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Progesterone suppresses the inflammatory response and nitric oxide synthase-2 expression following cerebral ischemia

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Abstract

Gender differences in outcome following cerebral ischemia have frequently been observed and attributed to the actions of steroid hormones. Progesterone has been shown to possess neuroprotective properties following transient ischemia, with respect to decreasing lesion volume and improving functional recovery. The present study was designed to determine the mechanisms of progesterone neuroprotection, and whether these relate to the inflammatory response. Male mice underwent either 60 min or permanent middle cerebral artery occlusion (MCAO) and received progesterone (8 mg/kg ip) or vehicle 1 h, 6 h and 24 h post-MCAO. Forty-eight hours following transient MCAO, structural magnetic resonance imaging revealed a significant decrease in the amount of edematous tissue present in progesterone-treated mice as compared with vehicle. Using real-time PCR we found that progesterone treatment significantly suppressed the injury-induced upregulation of interleukin (IL)-1β, transforming growth factor (TGF)β_2, and nitric oxide synthase (NOS)-2 mRNAs in the ipsilateral hemisphere while having no effect on tumor necrosis factor (TNF)-α mRNA expression. Progesterone treatment following permanent MCAO also resulted in a significant decrease in lesion volume. This was not apparent in mice lacking a functional NOS-2 gene. Thus, progesterone is neuroprotective in both permanent and transient ischemia, and this effect is related to the suppression of specific aspects of the inflammatory response.

Keywords: Progesterone; Stroke; Inflammation; Mouse; Middle cerebral artery occlusion; Nitric oxide synthase-2; Interleukin-1β

Introduction

Premenopausal women have a lower risk of stroke relative to men of the same age (Barrett-Connor and Bush, 1991; Kannel and Thom, 1994). Post-menopause, the incidence of stroke in females rapidly increases (Wenger et al., 1993), coincident with diminished circulating levels of estrogen and progesterone. Thus, hormone replacement therapy has been widely used in clinical trials aimed at reducing stroke occurrence. However, routine estrogen use, particularly if chronic administration is required (Sayed and Taxel, 2003), may not be advisable in males who are at a similar risk of stroke to postmenopausal women. Progesterone may be more desirable in terms of clinical practice, and it is neuroprotective following experimental traumatic brain (Roof et al., 1996; Shear et al., 2002) and spinal cord injury (Deniselle et al., 2002).

Following permanent ischemia, we reported that female mice are protected compared to males with respect to lesion volume (Loihl et al., 1999a). More recently we demonstrated the effectiveness of progesterone in reducing lesion volume, and preserving motor and cognitive abilities, following transient ischemia in male mice (Gibson and

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Murphy, 2004). This is in accordance with other studies that have demonstrated progesterone to be neuroprotective following global ischemia in cats (Cervantes et al., 2002; Gonzalez-Vidal et al., 1998) and focal ischemia in rats (Chen et al., 1999; Jiang et al., 1996; Murphy et al., 2002). However, as yet, little insight has been gained into the mechanism(s) of progesterone action following stroke.

Cerebral ischemia triggers a complex series of events including excitotoxicity, inflammation, edema formation, apoptosis and necrosis (Danton and Dietrich, 2003; Dirnagl, 1999). The nitric oxide synthase (NOS)-2 gene (also known as inducible NOS) is also transcriptionally activated, probably in response to the surge in proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Murphy, 2000), and male mice lacking a functional NOS-2 gene display smaller infarcts than wild type (Iadecola et al., 1997; Loihl et al., 1999a). Recently, progesterone treatment was found to suppress the inflammatory response following traumatic brain injury (Grossman et al., 2004), including the expression of specific proinflammatory cytokines (He et al., 2004). Here we describe similar effects of progesterone on the inflammatory response that results from cerebral ischemia in male mice. Furthermore, we show that the transcriptional activation of NOS-2 is also suppressed, and that the neuroprotective effects of progesterone are no longer evident in male mice deficient in the NOS-2 gene.

Materials and methods

Mice and genotyping

This study was conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986 (Project Licence 40/2206). All mice were housed in a pathogen-free facility at the University of Nottingham on a 12-h light/dark cycle with ad libitum access to food and water.

Mice with the NOS-2 gene deletion (−/−) were originally obtained from Carl Nathan, John MacMicking and John Mudgett (MacMicking et al., 1995). These mice were out-crossed to C57 BL/6 wild-type (*wt*) mice to generate NOS-2 heterozygote (+/−) mice for breeding. The NOS-2 (−/−) mice were selected based upon genotyping, as previously described (Loihl et al., 1999b).

Focal cerebral ischemia

All mice were adult male C57 BL/6 (wild type or NOS-2 −/−) weighing between 24–32 g at the time of surgery. In total, 74 mice were used in the current study. A total of 40 mice underwent transient middle cerebral artery occlusion (MCAO); 2 died and 3 were excluded due to inadequate occlusion/reperfusion as determined using laser Doppler flowmetry (Moor Instruments). A total of 30 mice underwent permanent MCAO; 4 died and 2 were excluded due to inadequate occlusion. Anesthesia was induced by inhalation of 4% isoflurane (in an NO_2/O_2 70/30% mixture) and maintained by inhalation of 1.5% isoflurane. Body temperature was monitored throughout surgery (via a rectal probe) and maintained at 37.0 ± 0.6°C using a heating blanket (Harvard Apparatus Ltd). Cerebral blood flow was monitored for 5 min prior and 5 min following MCAO and, for transient MCAO, immediately before and after reperfusion. A small incision was made in the skin overlying the temporalis muscle and a 0.7-mm flexible laser Doppler probe (model P10) was positioned on the superior portion of the temporal bone (6 mm lateral and 2 mm posterior from bregma), secured with Superglue (Loctite). Focal cerebral ischemia was induced by occlusion of the right middle cerebral artery as previously described (Gibson and Murphy, 2004). During the 60-min period of MCAO, drug treatment was randomly assigned as detailed below. Following 60 min of MCAO, mice were reanesthetized and the occluding filament was withdrawn gently back into the common carotid artery in order to allow reperfusion to take place. Relative cerebral blood flow was monitored for a further 5 min prior to the wound being sutured and mice were allowed to recover from the anesthesia. Mice undergoing permanent MCAO had the Doppler probe removed 5 min following onset of occlusion, the wound was sutured, and mice were then allowed to recover from anesthesia. Sham-operated mice underwent the same surgical procedure (n = 4), except that the filament was not advanced far enough to occlude the middle cerebral artery.

Drug treatment

All mice subjected to MCAO were randomly assigned to receive either progesterone or vehicle treatment. In the case of transient MCAO, the progesterone (P) group (n = 17) received progesterone (USP, Sigma) dissolved in DMSO (16 mg/ml; Sigma), which was injected intraperitoneally (ip) in the amount of 8 mg/kg at the onset of reperfusion, i.e., 1 h post-MCAO. Additional injections of progesterone (all 8 mg/kg) were administered at 6 and 24 h post-MCAO. Mice in the vehicle (V) group (n = 18) underwent the same experimental protocol, except that they received the same volume/weight of vehicle only. For mice undergoing permanent MCAO, the progesterone group (n = 12) received the same dose of progesterone (8 mg/kg) and by the same route (ip) as mice undergoing transient MCAO. The first injection of progesterone was administered immediately following the onset of ischemia with mice receiving additional injections at 6 and 24 h post-MCAO. Mice in the vehicle group (n = 12) underwent the same experimental protocol, except that they received the equivalent volume/weight of vehicle only. The experimenter was blinded to the treatment the mice had received prior to all subsequent analyses.

Structural MRI (sMRI)

A subset of mice undergoing transient MCAO were subjected to sMRI 24 h (P, $n = 3$; V, $n = 4$) and 48 h (P, $n = 3$; V, $n = 4$) post-MCAO, as we have described previously (Jones et al., 2004). Briefly, anesthesia was induced by inhalation of 4% isoflurane (in an NO_2/O_2 70/30% mixture) and maintained by inhalation of 1.5% isoflurane. Throughout the MRI procedure breathing rate was monitored. TURBO RARE 3D coronal images (RARE factor = 16) were obtained on a Bruker Avance Biospec imaging system (Bruker Biospin MRI Inc.) at 2.35 T using an effective TE of 62.9 ms, TR of 4136 ms with an image resolution of 0.2 × 0.2 × 0.2 mm. The resulting images of animals scanned 48 h after injury depicted the formation of spreading edema as defined by hyperintense, or white regions. Volume of edema was calculated using the 'region of interest' tool on Paravision (Bruker Biospin MRI Inc.), isolating areas of T2-weighted abnormality. Since each voxel represented a volume of 0.08 mm^3, the volume of edema for each animal could be estimated.

Real-time PCR

All mice subjected to transient ischemia were used for gene analysis 6 h (P, $n = 5$; V; $n = 5$), 24 h (P, $n = 6$; V, $n = 6$), 48 h (P, $n = 4$; V, $n = 5$) and 7 days (P, $n = 2$; V, $n = 2$) post-MCAO. Sham-operated controls were also included from each time point. Mice were killed by cervical dislocation at the appropriate time point post-MCAO, brains were removed, separated into ipsilateral and contralateral hemispheres, snap frozen in liquid nitrogen and stored at −80°C until use. Total RNA was isolated from brain samples using the SV Total RNA isolation system (Promega) according to the manufacturer's instructions. First strand cDNA was synthesized using random primers (Promega, USA) and Moloney murine leukemia virus reverse transcriptase (MMLV, Promega), using the following conditions: 70°C for 5 min, 42°C for 60 min and 75°C for 10 min. Additional reactions were performed, in which the reverse transcriptase was omitted to allow for assessment, if any, of genomic DNA contamination. Multiplex real-time PCR was carried out using an ABI prism 7000 sequence detector (Applied Biosystems, USA), with 2 μl cDNA, 18 μM each primer, 5 μM probe, and Universal Taqman 2 × PCR Mastermix (Applied Biosystems) to a final volume of 25 μl. All samples were run in triplicate. Primers and MGB TaqMan probes (labeled with the fluorescent reporter FAM) for IL-1β, TGF-β_2, TNF-α and NOS-2 were designed by Applied Biosystems (Assay-on-Demand) to avoid genomic amplification. Cyclophilin E, labeled with the fluorescence dye VIC, was designed by Applied Biosystems (assay-by-design). The thermal cycling conditions used during the PCR were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A standard curve was obtained for relative expression of gene of interest (FAM labeled) and endogenous cyclophilin E (VIC labeled), and a linear relationship was observed over a range of up to 500-fold. The difference between the two standard curves was less than 0.1 over the range investigated. Cycling threshold (Ct) values of the target gene were normalized to Ct values of the endogenous cyclophilin, and final results were calculated according to the formula $2^{-\Delta\Delta Ct}$. Cyclophilin mRNA levels were not altered in response to either injury or drug treatment. In addition to each sample being run in triplicate, the value of mRNA expression for each gene in each sample was expressed as a ratio of the expression in each hemisphere of the four shams. Thus, for each sample, eight levels of mRNA expression for one gene were obtained and the mean of these was calculated for each sample. In the case of IL-1β, only the 7 days sham expressed IL-1β at a detectable level; thus, all experimental samples were expressed as a ratio of both hemispheres of the 7 days sham.

Lesion volume

Mice undergoing permanent MCAO ($n = 6$ per group) were sacrificed at 48 h for lesion volume analysis. Following cervical dislocation, brains were removed and sectioned into 10 × 1 mm coronal slices using a mouse matrix (ASI Instruments, Houston, TX). In order to quantitate ischemic damage, slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, UK) in saline for 30 min at room temperature in the dark. They were then stored at 4°C in 10% formalin prior to analysis. TTC is a marker for mitochondrial function and has been shown to be a reliable indicator of ischemic areas for up to 3 days post-ischemia (Bederson et al., 1986; Lin et al., 1993). Digital photographs of all stained slices were taken and the unstained area of infarction was measured on the posterior surface of each coronal section using Scion Image Software. Infarct areas were calculated as previously described (Loihl et al., 1999a), which uses an indirect method whereby overestimation of the infarct area due to the contribution of edema is avoided.

Statistical analyses

All data are expressed as mean ± SEM and were analyzed by two-way analysis of variance (ANOVA). The edema volume and gene expression data were analyzed for differences according to time and treatment. Lesion volume data were analyzed for differences according to treatment (or genotype) and location of lesion. Post hoc analyses were carried out with Bonferroni's test. All data were analyzed using GraphPad Prism Version 4.00 for Windows (GraphPad Software, San Diego, USA). The criterion for statistical significance was set at $P < 0.05$.

Results

Doppler monitoring showed that, in all mice subjected to transient MCAO, relative CBF was reduced to 20.7% (±2.05) and 21.05% (±1.67) of pre-ischemic values within 5 min of advancing the filament and induction of MCAO in animals that subsequently received progesterone or vehicle treatment, respectively. Following 60 min of MCAO and withdrawal of the filament, relative CBF was increased to at least 50% of pre-ischemic values in order for the mice to be included in the study. There were no significant differences in the increase in relative CBF following withdrawal of the filament in the progesterone-treated group (90.72% ± 9.14) compared to the vehicle-treated group (99. 9% ± 11.09), at least for the 5 min following withdrawal of the filament (P = 0.839). For mice undergoing permanent MCAO, relative CBF was reduced to 19.68% (±2.04) and 20.92% (±2.31) of pre-ischemic values within 5 min of induction of MCAO in animals that subsequently received progesterone or vehicle treatment, respectively (P = 0.388).

Progesterone reduces volume of edematous tissue following transient MCAO

Fig. 1 depicts T2-weighted MRI images following transient MCAO obtained after 24 h (Figs. 1A and B) and 48 h (Figs. 1C and D) in progesterone (Figs. 1A and C) and vehicle (Figs. 1B and D) treated mice. Volume measurements (Fig. 1E) indicated a significant difference in edema following progesterone treatment over time ($F(1,14)$ = 26.67, P = 0.0004), and post hoc analysis revealed a significant reduction at 48 h ($P < 0.05$). There was also a significant expansion ($P < 0.05$) in edema volume between 24 h and 48 h post-MCAO in vehicle-treated mice, which was prevented by progesterone treatment.

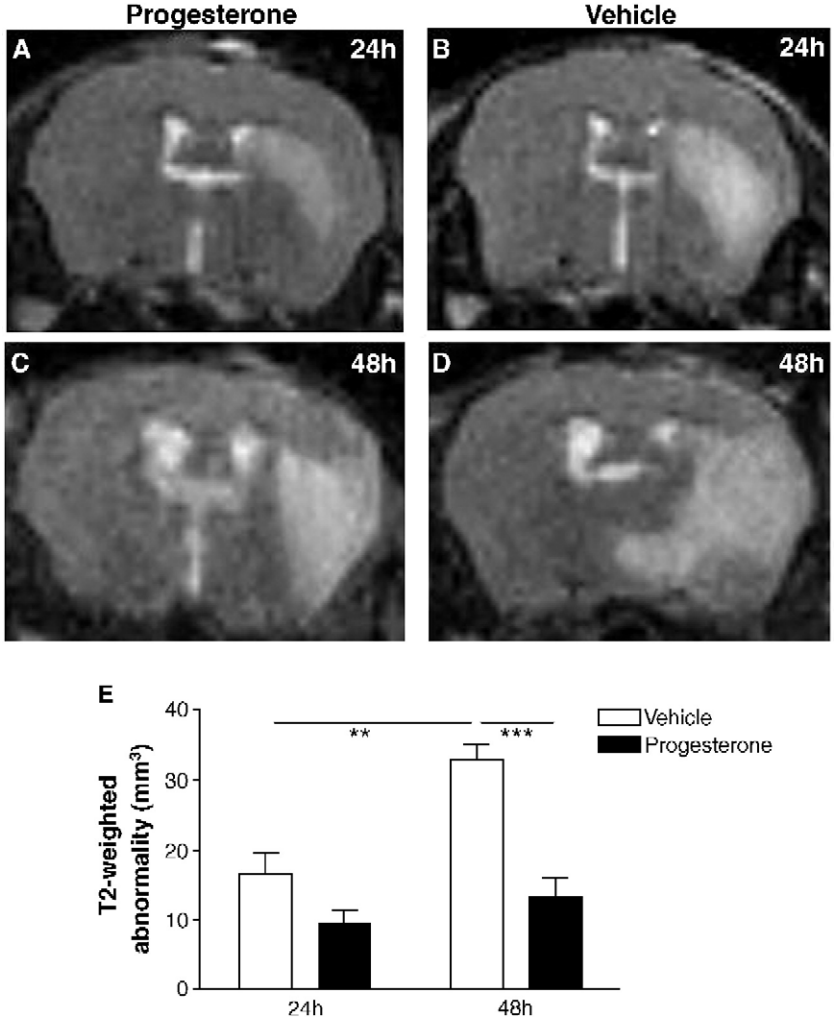

Fig. 1. T2-weighted MRI images reveal the presence of edematous tissue, as represented by areas of hyperintensity (white) 24 h (A, B) and 48 h (C, D) following transient MCAO. Measurements of the volume of edematous tissue revealed a decrease following progesterone treatment compared to vehicle treatment over time, $P < 0.001$ (E). Post hoc analysis revealed a significant difference between progesterone and vehicle treatment at 48 h, $P < 0.05$. There was a significant expansion ($P < 0.05$) of the volume of edematous tissue in vehicle-treated mice between 24 h and 48 h, which was not apparent in progesterone-treated mice (n = 3/4 per group).

Progesterone suppresses specific elements of the inflammatory response following transient MCAO

Real-time PCR revealed the levels of expression of mRNA for NOS-2, IL-1β, TGFβ$_2$ and TNF-α in the ipsilateral hemisphere at various times following transient MCAO, normalized to internal cyclophilin mRNA. Following transient MCAO, there was a rapid and significant upregulation of IL-1β mRNA in the ipsilateral compared to the contralateral hemisphere in mice that had received either vehicle ($F(1,36) = 1143$, $P = 0.0029$) or progesterone treatment ($F(1,34) = 129.1$, $P = 0.0306$, data not shown). The increase in ipsilateral IL-1β mRNA was significantly reduced following progesterone treatment ($F(1,35) = 537.0$, $P = 0.0459$; Fig. 2A), and post hoc analysis revealed a significant reduction at 6 h ($P < 0.05$). Progesterone treatment did not affect the level of contralateral IL-1β mRNA expression ($P = 0.7310$).

Transient MCAO also resulted in a significant and more sustained increase in the expression of TNF-α mRNA (Fig. 2B) in the injured hemisphere of mice that had received either vehicle ($F(1,36) = 19040.0$, $P = 0.0054$) or progesterone treatment ($F(1,34) = 4374.0$, $P = 0.0017$). However, this increase in TNF-α mRNA was not affected by progesterone, either in the ipsilateral ($P = 0.176$; Fig. 2C) or contralateral hemisphere ($P = 0.945$, data not shown).

The level of TGFβ$_2$ mRNA in the ipsilateral hemisphere (Fig. 2C) was significantly elevated in mice that had received vehicle ($F(1,36) = 153.9$, $P = 0.0477$), and progesterone treatment significantly reduced this ($F(1,35) = 148.5$, $P = 0.0464$; Fig. 2C). Progesterone had no effect on the level of TGFβ$_2$ mRNA present in the contralateral hemisphere ($P = 0.2143$, data not shown).

Transient MCAO resulted in a significant upregulation of NOS-2 mRNA in the ipsilateral compared to the contralateral hemisphere (data not shown), and this was significant in mice that had received either vehicle ($F(1,36) = 8.310$, $P = 0.0075$) or progesterone ($F(1,34) = 4.848$, $P = 0.0368$). However, progesterone treatment caused a significant suppression of ipsilateral NOS-2 mRNA expression ($F(1,35) = 6.541$, $P = 0.0165$; Fig. 2D). Post hoc analysis revealed a significant reduction following progesterone at 24 h post-MCAO ($P < 0.05$). On the contralateral side,

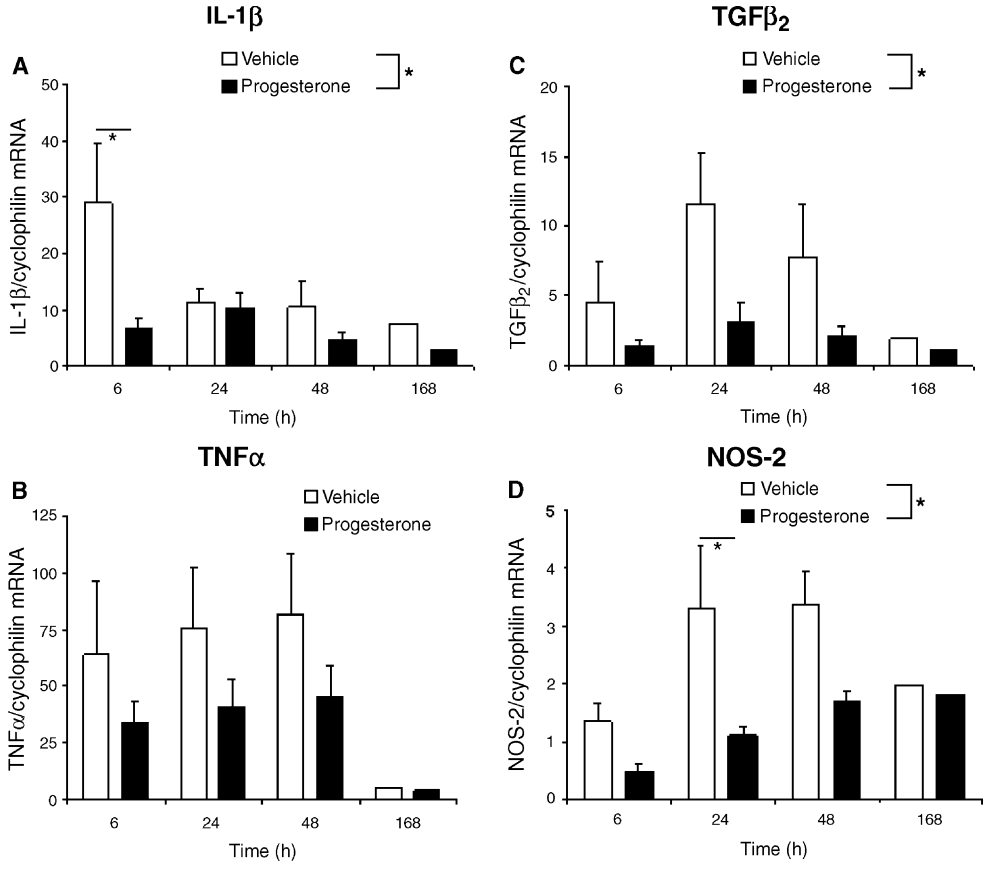

Fig. 2. Real-time PCR revealed the levels of expression of IL-1β (A), TNFα (B), TGFβ$_2$ (C) and NOS-2 (D) mRNAs at 6, 24, 48 h and 7 days following transient MCAO. Values obtained in the ipsilateral hemisphere were normalized to cyclophilin and then expressed as a ratio to both hemispheres of all shams (given a nominal value of 1). Two-way ANOVA revealed a significant reduction following progesterone treatment in the level of IL-1β, TGFβ$_2$ and NOS-2 (*$P < 0.05$) mRNAs, while there was no significant effect of progesterone treatment on the level of expression of TNFα. Post hoc analysis revealed a significant reduction in IL-1β mRNA expression at 6 h (*$P < 0.05$; A) and a significant reduction in NOS-2 mRNA expression at 24 h (*$P < 0.05$; D) post-MCAO in response to progesterone treatment ($n = 4$–6 per group, except for 7 days where bars represent mean of $n = 2$).

progesterone treatment had no effect on the level of NOS-2 mRNA present ($P = 0.7250$, data not shown).

Progesterone treatment does not further benefit male NOS-2 −/− mice

Fig. 3 shows representative brain slices stained with TTC 48 h after permanent MCAO in vehicle- and progesterone-treated wild-type and NOS-2 −/− mice. In all mice the lesion is located within the striatal and cortical areas of the brain, as indicated by the white area. Measurements from TTC sections (Fig. 4A) indicated an overall reduction in lesion volume following progesterone treatment in wild-type mice at 48 h post-MCAO ($F(1,12) = 8.355$, $P = 0.0071$). Post hoc analysis revealed a significant reduction in total ($P < 0.05$) and cortical ($P < 0.05$) lesion volume following progesterone treatment, while there was no effect on striatal lesion volume. In NOS-2 −/− mice that had received vehicle there was a significant reduction in lesion volume ($F(1,12) = 5.854$, $P = 0.0218$) compared to vehicle-treated wild-type mice 48 h post-MCAO (Fig. 4B). Post hoc analysis revealed a significant reduction in the cortex ($P < 0.05$). However, progesterone treatment had no additional benefit for NOS-2 −/− mice in terms of reducing lesion volume ($P = 0.6011$; Fig. 4C).

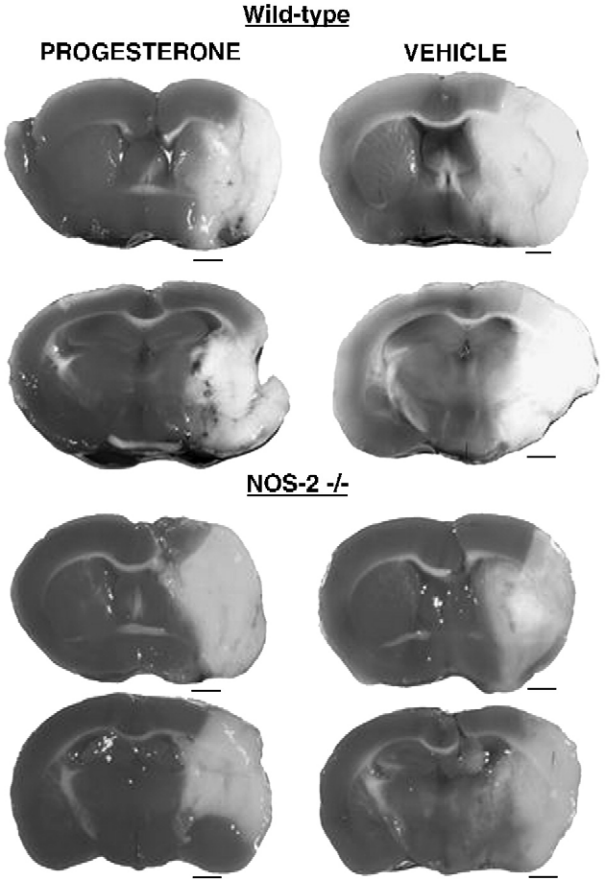

Fig. 3. TTC-stained brain slices show that the lesion at 48 h in both vehicle- and progesterone-treated mice is located within striatal and cortical areas, in both wild-type and NOS-2 −/− mice. Scale bars represent 1 mm.

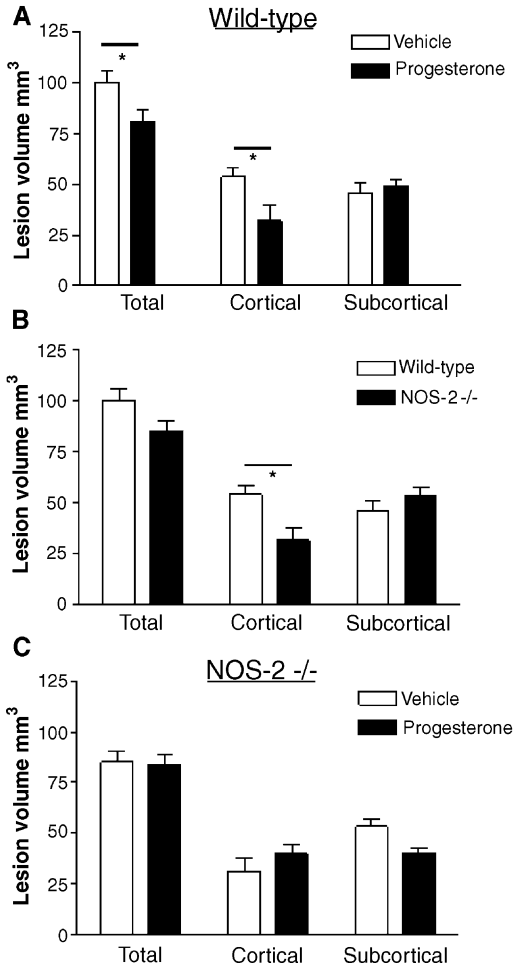

Fig. 4. Measurements of lesion volume indicate a significant reduction in lesion volume in wild-type mice following progesterone treatment 48 h post-MCAO, $P < 0.05$. Post hoc analysis revealed a significant (*$P < 0.05$) reduction in total and cortical lesion volume following progesterone treatment, but no differences in striatal lesion volume (A). In NOS-2 −/− mice that had received vehicle treatment there was a significant reduction in lesion volume compared to wild-type mice ($P < 0.05$), and post hoc analysis revealed a significant reduction in cortical lesion volume, *$P < 0.05$ (B). Following progesterone treatment in NOS-2 −/− mice there were no differences in lesion volume (C), $n = 6$ per group.

Discussion

The present study demonstrates that progesterone treatment following transient MCAO reduced the amount of edematous tissue present and modulated the expression of genes involved in the inflammatory cascade. Progesterone treatment reduced the expression of IL-1β, TGFβ$_2$ and NOS-2 but had no affect on the level of expression of TNF-α. The neuroprotective potential of progesterone treatment was also explored following permanent ischemia and was seen to reduce the total amount of lesion volume present. This effect was restricted to cortical areas, and progesterone

treatment had no effect on the volume of the striatal lesion. While NOS-2 −/− mice exhibited less cortical damage than wild-type mice, progesterone treatment did not further enhance this protection. Apart from relative cerebral blood flow immediately following reperfusion, we did not monitor the effects of progesterone on physiological parameters. However, others (Chen et al., 1999; Jiang et al., 1996; Kumon et al., 2000) have reported no effects of a similar dose of progesterone on pH, PCO_2, PO_2, hematocrit, blood glucose, heart rate and mean arterial pressure in the rat.

In the present study we administered progesterone at a dose of 8 mg/kg, which we have shown to be effective in reducing lesion volume and functional deficits in mice following MCAO (Gibson and Murphy, 2004). In rats, a dose of 8 mg/kg reduces lesion volume following cerebral ischemia (Chen et al., 1999; Kumon et al., 2000), while lower (4 mg/kg) or higher doses (32 mg/kg) failed to have the same effect (Chen et al., 1999). Exogenous progesterone has a dose- and time-dependent neuroprotective action in experimental stroke. Following administration of a high chronic dose (30 mg/kg, 7–10 days), progesterone treatment pre-injury exacerbates striatal injury (Murphy et al., 2000), although the same group observe a neuroprotective effect of lower dose progesterone (20 mg/kg) when treatment was initiated immediately prior to ischemia (Murphy et al., 2002). It is possible that high dose and abrupt termination of progesterone treatment result in withdrawal effects that exacerbate excitotoxicity and ischemic cell death (Goss et al., 2003). This could be caused by GABA current modulation and alterations in $GABA_A$ receptor α4 subunit levels as a result of sharply declining plasma progesterone levels after ischemia (Moran et al., 1998; Reilly et al., 2000).

The observation that progesterone treatment following transient MCAO reduces edema formation is in accordance with the reported mechanism of neuroprotection following traumatic brain injury (Roof et al., 1993), where progesterone is still effective when administered 24 h after insult (Roof et al., 1996). We used T2-weighted MRI to visualize edema formation. This was characterized by an area of hyperintense pixels as a result of T2 prolongation, which is a sensitive measure of cerebral edema (Brandt-Wadzki et al., 1987; Kato et al., 1986) and used commonly following cerebral ischemia (Gerriets et al., 2004; Neumann-Haefelin et al., 2000). There are several possibilities with respect to how progesterone can reduce edema formation, which is a consequence of the inflammatory response. The pro-inflammatory cytokine IL-1β has been reported, as in the current study in mice, to be elevated following transient ischemia in the rat (Minami et al., 1992; Yabuuchi et al., 1994). In fact, the contribution of IL-1β to the damage occurring after ischemia has been further demonstrated by the fact that exogenous administration of IL-1β itself exacerbates ischemic damage (Loddick and Rothwell, 1996). Administration of an endogenous IL-1β receptor antagonist (Betz et al., 1995; Relton and Rothwell, 1992), or a neutralizing antibody (Yamasaki et al., 1995), reduces brain damage and edema when administered before a stroke in rats. Thus, as in the case of traumatic brain injury (He et al., 2004), the suppression of IL-1β could account for the reduction in edema formation and lesion volume we observed following progesterone treatment.

In addition, progesterone may affect edema formation through its actions on the blood–brain barrier (BBB). The rate of sodium transport from blood to brain, via Na, K-ATPase, appears to determine the rate of edema accumulation during the early stages when the BBB is still intact. In isolated brain capillaries high concentrations of steroids, such as progesterone (Chaplin et al., 1981), can inhibit active ion transport (Betz, 1986). Thus, it may be possible that progesterone reduces the accumulation of brain edema during ischemia by reducing BBB permeability to sodium, either through a direct effect on brain capillary Na, K-ATPase or through a generalized effect on BBB permeability. However, although steroids are capable of reducing intact-barrier edema, they were shown to have no effect on BBB sodium transport (Betz and Coester, 1990). Steroids such as progesterone also act as free radical scavengers (Demopoulos et al., 1972) and improve cellular viability and reduce edema formation (Martz et al., 1989, 1990; Roof et al., 1997).

In the current study progesterone suppressed the ischemia-induced expression of IL-1β, NOS-2 and $TGFβ_2$. In vitro studies have also described the ability of progesterone to modulate expression of these genes. For example, progesterone can reduce IL-1β mRNA levels in monocytes (Polan et al., 1989) and reduce IL-1β induced behavior in rats (Avitsur et al., 1995). Progesterone may also act directly on NOS-2 and $TGFβ_2$ expression. In vitro, progesterone decreases NOS-2 mRNA expression and NOS-2 promoter activity in macrophages (Miller et al., 1996) and decreases NOS-2 protein synthesis in microglial cells (Lieb et al., 2003). However, in vitro studies suggest an increase in $TGFβ_2$ expression in response to progesterone (Luo et al., 2002). While we found that progesterone had no affect on TNFα expression, it has been reported to decrease TNFα mRNA and protein expression in vitro and in vivo (He et al., 2004; Hunt et al., 1997; Kurachi et al., 2001), and also increase TNFα secretion (Jain et al., 2004).

Our study provides no evidence for a direct action of progesterone on IL-1β, $TGFβ_2$ and NOS-2, and the effects could be indirect. For example, the observed decreases in NOS-2 and $TGFβ_2$ expression may be secondary to suppression of IL-1β, as both genes have been shown to be transcriptionally upregulated by IL-1β in vitro (Murphy et al., 1993; Offner et al., 1996). In vitro, IL-1β causes an upregulation in both the production of NO via NOS-2, and the levels of NOS-2 mRNA and protein expression (Beauregard et al., 2003; Estevez et al., 2004). This induction of NOS-2 expression by IL-1β is further demonstrated by direct injection of IL-1β into the spinal

cord, which results in increased expression of NOS-2 protein (Sung et al., 2004).

Progesterone treatment reduces the lesion volume present after transient ischemia (Gibson and Murphy, 2004) and permanent ischemia (this study). There is expression of NOS-2 following permanent cerebral ischemia and this evidently contributes to damage, because mice lacking a functional NOS-2 gene exhibit some protection with respect to lesion volume (Iadecola et al., 1997). We reported previously that mice lacking a functional NOS-2 gene are protected with respect to lesion volume at 72 h, but not at 24 h post-MCAO (Loihl et al., 1999a). The current study extends that observation as NOS-2 −/− mice also exhibit reduced ischemic damage at 48 h following permanent MCAO. Interestingly, we find that progesterone administration does not affect lesion volume in NOS-2 −/− mice. This could suggest that a functional NOS-2 gene is required for progesterone to have neuroprotective effects, acting either directly or indirectly via suppressing IL-1β expression. Alternatively, the extent of cortical tissue that can be salvaged by either progesterone treatment or NOS-2 deletion may be the same, and the NOS-2 −/− mice are already protected. We have yet to establish a causal or correlative link.

In summary, we show that at least part of the explanation for the neuroprotective properties of progesterone following cerebral ischemia is the reduction in edema, probably related to the suppression of IL-1β. These effects could be mediated by either membrane associated (Li and O'Malley, 2003) and/or intracellular progesterone receptors (Zhu et al., 2003a, 2003b), and it will be of interest to identify the underlying pathways.

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References

Avitsur, R., Donchin, O., Barak, O., Cohen, E., Yirmiya, R., 1995. Behavioral effects of interleukin-1β: modulation by gender, estrus cycle, and progesterone. Brain Behav. Immun. 9, 234–241.

Barrett-Connor, E., Bush, T.L., 1991. Estrogen and coronary heart disease in women. JAMA 265, 1861–1867.

Beauregard, C., Brandt, P.C., Chiou, G.C.Y., 2003. Induction of nitric oxide synthase and over-production of nitric oxide by interleukin-1β in cultured lacrimal gland acinar cells. Exp. Eye Res. 77, 109–114.

Bederson, J.B., Pitts, L.H., Germano, S.M., Nishimura, M.C., Davis, R.L., Bartowski, H.M., 1986. Evaluation of 2, 3, 5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 17, 1304–1308.

Betz, A.L., 1986. Transport of ions across the blood–brain barrier. Fed. Proc. 45, 2050–2054.

Betz, A.L., Coester, H.C., 1990. Effects of steroids on edema and sodium uptake of the brain during focal ischemia in rats. Stroke 21, 1199–1204.

Betz, A.L., Yang, G.Y., Davidson, B.L., 1995. Attenuation of stroke size in rats using an adenoviral vector to induce over expression of interleukin-1 receptor antagonist in brain. J. Cereb. Blood Flow Metab. 15, 547–551.

Brandt-Wadzki, M., Weinstein, P., Bartkowski, H., Moseley, M., 1987. MR imaging and spectroscopy in clinical and experimental cerebral ischemia: a review. Am. J. Neuroradiol. 148, 579–588.

Cervantes, M., Gonzalez-Vidal, M.D., Ruelas, R., Escobar, A., Morali, G., 2002. Neuroprotective effects of progesterone on damage elicited by acute global ischemia in neurons of the caudate nucleus. Arch. Med. Res. 33, 6–14.

Chaplin, E.R., Free, R.G., Goldstein, G.W., 1981. Inhibition by steroids of the uptake of potassium by capillaries isolated from rat brain. Biochem. Pharmacol. 30, 241–245.

Chen, J., Chopp, M., Li, Y., 1999. Neuroprotective effects of progesterone after transient middle cerebral artery occlusion in rat. J. Neurol. Sci. 171, 24–30.

Danton, G.H., Dietrich, W.D., 2003. Inflammatory mechanisms after ischemia and stroke. J. Neuropath. Exp. Neurol. 62, 127–136.

Demopoulos, H.B., Milvy, P., Kakari, S., Ransohoff, J., 1972. Molecular aspects of membrane structure in cerebral edema. In: Reulen, H., Schurmann, K. (Eds.), Steroids and Brain Edema. Springer-Verlag, New York, pp. 29–39.

Deniselle, M.C.G., Lopez-Costa, J.J., Saavedra, J.P., Garay, L., Guennon, R., Schumacher, M., De Nicola, A.F., 2002. Progesterone neuroprotection in the Wobbler mouse, a genetic model of spinal cord motor neuron disease. Neurobiol. Dis. 11, 457–468.

Dirnagl, U., 1999. Pathobiology of ischemic stroke: an integrated view. Trends Neurosci. 22, 391–397.

Estevez, A., Farina, M., Franchi, A., Johnson, C., Vega, A., Motta, A.B., 2004. Interleukin-1β up-regulates nitrite production: effects on ovarian function. Nitric Oxide 10, 92–100.

Gerriets, T., Stolz, E., Walberer, M., Muller, C., Kluge, A., Bachmann, A., Fisher, M., Kaps, M., Bachmann, G., 2004. Non-invasive quantification of brain edema and the space-occupying effect in rat stroke models using magnetic resonance imaging. Stroke 35, 566–571.

Gibson, C.L., Murphy, S.P., 2004. Progesterone enhances functional recovery following middle cerebral artery occlusion in male mice. J. Cereb. Blood Flow Metab. 24, 805–813.

Gonzalez-Vidal, M.D., Cervera-Gaviria, M., Ruelas, R., Escobar, A., Morali, G., Cervantes, M., 1998. Progesterone: protective effects on cat hippocampal damage due to acute global cerebral ischemia. Arch. Med. Res. 29, 117–124.

Goss, C.W., Hoffman, S.W., Stein, D.G., 2003. Behavioral effects and anatomic correlates after brain injury: a progesterone dose-response study. Pharmacol. Biochem. Behav. 76, 231–242.

Grossman, K.J., Goss, C.W., Stein, D.G., 2004. Effects of progesterone on the inflammatory response to brain injury in the rat. Brain Res. 1008, 29–39.

He, J., Evans, C.-O., Hoffman, S.W., Oyesiku, N.M., Stein, D.G., 2004. Progesterone and allopregnanolone reduce inflammatory cytokines after traumatic brain injury. Exp. Neurol. 189, 404–412.

Hunt, J.S., Miller, L., Roby, K.F., Huang, J., Platt, S., DeBrot, B.L., 1997. Female steroid hormones regulate production of pro-inflammatory molecules in uterine leukocytes. J. Reprod. Immunol. 35, 87–99.

Iadecola, C., Zhang, F., Casey, R., Nagayama, M., Ross, M.E., 1997. Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. J. Neurosci. 17, 9157–9164.

Jain, S.K., Kannan, K., Prouty, L., Jain, S.K., 2004. Progesterone, but not 17β estradiol, increases TNFα secretion in U937 monocytes. Cytokine 26, 102–105.

Jiang, N., Chopp, M., Stein, D., Feit, H., 1996. Progesterone is neuroprotective after transient middle cerebral artery occlusion in male rats. Brain Res. 735, 101–107.

Jones, N.C., Constantin, D., Gibson, C.L., Prior, M.J.W., Morris, P.G., Marsden, C.A., Murphy, S., 2004. A detrimental role for nitric oxide synthase-2 in the pathology resulting from acute cerebral injury. J. Neuropathol. Exp. Neurol. 63, 708–720.

Kannel, W.B., Thom, T.J., 1994. The incidence, prevalence and mortality of cardiovascular disease. In: Schlant, R.C., Alexander, R.W. (Eds.), The Heart. McGraw-Hill Inc., New York, pp. 185–197.

Kato, H., Kogure, K., Ohtoma, H., Izumiyama, M., Tobita, M., Matsui, S., Yamamoto, E., Kohno, H., Ikene, Y., Watanabe, T., 1986. Characterization of experimental ischemic brain edema utilizing proton nuclear magnetic resonance imaging. J. Cereb. Blood Flow Metab. 6, 212–221.

Kumon, Y., Kim, S.C., Tompkins, P., Stevens, A., Sakaki, S., Loftus, C.M., 2000. Neuroprotective effect of postischemic administration of progesterone in spontaneously hypertensive rats with focal cerebral ischemia. J. Neurosurg. 92, 848–852.

Kurachi, O., Matsuo, H., Samoto, T., Maruo, T., 2001. Tumor necrosis factor-α expression in human uterine leiomyoma and its down-regulation by progesterone. J. Clin. Endocrinol. Metab. 86, 2275–2280.

Li, X., O'Malley, B.W., 2003. Unfolding the action of progesterone receptors. J. Biol. Chem. 278, 39261–39264.

Lieb, K., Engels, S., Fiebich, B.L., 2003. Inhibition of LPS-induced iNOS and NO synthesis in primary rat microglial cells. Neurochem. Int. 42, 131–137.

Lin, T.-N., He, Y.Y., Wu, G., Khan, M., Hsu, C.Y., 1993. Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. Stroke 24, 112–117.

Loddick, S.A., Rothwell, N.J., 1996. Neuroprotective effects of human recombinant interleukin-1 receptor antagonist in focal ischemia in the rat. J. Cereb. Blood Flow Metab. 16, 932–940.

Loihl, A.K., Asensio, V., Campbell, I.L., Murphy, S.P., 1999a. Expression of nitric oxide synthase (NOS)-2 following permanent focal ischemia and the role of nitric oxide in infarct generation in male, female and NOS-2 gene-deficient mice. Brain Res. 830, 155–164.

Loihl, A.K., Whalen, S., Campbell, I., Mudget, J.S., Murphy, S., 1999b. Transcriptional activation following cerebral ischemia in mice of a promoter-deleted NOS-2 gene. J. Biol. Chem. 274, 8844–8849.

Luo, X.-H., Liao, E.-Y., Su, X., 2002. Progesterone upregulates TGF-β isoforms (β_1, β_2, and β_3) expression in normal human osteoblast-like cells. Calcif. Tissue Int. 71, 329–334.

MacMicking, J.D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D.S., Trumbauer, M., Stevens, K., Xie, Q.W., Sokol, K., Hutchinson, N., Chen, H., Mudgett, J.S., 1995. Altered responses to bacterial-infection and endotoxic-shock in mice lacking inducible nitric-oxide synthase. Cell 81, 641–650.

Martz, D., Rayos, G., Schielke, G.P., Betz, A.L., 1989. Allopurinol and dimethylthiourea reduce brain infarction following middle cerebral artery occlusion in rats. Stroke 20, 488–494.

Martz, D., Beer, M., Betz, A.L., 1990. Dimethylthiourea reduces ischemic brain edema without affecting cerebral blood flow. J. Cereb. Blood Flow Metab. 10, 352–357.

Miller, L., Alley, E.W., Murphy, W.J., Russell, S.W., Hunt, J.S., 1996. Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. J. Leukocyte Biol. 59, 442–450.

Minami, M., Kuraishi, Y., Yabuuchi, K., Yamazaki, A., Satoh, M., 1992. Induction of interleukin-1β mRNA after transient forebrain ischemia. J. Neurochem. 58, 390–392.

Moran, M.H., Goldberg, M., Smith, S.S., 1998. Progesterone withdrawal: II. Insensitivity to the sedative effects of a benzodiazepine. Brain Res. 807, 91–100.

Murphy, S., 2000. Production of nitric oxide by glial cells. Glia 29, 1–14.

Murphy, S., Simmons, M.L., Agullo, L., Garcia, A., Feinstein, D.L., Galea, E., Reis, D.J., Minc-Golomb, D., Schwartz, J.P., 1993. Synthesis of nitric oxide in CNS glial cells. Trends Neurosci. 16, 323–328.

Murphy, S.J., Traystman, R.J., Hurn, P.D., 2000. Progesterone exacerbates striatal stroke injury in progesterone-deficient female animals. Stroke 31, 1173–1178.

Murphy, S.J., Littleton-Kearney, M.T., Hurn, P.D., 2002. Progesterone administration during reperfusion, but not preischemia alone, reduces injury in ovariectomized rats. J. Cereb. Blood Flow Metab. 22, 1181–1188.

Neumann-Haefelin, T., Kastrup, A., de Crespigny, A., Yenari, M.A., Ringer, T., Sun, G.H., Moseley, M.E., 2000. Serial MRI after transient focal cerebral ischemia in rats. Stroke 31, 1965–1973.

Offner, F.A., Feichtinger, H., Stadlmann, S., Obrist, P., Marth, C., Klinger, P., Grage, B., Schmahl, M., Knabbe, C., 1996. Transforming growth factor-beta synthesis by human peritoneal mesothelial cells. Am. J. Pathol. 148, 1679–1688.

Polan, M.L., Loukides, J., Nelson, P., Carding, S., Diamond, M., Walsh, A., Bottomly, K., 1989. Progesterone and estradiol modulate interleukin-1β messenger ribonucleic acid levels in cultured human peripheral monocytes. J Clin. Endocrinol. Metab. 69, 1200–1206.

Reilly, M.T., Crabbe, J.C., Rustay, N.R., Finn, D.A., 2000. Acute neuroactive steroid withdrawal in withdrawal seizure-prone and withdrawal seizure-resistant mice. Pharmacol. Biochem. Behav. 67, 709–717.

Relton, J.K., Rothwell, N.J., 1992. Interleukin-1 receptor antagonist inhibits ischaemic and excitotoxic neuronal damage in the rat. Brain Res. Bull. 29, 243–246.

Roof, R.L., Duvdevani, R., Stein, D.G., 1993. Progesterone treatment attenuates brain edema following contusion injury in male and female rats. Restor. Neurol. Neurosci. 4, 425–427.

Roof, R.L., Duvdevani, R., Heyburn, J.W., Stein, D., 1996. Progesterone rapidly decreases brain edema: treatment delayed up to 24 hours is still effective. Exp. Neurol. 138, 246–251.

Roof, R.L., Hoffman, S.W., Stein, D.G., 1997. Progesterone protects against lipid peroxidation following traumatic brain injury in rats. Mol. Chem. Neuropathol. 31, 1–11.

Sayed, Y., Taxel, P., 2003. The use of estrogen therapy in men. Curr. Opin. Pharmacol. 3, 650–654.

Shear, D.A., Galani, R., Hoffman, S.W., Stein, D.G., 2002. Progesterone protects against necrotic damage and behavioural abnormalities caused by traumatic brain injury. Exp. Neurol. 178, 59–67.

Sung, C.-S., Wen, Z.-H., Chang, W.-K., Ho, S.-T., Tsai, S.-K., Chang, Y.-C., Wong, C.-S., 2004. Intrathecal interleukin1β administration induces thermal hyperalgesia by activating inducible nitric oxide synthase expression in the rat spinal cord. Brain Res. 1015, 145–153.

Wenger, N.K., Speroff, L., Packard, B., 1993. Cardiovascular health and disease in women. N. Engl. J. Med. 329, 247–256.

Yabuuchi, K., Minami, M., Katsumata, S., Yamzaki, A., Satoh, M., 1994. An in situ hybridisation study on interleukin-1β mRNA induced by transient forebrain ischemia in the rat brain. Mol. Brain Res. 26, 135–142.

Yamasaki, Y., Matsuura, N., Shozuhara, H., Onodera, H., Itoyama, Y., Kogure, K., 1995. Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. Stroke 26, 676–681.

Zhu, Y., Rice, C.D., Pang, Y., Pace, M., Thomas, P., 2003a. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc. Natl. Acad. Sci. U. S. A. 100, 2231–2236.

Zhu, Y., Bond, J., Thomas, P., 2003b. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc. Natl. Acad. Sci. U. S. A. 100, 2237–2242.