.0798 ± 0.00723 and 0.0564 ± 0.00374, respectively), p47phox expression and IL-1β expression were significantly lower than in untreated ovariectomized rats (Fig. 4A and B). Interestingly, however, comparison between young ovariectomized rats subjected to either immediate or delayed oestrogen therapy revealed significant differences in the expression of AT2 receptors, which was significantly lower in the rats subjected to a 3-week period of hypo-oestrogenicity (Fig. 4C and D).

As it has been reported that significant levels of androgens are produced in the ovaries of aged rats, and androgens may affect renin-angiotensin system activity (see ‘Discussion’ section), a group of aged rats were ovariectomized before oestrogen therapy and compared with intact aged rats treated with oestrogen therapy. However, no significant differences were observed in the expression of AT1, AT2, p47, IL-1β and angiotensin converting enzyme (0.0794 ± 0.00667 and 0.0666 ± 0.00182, respectively) between the two groups of aged menopausal rats treated with oestrogen therapy (Fig. 5A and B).

**Effect of treatment with candesartan in young menopausal and aged menopausal rats**

Contrary to what was observed after oestrogen therapy, treatment with the AT1 antagonist candesartan induced similar effects in
young ovariectomized and aged menopausal rats. In young menopausal rats, candesartan induced a significant increase in the messenger RNA and protein expression of AT2 receptors, as well as a decrease in the expression of the NADPH oxidase subunit p47phox and the pro-inflammatory cytokine IL-1β. Changes in angiotensin converting enzyme activity were statistically not significant (0.0798 ± 0.0072 and 0.0650 ± 0.0111; Fig. 6A and B).

In aged menopausal rats, treatment with the AT1 blocker candesartan also induced a significant increase in the messenger RNA and protein expression of AT2 receptors, as well as a marked decrease in the expression of the NADPH oxidase subunit p47phox and the pro-inflammatory cytokine IL-1β. In aged rats, treatment with candesartan, therefore, induced higher anti-inflammatory and antioxidant effects than treatment with oestrogen. Changes in angiotensin converting enzyme activity were statistically not significant (0.0890 ± 0.0117 and 0.0889 ± 0.00805, respectively; Fig. 6C and D).

**Discussion**

The present results confirm that oestrogen therapy protects against dopaminergic degeneration induced by neurotoxins in young rats subjected to surgical menopause and immediate oestrogen therapy, and that this effect is lower in aged rats. Interestingly, oestrogen administered to young rats after a period of hypo-oestrogenicity was also neuroprotective, although the rats showed some changes in renin-angiotensin system activity that suggest that the effectiveness of oestrogen therapy may
decrease after very long periods of hypo-oestrogenic state in young animals or in older animals subjected to hypo-oestrogenicity. The present results may explain discrepancies among previous studies in which neither the age of the subjects nor the time postmenopause prior to oestrogen therapy were taken into account. The mechanism involved in the beneficial effects of oestrogen has not been totally clarified. However, a growing body of evidence from preclinical and clinical studies suggests that anti-inflammatory actions are fundamental for the protective actions of oestrogen (Turgeon et al., 2006; Suzuki et al., 2007; Chen et al., 2008). Recent studies have also shown that anti-inflammatory actions are fundamental for the protective actions of oestrogen (Turgeon et al., 2006; Tripanichkul et al., 2006); it is also known that neuroinflammation and microglial activation play a major role in the progression of Parkinson's disease (Wu et al., 2002, 2003a; Rodriguez-Pallares et al., 2007). Interestingly, however, recent studies have shown that oestrogen attenuates inflammatory cytokine production following brain lesions in young adult female rats but not in acyclic reproductive senescent females (Johnson et al., 2006), and that oestrogen suppresses microglial activation and cytokine expression in cultured microglia derived from neonatal or foetal sources, but failed to do so in cultures of microglia from senescent females (Sohrabji, 2005; Johnson et al., 2006). The present results support this view, and suggest that the brain renin-angiotensin system plays a major role in these effects.

Figure 4  Real-time quantitative reverse transcriptase–polymerase chain reaction (A and C) and western blot (B and D) analysis of changes induced by treatment with 17-β-oestradiol (E2) in the expression of AT1, AT2, p47phox and IL-1β, and the activity of the angiotensin converting enzyme (ACE; B and D) in young ovariectomized rats subjected to a 3-week period of hypo-oestrogenicity (ovx + t + E2) in comparison with untreated young menopausal rats (ovx; A and B) and young menopausal rats immediately treated with oestrogen (ovx + E2; C and D). Protein expression was obtained as relative to the GAPDH band value and the expression of each gene was obtained relative to the housekeeping transcripts (β-actin). The results were then normalized to the values for ovariectomized (A and B) or ovx + E2 (C and D) rats (100%). Data are means ± SEM; n = 6. *P < 0.05 (Student's t-test).
In the present study, we observed increased renin-angiotensin system activity in young rats subjected surgical menopause (ovariectomized) relative to rats with stable high levels of oestrogen (i.e. surgical menopause treated with oestrogen therapy). Increased angiotensin converting enzyme activity, increased AT1 expression and decreased AT2 expression, as well as increased NADPH activity and IL-1β expression were observed in untreated surgical menopausal rats. Comparison between young surgical menopausal rats and aged menopausal rats revealed that renin-angiotensin system activity was also exacerbated in aged menopausal rats. Both groups of menopausal rats showed similar levels of NADPH activity and AT2 receptor expression. The AT1 messenger RNA expression was higher in aged rats than in young ovariectomized rats. Since oestrogen affects AT1 expression at post-transcriptional levels, this suggests that additional factors increase renin-angiotensin system activity in aged rats (see below). However, AT1 protein expression was higher in young ovariectomized rats than in aged menopausal rats, suggesting that the effect of the presence/absence of oestrogen is more important in surgical menopause than in natural menopause, which was confirmed in menopausal rats treated with oestrogen. Similar levels of AT2 messenger RNA and protein expression in young ovariectomized and aged rats also suggest that ageing-related factors other than low levels of oestrogen reduced the expression of AT2 receptors in aged rats, because the lack of oestrogen decreased AT2 receptor levels in young rats but did not significantly change AT2 receptor levels in aged rats.

The observed upregulation of renin-angiotensin system activity may contribute to increased dopaminergic cell vulnerability. It is well known that angiotensin acts via AT1 receptors inducing inflammatory responses and releasing high levels of reactive oxygen species, mainly by activation of the NADPH complex in vascular degenerative disease and other diseases mediated by oxidative stress and chronic inflammation (Touyz, 2002; Qin et al., 2004). In the nigrostriatal system in animal models of Parkinson’s disease, we have previously shown that brain angiotensin induces activation of the NADPH complex via AT1 receptors, leading to increased neuroinflammation, reactive oxygen species and dopaminergic cell death (Rey et al., 2007; Rodriguez-Pallares et al., 2008; Joglar et al., 2009). We have shown NADPH expression in dopaminergic neurons and microglial cells. However, it is known that in non-inflammatory cells, such as neurons, the NADPH complex produces only low rates of reactive oxygen species for signalling function. In inflammatory cells such as microglia, NADPH activation produces high concentrations of reactive oxygen species that are released extracellularly to kill invading microorganisms or cells (Babior, 1999, 2004). In accordance with this, we observed that angiotensin was not able to increase dopaminergic neuron death in the absence of microglial cells (Rodriguez-Pallares et al., 2008; Joglar et al., 2009). The increased angiotensin converting enzyme activity in menopausal rats leads to increased angiotensin production. Increased angiotensin converting enzyme activity has also been observed in other tissues in postmenopausal females (Nogawa et al., 2001) and in experimental models of cardiovascular disease after ovariectomy (Wu et al., 2003b; Dean et al., 2005). The observed upregulation of AT1 receptors may also contribute to NADPH activation and increased dopaminergic cell vulnerability. This is supported by the present experiments in which we have observed that the enhanced susceptibility of dopaminergic neurons was significantly decreased by AT1 receptor inhibition with candesartan. Furthermore, it is particularly interesting that untreated surgically menopausal rats had significantly fewer AT2 receptors than young rats treated with...
Oestrogen, which may also enhance dopaminergic cell loss. AT1 and AT2 receptors have opposing effects and AT2 receptors counterbalance the deleterious effect of AT1 receptor stimulation, so that functional interactions between the two receptor subtypes and their specific distribution determine the angiotensin-induced effects (Sohn et al., 2000), which in the case of untreated menopausal rats resulted in a pro-oxidative and pro-inflammatory state, as suggested by the increased NADPH activity and IL-1β expression in comparison with rats treated with oestrogen. The detailed mechanisms involved in the above described regulation of renin-angiotensin system components by oestrogen have not been clarified for the brain or peripheral tissues. Oestrogen has been suggested to inhibit the angiotensin-induced NADPH activation and production of reactive oxygen species (Xue et al., 2007, 2008), to inhibit AT1 receptor-mediated extracellular signal-regulated kinase activation (Liu et al., 2002), and to inhibit angiotensin-induced activation of mitogen-activated protein kinases (Imanishi et al., 2005) in different tissues.

In the present study, it is particularly interesting that the response to oestrogen therapy was different in young (i.e. surgically induced menopause) and aged menopausal rats. The effects of treatment with oestrogen on AT1, AT2 receptor expression and NADPH activity were lower in aged rats than in young menopausal rats. In accordance with this, oestrogen therapy significantly reduced 6-hydroxydopamine-induced dopaminergic cell loss in young rats but not in aged rats. Oestrogen regulation of AT2 levels appears particularly important (Okumura et al., 2005; Chakrabasty et al., 2008; Sakata et al., 2009). AT2 receptors are particularly abundant in tissues during development and decrease after birth, although they are upregulated under pathological conditions to counter-regulate pro-inflammatory effects mediated by AT1 receptors. The present results suggest that

Figure 6 Real-time quantitative reverse transcriptase–polymerase chain reaction (A and C) and western blot (B and D) analysis of changes induced by treatment with the AT1 antagonist candesartan in the expression of AT2, p47phox and IL-1β, and the activity of the angiotensin converting enzyme (ACE; B and D) in young ovariectomized rats (ovx + candesartan; A and B) and aged acyclic rats (aged + candesartan; C and D) in comparison with the corresponding untreated rats (ovx or aged rats). Protein expression was obtained relative to the GAPDH band value and the expression of each gene was obtained relative to the housekeeping transcripts (β-actin). The results were then normalized to the values for ovariectomized (A and B) or aged (C and D) rats (100%). Data are means ± SEM; n = 6. *P < 0.05 (Student’s t-test).
ageing disrupts the ability of the cell to respond to oestrogen by upregulation of AT2 receptors (Armando et al., 2002; Suarez et al., 2004). Interestingly, the results observed in rats subjected to delayed oestrogen therapy indicate that the treatment is able to reverse changes induced by a period of hypo-oestrogenicity in young animals (i.e. the increase in renin-angiotensin system activity, and in markers of oxidative stress, neuroinflammation and dopaminergic neuron vulnerability). However, the oestrogen-induced upregulation of AT2 receptor expression was significantly lower than in young ovariectomized rats treated immediately with oestrogen therapy. It is therefore possible that very long periods of hypo-oestrogenicity in young animals and/or delayed oestrogen therapy in older rats decreases the neuroprotective effects of oestrogen therapy.

Different responses to oestrogen therapy in young menopausal rats and aged rats have also been observed in other experimental models. Thus, oestrogen therapy has been shown to protect against other diseases such as atherosclerosis, although only when the vessels are healthy and not when atherosclerosis is already established (Clarkson, 2007, 2009; Antonicelli et al., 2008). However, the mechanism responsible for the different response to oestrogen therapy in young and aged animals (i.e. surgical and natural menopause) has not been clarified. In the present study, young ovariectomized rats and aged rats treated with oestrogen did not show significant differences in blood levels of oestrogen, which were similar to those previously observed with similar implants (Febo et al., 2002; Sandoval and Witt, 2011), and similar to those observed in intact rats in prooestrus (Nequin et al., 1979). The above reported differences are therefore due to differences in the response to oestrogen between young and aged rats. Several recent studies have reported that significant levels of androgens are produced in the ovaries of aged rats (Fogle et al., 2009; Korke et al., 2009), but not in young ovariectomized rats (surgically induced menopause), and it has been suggested that administration of androgens induce activation of renin-angiotensin system in several tissues (Fisher et al., 2002; Henriques et al., 2008; Ojeda et al., 2010). Androgens derived from aged ovaries may therefore counteract the inhibitory effect of oestrogen therapy on the nigral renin-angiotensin system. However, we have observed that oestrogen therapy did not have significantly different effects in aged ovariectomized rats and intact aged rats. Furthermore, renin-angiotensin system activity has also been found to be higher in aged males than in young males (Villar-Cheda et al., 2012). Interestingly in the case of the dopaminergic system, it has been shown that loss of oestrogen decreases striatal dopaminergic levels and oestrogen administration increases dopaminergic release (Becker, 1999; Ohtani et al., 2001; Shulman, 2002; Liu and Dluzen, 2007), and that there is a counter-regulatory mechanism between the dopaminergic and renin-angiotensin system (Gildea, 2009; Villar-Cheda et al., 2010), which may also explain the increased renin-angiotensin system activity in menopausal rats and the decrease in renin-angiotensin system activity by oestrogen therapy. Decreased dopaminergic levels have also been observed in aged male animals (i.e. independently of oestrogen depletion; McCormack et al., 2004; Cruz-Muros et al., 2009), which may also induce increased renin-angiotensin system activity in males. However, different responses to oestrogen in young and aged animals have also been observed in other tissues (independently of dopaminergic levels; see above) and decreased levels of dopamine with age is not the only factor involved.

It has been suggested that prolonged hypo-oestrogenicity disrupts the ability of injured tissue to upregulate oestrogen receptors that mediate the anti-inflammatory and neuroprotective actions of oestrogen, thus eliminating the beneficial effects of oestrogen treatment (Suzuki et al., 2007). However, it has also been suggested that a prolonged period of oestrogen deprivation may upregulate oestrogen receptors in senescent females, and exogenous oestrogen therapy may be ineffective or neurotoxic when acting on supraphysiological receptor substrates (Bake et al., 2008; Selvamani and Sohrabi, 2010). The present results in young ovariectomized rats subjected to delayed oestrogen therapy reveal that a prolonged hypo-oestrogenic state may modify the effects of oestrogen on renin-angiotensin system activity, particularly the oestrogen-induced upregulation of AT2 receptors. However, several studies in different tissues suggest that prolonged hypo-oestrogenicity is not the only factor responsible for the different responses to oestrogen therapy in young and aged rats. Normal ageing is associated with a proinflammatory, pro-oxidant state that may favour an exaggerated response to injury and degenerative diseases, in males as well as in females (Csiszar et al., 2003; Ungvari et al., 2004; Choi et al., 2008); particularly, we have observed increased renin-angiotensin system activity in the substantia nigra of aged males in comparison with young males (i.e. independently of oestrogen levels or hypo-oestrogenic periods; Villar-Cheda et al., 2012). Factors other than oestrogen are therefore involved in the upregulation of renin-angiotensin system in aged rats, an effect that cannot be counteracted by oestrogen therapy alone.

Parkinson’s disease is usually considered a multifactorial process in which low and apparently non-toxic doses of several pathogenic factors can act synergistically to cross the threshold of dopaminergic cell degeneration (Gao et al., 2003), and oxidative stress and inflammation play major roles in the synergistic process (Wu et al., 2003a; Andersen, 2004). A pro-oxidative and proinflammatory state in both groups of menopausal rats may therefore be related to the increased risk of Parkinson’s disease reported in menopausal females in several epidemiological studies (Currie et al., 2004; Ragonese et al., 2006a, b). Furthermore, untreated surgical or natural menopause may increase the progression of the dopaminergic degeneration in patients with initial or moderate stages of Parkinson’s disease.

In conclusion, the results suggest that oestrogen constitutes a major factor for inhibition of renin-angiotensin system activity in young females. The loss of oestrogen after ovariectomy (surgical menopause) increases renin-angiotensin system activity and the susceptibility to neuroinflammation oxidative damage and dopaminergic neuron neurodegeneration, and oestrogen therapy induces pronounced renin-angiotensin system inhibition and neuroprotection. In aged animals, other factors in addition to the lack of oestrogen contribute to the increase in renin-angiotensin system activity, and oestrogen therapy can only partially counteract the renin-angiotensin system hyperactivity, leading to no significant neuroprotection in the present experimental conditions. In addition, changes in oestrogen receptors or...
oestrogen-induced intracellular signalling with ageing may also lead to partial or ineffective renin-angiotensin system inhibition by oestrogen therapy in aged animals. In any case, it is particularly interesting that direct manipulation of the renin-angiotensin system with the AT1 antagonist candesartan led to remarkable reduction in renin-angiotensin system activity (increased either by the lack of oestrogen in young surgically induced menopausal rats or by the lack of oestrogen and additional factors in aged rats) and significantly protected against dopaminergic neuron loss in both types of menopausal rats. This suggests that manipulation of the brain renin-angiotensin system may be an efficient approach for the prevention or treatment of Parkinson’s disease in oestrogen-deficient females together with or instead of oestrogen replacement therapy.

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