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The enantiomer of progesterone acts as a molecular neuroprotectant after traumatic brain injury

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Abstract

Previous work shows that neurosteroid enantiomers activate specific molecular receptors that relay neuroprotection. However, the actions of the enantiomer of progesterone (*ent*-PROG) at the PROG receptor (PR) are unknown. PR binding and transcriptional assays were performed to determine the actions of *ent*-PROG at the classical PR. Additionally, the neuroprotective effects of *ent*-PROG in traumatic brain injury (TBI) were investigated and compared to the actions of PROG and its metabolite allopregnanolone (ALLO), both of which have been shown to have neuroprotective properties when given after TBI. Binding studies performed in COS cells over-expressing the PR showed that *ent*-PROG inhibited PROG binding to the PR. In contrast, *ent*-PROG did not activate PR-mediated transcription. Rats received bilateral medial frontal cortex injury followed by treatments at 1, 6, 24 and 48 h with PROG, ALLO or *ent*-PROG. Brains were processed for edema, protein and enzyme activity. *ent*-PROG treatment *in vivo* decreased cerebral edema, cell death mediators, inflammatory cytokines, and reactive gliosis, and increased antioxidant activity. These findings suggest that the progestin-mediated pro-survival response seen with TBI is regulated either independently of the classical PR or via nongenomic PR-regulated actions.

Keywords: Progesterone; Enantiomer; Allopregnanolone; Traumatic brain injury; Neuroprotection; Progesterone receptor

1. Introduction

It is estimated that over 1.5 million people suffer from traumatic brain injury (TBI) each year. Of these, over 50,000 die and another 80,000 become impaired or disabled for life (Thurman et al., 1999; Langlois et al., 2004). Research seeking new treatments for TBI has shown that the neurosteroid progesterone (PROG) and its metabolite, allopregnanolone (ALLO), improve neuronal survival and functional recovery after brain injury in rodent models (Djebaili et al., 2004). These neurosteroids have been shown to decrease cell death while improving behavioral aspects of cognition such as learning and memory (Roof and Stein, 1999; Galani et al., 2001; Shear et al., 2002). Furthermore, deleterious inflammatory cytokines including tumor necrosis factor (TNF-α) (Vitarbo et al., 2004) and interleukin-1β (IL-1β) (Pearson et al., 1999) are diminished following treatment with PROG or ALLO after bilateral medial frontal cortex (MFC) injury (He et al., 2004).

Much of the morbidity and mortality associated with head trauma is a direct result of brain edema (Papadopoulos et al., 2002). Serum levels of PROG are inversely correlated with the degree of cerebral edema following TBI (Wright

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et al., 2001). PROG and ALLO diminish brain water content compared to untreated TBI controls, reflecting a decrease in both vasogenic and cytotoxic edema (Galani et al., 2001; Hoffman et al., 2001). Vasogenic edema occurs when the blood–brain barrier (BBB) is compromised and plasma proteins enter the brain parenchyma, causing fluid to build up in the extracellular space (Unterberg et al., 2004). Cytotoxic edema occurs somewhat later than the vasogenic variety and is caused by accumulation of fluid primarily within the astrocytes (He et al., 2004). These swollen astrocytes become dysfunctional and lose the ability to buffer the intracellular milieu in the injured areas of the brain (Chen and Swanson, 2003). PROG treatment results in a significant decrease in the accumulation of reactive astrocytes in the proximity of the cortical wound after penetrating (Garcia-Estrada et al., 1999) and cortical impact (Djebaili et al., 2005) head injury.

In the case of estrogen (E), its E2 enantiomer (*ent*-E2) was shown to prevent *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity in cortical neurons and HT-22 cell cultures (Xia et al., 2002; Green et al., 2001). The *ent*-E2 was also reported to decrease infarct volumes after cerebral ischemia with effectiveness comparable to natural E2 (Green et al., 2001). These findings suggest that synthetic steroids with physicochemical properties identical to those of the natural forms can function at the molecular level by mechanisms other than classical receptor-mediated transcription.

The enantiomer of pregnenolone sulfate (*ent*-PS), which inhibits GABA-ergic activity through chloride channel blockade and stimulates NMDA receptors, has been shown to have a promnestic effect in non-injured rodents that is 10-fold higher than that of natural PS (Akwa et al., 2001). PS has been shown to improve memory after penetrating brain injury, but its effects have not been compared to those of the PS enantiomer (Garcia-Estrada et al., 1999). In a similar fashion, *ent*-PROG (Fig. 1) has recently been shown to bind with higher affinity than PROG to the hydroxylating enzyme CYP17, potentially inhibiting biosynthesis of steroids such as cortisol (Auchus et al., 2003). Interestingly, to date, no work has been done to determine the binding affinity of *ent*-PROG to the PR; nor do we know the effects of *ent*-PROG treatment on PR-mediated transcription. The current experiments were designed to test not only PR binding affinity and transcription induction of the enantiomer compared to natural PROG, but also to compare the effects of *ent*-PROG treatment to those of natural PROG and ALLO on cerebral edema and cellular markers of neuronal rescue after TBI.

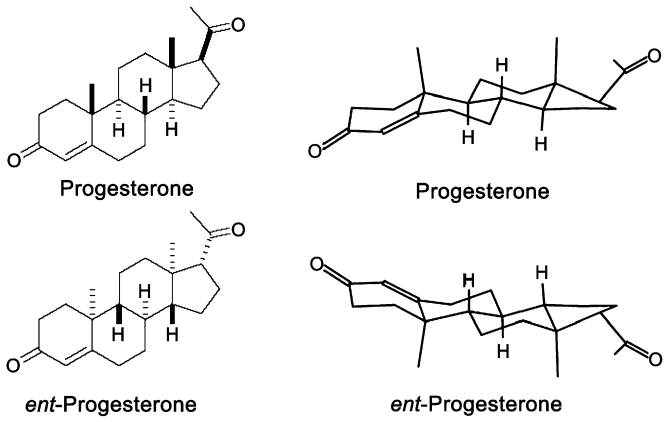

Fig. 1. Neurosteroid structures. Structures of PROG and *ent*-PROG. Left column, two-dimensional line drawings. Right column, three-dimensional drawlings showing the mirror image relationship between the two steroids.

ent-PROG should be studied as a potential neuroprotectant to help define molecular mechanisms of PROG and improve clinical treatment for TBI. We hypothesized that enantiomeric effects would parallel those of natural neurosteroids with respect to neuroprotection, but would have diminished intensity due to lower PR receptor affinity. We used molecular assays for cerebral edema, oxidative stress, cell death, and inflammation as our outcome measures. To our knowledge, our study may be the first to report on the binding affinity of *ent*-PROG to the PR as well as its ability to activate PR-mediated transcription. Furthermore, this research represents the first comparison of *ent*-PROG to PROG and PROG-derived neurosteroids on their role in neuroprotection after TBI.

2. Methods

2.1. Cell culture assays

Ligand binding assays were performed using COS (green monkey kidney) cells in 24-well plates pretreated with 0.1% gelatin (Sigma, St. Louis, MO). Cells were transfected with lipofectamine (Invitrogen, Carlsbad, CA) with either an empty pcDNA 3.1 vector, or the human PROG receptor B (HuPRB) in pLEN. After 48 h cells were washed once at 4 °C with DMEM containing 1 mg/ml bovine serum albumin (BSA) and 20 mmol HEPES (H) (DMEM/BSA/H). The cells were then incubated in DMEM/BSA/H containing 1 nmol PROG (PerkinElmer, Boston, MA) and in varying concentrations of *ent*-PROG for 1 h at 4 °C. Cells were then washed three times with cold DMEM/BSA/H and steroids extracted by incubating the cells with 100% EtOH for 30 min. Total counts bound to cells were measured by liquid scintillation and the K_d values were determined using Prism software (GraphPad Software, Inc., San Diego, CA).

For transcription assays, COS cells were first transfected in 12-well plates using lipofectamine (Invitrogen). Cells were then transfected with 0.8 μg HuPRB in the mammalian expression vector pLEN, 1.2 μg of the mouse mammary tumor virus-luciferase plasmid, and 5 ng of cytomegalovirus-β-galactosidase plasmid. The cells were incubated with the mixture for 4 h and placed in complete medium with 5% charcoal-filtered serum with either PROG (20 nmol) or *ent*-PROG at 0, 100, or 1000 nmol. After 48 h incubation the cells were lysed and extracts analyzed by the luciferase assay system (Promega, Madison, WI) and Galactostar Kit (Perkin-Elmer, Wellesley, MA).

2.2. Animals

Seventy-two male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 290–315 g prior to surgery, were subjects. Eighteen rats were used for sham surgeries and received the same exposure to anesthetics as the remaining 90 rats which were given bilateral lesions of the MFC. The rats with lesions were randomly assigned to one of five treatment groups: PROG (16 mg/kg), ALLO (8 mg/kg), vehicle, and one of two doses of *ent*-PROG (4 and 16 mg/kg). The doses chosen were based on molecular findings reported previously by our group (Pettus et al., 2005). All rats were assigned to a survival time of either 6 ($n = 6$) or 72 h ($n = 12$). The animals were housed in standing plastic cages and maintained on a 12 h dark/light reversed light cycle. Rats were provided with food and water ad libitum. All procedures were approved by the Emory University Institutional Animal Care and Use Committee (Protocol #098-2001).

2.3. Surgery protocol

Sterile surgical procedures were used to prevent animal infection. Rats were anesthetized and maintained on isoflurane and an equivalent amount of NO_2 and O_2 for 3 min prior to surgery. After brain contusion O_2 levels were doubled compared to NO_2 and maintained through the remainder of the surgery procedure. A stereotaxic apparatus was used to stabilize the head in a horizontal position. Core body temperature was monitored and maintained at 37 °C using a Harvard™ homeothermic blanket (Harvard Apparatus, Holliston, MA). There was no direct measure used to detect brain temperature. Blood oxygen and heart rate were maintained using a SurgiVet monitor (SurgiVet, Waukesha, WI) and maintained above 90% and 340 bpm respectively. A midline incision was made and the scalp retracted. A bilateral 6-mm craniotomy was performed with surgical drill centered at 3 mm rostral to bregma. The stainless steel impactor was positioned over the MFC at 3.0 mm A/P and 0.0 M/L. These coordinates represent the MFC as described by Paxinos and Watson (1986). The cortical injury was induced using a pneumatically controlled device (Hoffman et al., 1994). Brain impact duration was 0.5 s using a 5-mm impactor tip with a velocity set at 2.25 m/s and a cortical depth of 2 mm. Following the contusion, bleeding was halted and the fascia and scalp were sutured shut. After surgery animals were allowed to recover from anesthesia on a homeothermic heating blanket in a holding cage until awake. Sham surgeries controlled for anesthesia and stress. All surgical procedures were the same, except that sham rats were not given a craniotomy or cortical injury. Previous studies using craniotomy as a control found no differences between shams with or without this procedure (Goss et al., 2003).

2.4. Treatment protocol

All injections were done at the same time with brain harvesting at 6 or 72 h post-injury. Rats in each group were weighed prior to treatment to ensure proper dosage. The first injection at 1 h after surgery was given intraperitoneally to ensure rapid absorption. All subsequent injections were made subcutaneously for gradual absorption at 6, 24 and 48 h. Injection times and neurosteroid doses were based on previous results of neurosteroid treatment (Roof et al., 1994; He et al., 2004). The steroids were dissolved in vehicle (22.5% 2-hydroxypropyl-β-cyclodextrin solution) at the following concentrations: PROG (Sigma; P-0130): 16 mg/kg; ALLO (Calbiochem; 127100; San Diego, CA, USA): 8 mg/kg; and two doses of *ent*-PROG, prepared as described previously (Auchus et al., 2003): 4 mg/kg and 16 mg/kg. The sham group received no treatment and injury control group received vehicle only.

2.5. Cerebral edema analysis

At 72 h after TBI fresh brains were extracted from the skull and the dorsal cerebrum was separated along the line of the lateral fissure. Four 3-mm coronal sections were cut rostral to caudal, placed in pre-weighed 1.5-ml tubes and reweighed (wet weight). Tubes were then left uncapped and placed in a vacuum oven set at 60 °C with an atmospheric pressure of 0.3 for 48 h. Following tube recapping, the tissue samples were again weighed (dry weight). Cerebral edema (% water content) was determined as the difference in wet and dry weights divided by wet weight (Roof and Stein, 1992). Edema measures are reflective of the difference in water content between the average of the two most rostral (injury region) segments and most caudal (occipital cortex) segments of the dorsal sections of the brain (Roof and Stein, 1992).

2.6. Protein expression

Brain samples were prepared for analysis of the pro-apoptotic protein p53 at 6 h and the following proteins at 72 h post-injury: tumor necrosis factor alpha (TNF-α) (Santa Cruz Biotechnology, Santa Cruz CA), interleukin-1 beta (IL-1β) (Abcam Inc., Cambridge, MA) and glial fibrillary acidic protein (GFAP) (Santa Cruz Biotechnology). Brain tissue from the penumbral region of the injury was extracted and quickly placed on an ice-chilled glass plate. Using a cold brain-mold, a 3-mm thick coronal slab encompassing the injury site was excised. Using a 2-mm tissue punch, four samples of the cortical lesion site were collected and flash frozen in chilled 2-methylbutane. Respective regions were also collected for sham surgeries. Samples were transferred into tubes containing T-per (Pierce, Rockford, IL) with protease inhibitors (Sigma). Following homogenization, samples were centrifuged at 10,000 × *g* for 20 min. Then 200 μL were set aside for protein assay (Pierce BCA protein assay kit). An SDS lamellae sample buffer was added to sample aliquots and incubated at 90 °C for 10 min on a heating block. Samples were loaded on a 12% acrylamide Criterion gel (BioRad, Hercules, CA), and run at 200 V for 1 h. Gels were then rinsed in transfer buffer for 10 min and loaded against a PVDF nitrocellulose membrane (Millipore, Bedford, MA) for transfer. The transfer was conducted on a BioRad Criterion apparatus at 100 V for 30 min under cooling conditions. Membranes were then incubated in milk diluent blocker (KPL, Gaithersburg, MD, USA) for 3 h on a shaker at RT. Dilutions (1:1000) of primary antibodies for rabbit anti-rat p53, rabbit anti-rat TNF-α, goat anti-rat IL-1β and goat anti-rat GFAP were applied to separate membranes and left on a shaker overnight at 4 °C. Membranes were thoroughly rinsed in PBS/Tween, then incubated in appropriate species-specific HRP-conjugated secondary antibodies (KPL) for 90 min at 37 °C. Following thorough rinsing in PBS/Tween, chemiluminescent agents (Pierce) were used to detect the HRP tag on a chemiluminescent scanner (Kodak Image station 440CF) with analysis software under the same brand and model name. Band density measurements were compared among treatment groups.

2.7. Glutathione reductase activity

Brain tissue from the penumbral region of the injury was extracted at 72 h post-TBI as previously described and analyzed using a glutathione reductase assay kit (Trevigen, Gaithersburg, MD). Briefly, samples were homogenized in T-PER (Pierce) and centrifuged at 8500 × *g* for 10 min at 4 °C. The following components were added to a quartz cuvette: dH_2O, 25× assay buffer, sample, GSSG solution, NADPH. The spectrophotometer was set to an absorbance of 340 nm. Measurements were taken every 60 s for 5 min. Activity was determined for each sample by calculating the rate of decreased absorbance at 340 nm ((absorbance 340 nm at 1 min) − (absorbance 340 nm at 5 min)/4 min = ΔAbsorbance 340 nm/min)). Each sample was measured three times and the average ΔAbsorbance 340 nm/min was taken as the activity level of GR.

2.8. Statistical analysis

Experimental results were expressed as mean ± SD. Data were examined for normality and homoscedasticity before being analyzed by one-way analysis of variance (ANOVA). The Tukey test was performed for post hoc analyses. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. ent-PROG inhibits PROG binding to the PR

ent-PROG acts as a competitor for radiolabeled PROG-binding to HuPRB over-expressed in COS cells. The enantiomeric steroid binds to PRB with an equilibrium constant (K_d) of 74 nmol (Fig. 2).

3.2. ent-PROG does not activate PR-mediated transcription

In COS cells transfected with a plasmid encoding the HuPRB along with a plasmid containing the MMTV promoter driving luciferase production, 1000 nmol of *ent*-PROG showed minimal to no activation of transcription as measured by luciferase activity. PROG transcriptional activity was a significant

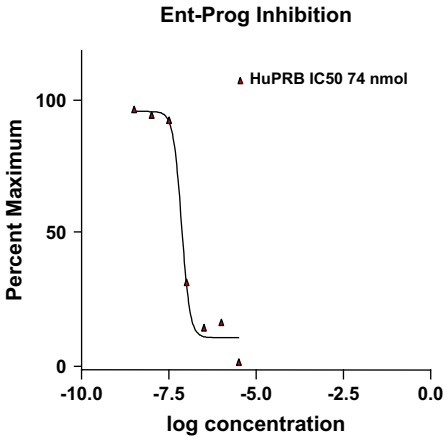

Fig. 2. PR-binding assays. *ent*-PROG binds to PRB. COS cells were transfected with a cDNA encoding HuPRB and binding studies were performed using 1 nM radiolabeled PROG in the presence of increasing concentrations of *ent*-PROG. The *y*-axis represents the percent of bound PROG relative to maximum binding (no *ent*-PROG). Each triangle represents the average of three points, and this experiment was performed three times with similar results.

fourfold higher than any measurable activity of *ent*-PROG (Fig. 3).

3.3. Cortical injury-induced edema is reduced by ent-PROG

As previously reported (Galani et al., 2001; Djebaili et al., 2004), exogenous treatments with PROG (16 mg/kg) and ALLO (8 mg/kg) significantly reduce post-injury mean % brain water content at 72 h compared to vehicle-injury (Fig. 4). There were no differences in cerebral edema compared to vehicle-injury when treating with 4 mg/kg *ent*-PROG. However, post hoc analysis showed a significant decrease in cerebral edema with *ent*-PROG at 16 mg/kg. Since *ent*-PROG at 4 mg/kg had no effect, we chose to use *ent*-PROG at 16 mg/kg for completion of the experiment.

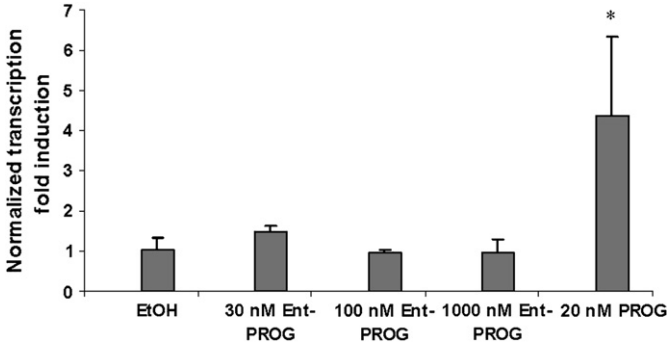

Fig. 3. PR-mediated transcription. *ent*-PROG does not activate HuPRB-mediated transcription. COS cells were transfected with a cDNA encoding HuPRB and *ent*-PROG was added at the indicated concentrations for 48 h. PROG and ethanol (EtOH) were added as positive and negative controls, respectively. The data are shown as fold-induction over EtOH, and represent the average ± standard deviation ($n = 3$). This experiment was performed three times with similar results.

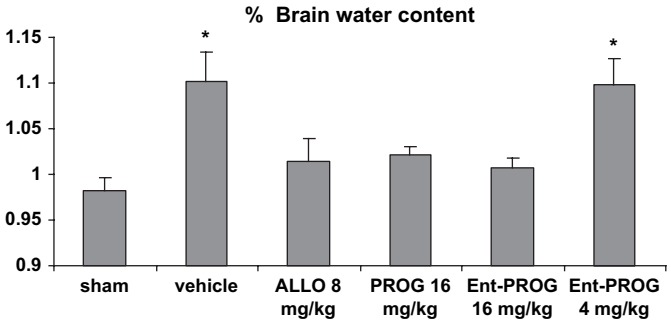

Fig. 4. Cerebral edema analysis. Brain-injured tissue was taken from PROG (16 mg/kg), ALLO (8 mg/kg), *ent*-PROG (16 mg/kg), *ent*-PROG (4 mg/kg) vehicle and sham groups ($n = 6$/group). Wet to dry weight analysis of water content showed a significant decrease in edema after all neurosteroid treatments except for *ent*-PROG (4 mg/kg) compared to vehicle. A dose above 4 mg/kg but not higher than 16 mg/kg of *ent*-PROG is required to lower brain edema after TBI. *Denotes significance at $p < 0.05$.

3.4. ent-PROG reduces reactive gliosis

PROG has been shown to diminish reactive astrocyte expression and astroglial swelling after TBI (Garcia-Estrada et al., 1999; Djebaili et al., 2005). Our parametric analysis of the data indicated that there were differences among groups with respect to GFAP levels as measured by Western blotting and densitometry. Post hoc analysis showed that *ent*-PROG, along with PROG and ALLO, significantly reduced protein expression of GFAP, compared to vehicle. However, the level of GFAP protein expression in the neurosteroid treated groups are still significantly higher than shams. These findings indicate that neurosteroids reduce injury-induced astrocytic hypertrophy (Fig. 5).

3.5. Cerebral inflammatory cytokines are inhibited by ent-PROG

PROG and ALLO partially protect against neuroinflammation after TBI by inhibiting expression of inflammatory

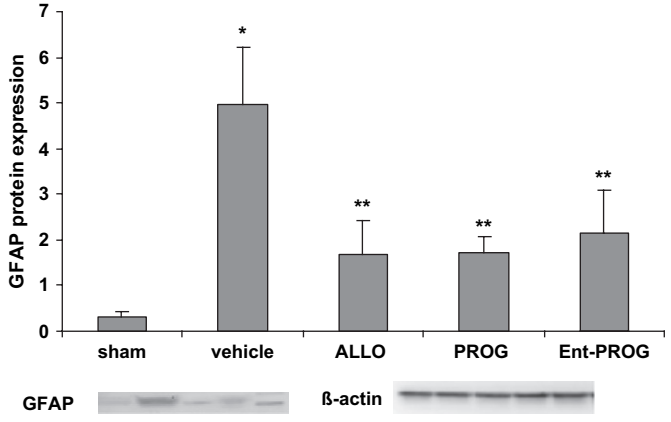

Fig. 5. Reactive gliosis analysis. Protein extraction and immunoanalysis of GFAP-reactive astrocytosis was performed at 72 h on brain-injured samples of rats treated with either PROG (16 mg/kg), ALLO (8 mg/kg), *ent*-PROG (16 mg/kg), or vehicle and sham operates ($n = 6$). Neurosteroid-treated groups showed a reduction in GFAP protein expression compared to vehicle. However these levels were still significantly higher than sham. *Denotes significance from all other groups at $p < 0.05$; **denotes significance from sham at $p < 0.05$.

cytokines such as TNF-α and IL-1β (Pettus et al., 2005). A one-way ANOVA showed that there were differences among groups with respect to both inflammatory cytokines. Subsequent post hoc analyses of Western blotting and densitometric data showed that 16 mg/kg *ent*-PROG induced a significant decline in TNF-α and IL-1β comparable to that of PROG and ALLO when compared to cytokine expression in the vehicle-treated injury group (Fig. 6A, B).

3.6. ent-PROG inhibits the induction of the neuronal cell death mediator p53 after cortical injury

Our laboratory has previously shown that both PROG and ALLO reduce apoptosis after TBI by increasing the expression of the anti-apoptotic protein bcl-2 and decreasing the pro-apoptotic protein caspase-3 (Djebaili et al., 2005). Here we used Western blotting and densitometric analysis to determine whether *ent*-PROG (16 mg/kg), PROG (16 mg/kg), or ALLO (8 mg/kg) affected pro-apoptotic p53 protein expression after TBI, given that p53 mediates both bcl-2 and caspase 3 activity. *ent*-PROG as well as PROG and ALLO treatment caused a significant reduction in p53 expression at 6 h compared to vehicle-treated injury (Fig. 7). There were no significant differences between sham and any of the treatments with progestins. We did not find any significant differences in p53 expression among the groups at 72 h post-TBI.

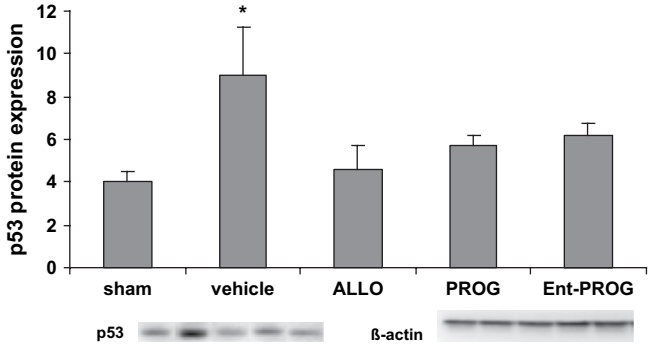

Fig. 7. Analysis of the cell death mediator, p53. Immunoanalysis of p53 protein abundance was done acutely at 6 h post-TBI. Brain tissue was collected from rats treated with PROG (16 mg/kg), ALLO (8 mg/kg), *ent*-PROG (16 mg/kg), vehicle and sham operates ($n = 5$/group). PROG, ALLO and *ent*-PROG reduced p53 expression compared to vehicle and were comparable to sham. *Denotes significance at $p < 0.05$.

3.7. ent-PROG increases anti-oxidant activity in the injured brain

We have previously reported that PROG reduces lipid peroxidation and oxidative stress when administered after TBI (Roof et al., 1997). In this current study, we show that anti-oxidant activity was significantly different among treatment groups. Post hoc analyses of the data indicated that all neurosteroid treatments significantly increased GR activity compared to vehicle (Fig. 8). However, *ent*-PROG administration showed the most potent effect, significantly increasing GR activity in comparison not only to vehicle-injured rats but also to those treated with PROG and ALLO (Fig. 8).

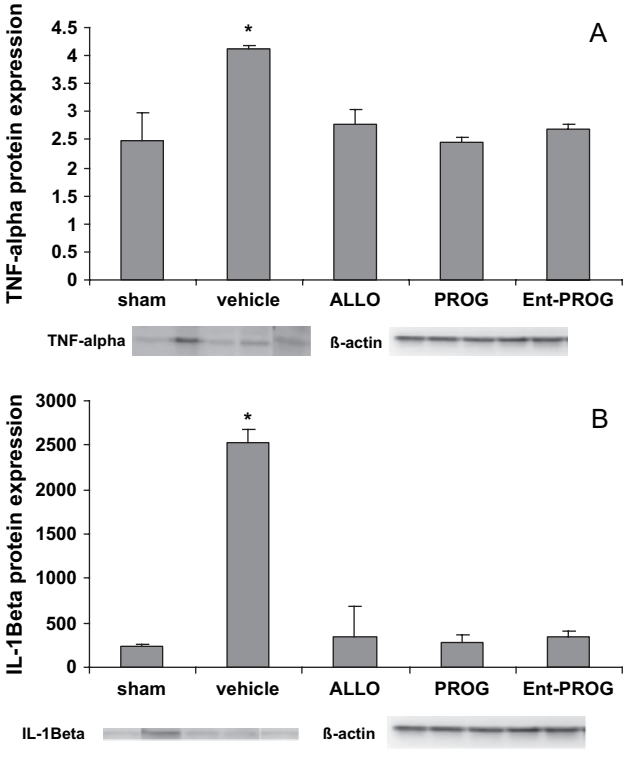

Fig. 6. (A,B) Progestins reduce inflammatory cytokines after brain injury. Immunoassays were used to determine the protein expression of (A) TNF-α and (B) IL-1β at 72 h after brain injury. Treatments included PROG (16 mg/kg), ALLO (8 mg/kg), *ent*-PROG (16 mg/kg) or vehicle and untreated sham operates ($n = 6$). Results show a significant and comparable decrease in both cytokines following treatment with neurosteroids compared to vehicle. Expression levels following neurosteroid treatments were equivalent to shams. *Denotes significance at $p < 0.05$.

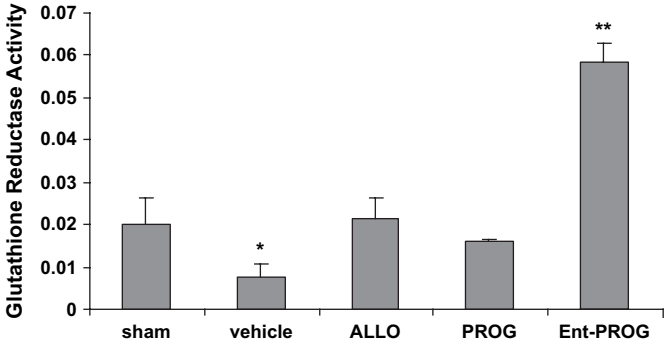

Fig. 8. Measurement of brain anti-oxidant capacity. Protein extracts were taken from injured and sham operate brains 72 h post-TBI. GR activity was measured by spectrophotometric analysis as a reflection of NADPH oxidation over a 5 min interval at an absorbance of 340 nM. Readings show a significant increase in GR activity in samples taken from sham rats and those treated with neurosteroids compared to vehicle. The GR activity of *ent*-PROG samples were significantly increased compared to all other neurosteroids and sham treatments ($n = 6$). *Denotes significance at $p < 0.05$ from vehicle. **Denotes significance at $p < 0.01$ from vehicle and $p < 0.05$ from PROG, ALLO and sham operates.

4. Discussion

Our previous findings have shown that bilateral contusion injury to the MFC increases GFAP, TNF-α, IL-1β, p53 and brain water content while reducing anti-oxidant capacity (Djebaili et al., 2005; Roof and Stein, 1992). Conversely, the results presented here show that PROG, its metabolite ALLO, and *ent*-PROG (16 mg/kg) all reduced edema, inflammation, and reactive gliosis, while increasing the production of anti-oxidants in the injured brain. We show in this experiment that PROG's enantiomer is neuroprotective after brain injury and further demonstrate that its neuroprotective actions are probably independent of PR-mediated transcriptional activity. Here we discuss the known common molecular mechanisms through which selected steroids act to mediate neuroprotection after TBI.

4.1. Neurosteroids and TBI

In both TBI and stroke, PROG and its metabolite ALLO have been shown to be neuroprotective (He et al., 2004; Djebaili et al., 2005; Sayeed et al., 2006). Serum levels of PROG are inversely proportional to cerebral edema (Wright et al., 2001). Cerebral edema is reduced by both PROG and ALLO within the first 72 h following injury and this reduction extends out to 5 days (Roof et al., 1994; Galani et al., 2001; Hoffman et al., 2001). Previous reports have shown that at 48 h, PROG reduces the production of inflammatory cytokines (Pettus et al., 2005). Glutathione is a known antioxidant that decreases with TBI, and this decrease may exacerbate the injury cascade (Bayir, 2004). As noted above, studies have revealed that PROG acts as a neuroprotectant by reducing the production of reactive oxygen species (Goodman et al., 1996; Moorthy et al., 2005), and biologically active lipid peroxidation by-products (Roof et al., 1997). We have shown that PROG's GABAergic metabolite ALLO, which lacks PR affinity (Rupprecht and Holsboer, 1999), reduces inflammation and cell death following treatment.

We cannot ignore the possibility that the effects of neurosteroids on the injured brain may be due, in part, to systemic actions in other systems of the body. For example, recent studies have shown that there is a systemic increase in reactive oxygen species combined with a decrease in many low molecular weight antioxidants up to 24 h post-TBI. These effects have been noted in the kidney, liver and lungs, and include decreases in ascorbic acid and α-tocopherol (Shohami et al., 1999; Bayir et al., 2004). There are also reports showing that treatment with 17β-estradiol (E2), as with PROG and its metabolites, provides systemic protection from free radicals in both cardiovascular tissue and mononuclear leukocytes (Telci et al., 2002; Cassidy, 2003). However, there are mixed results in studies of PROG's ability to act as an anti-oxidant, since its structure and those of ALLO and *ent*-PROG do not indicate free radical scavenging properties. Nonetheless, treatment with PROG reduces free radical damage and lipid peroxidation (Roof et al., 1997; Kuebler et al., 2003).

4.2. Interactions of ent-PROG with the PR

ent-PROG is the mirror image stereoisomer (enantiomer) of PROG, with chemical and physical properties identical to those of natural PROG, but, as shown here, although it binds with moderate affinity to the classical PR, *ent*-PROG does not activate PR-mediated transcription. Thus, *ent*-PROG may be regulating its neuroprotective effects via transcription-independent PR-mediated signaling. Alternatively, *ent*-PROG may work in a PR-independent fashion, much like the estrogen enantiomer, *ent*-E2, which has minimal binding affinity to the ER but nonetheless provides neuroprotection after head injury (Green et al., 2001). There may also be differences in the mechanisms of neuroprotection, since *ent*-E2 as a phenolic compound has antioxidant properties, whereas *ent*-PROG does not.

Another potential regulator of PROG-mediated neuroprotection is the $GABA_A$ receptor, which can be activated by the PROG metabolite ALLO to increase inhibitory tone and subsequently decrease excitotoxic cell death in the injured brain (Lockhart et al., 2002). However, *ent*-PROG metabolism does not produce significant GABAergic metabolites, nor does *ent*-ALLO affect GABAergic transmission (Wittmer et al., 1996, Zorumski et al., 1998).

4.3. Effects of ent-PROG on TBI-induced edema, inflammation and reactive gliosis

By treating TBI rats with *ent*-PROG at 16 mg/kg, we were able to obtain several positive outcomes. *ent*-PROG reduced brain water content 72 h after TBI compared to vehicle-injured animals and reduced reactive gliosis as measured by decreased expression of GFAP compared to vehicle-injured animals. *ent*-PROG concomitantly reduced the cytokines TNF-α and IL-1β to about the same levels we saw after treatments with PROG and ALLO. TNF-α and IL-1β are known to impair BBB integrity and cause neuronal cell death (Morganti-Kossmann et al., 2002), and thus their neurosteroid-mediated decline contributes to the reduced cerebral edema and subsequent neuronal protection reported in our previous studies (He et al., 2004).

4.4. Effect of ent-PROG on anti-oxidant activity after TBI

The brain's primary defense mechanisms against reactive oxygen species are antioxidants which include reduced glutathione (GSH). The glutathione reductase (GR) enzyme functions to increase the ratio of reduced GSH to that of glutathione disulfides (GSSG) (Kirsch and De Groot, 2001). PROG, ALLO and *ent*-PROG significantly increased GR activity after brain injury compared to the vehicle-injured animals. Interestingly, *ent*-PROG showed the most robust increase, suggesting it may be a more potent anti-oxidant than its natural form. Past studies have suggested a role for progestins in anti-oxidant defense, which is mediated through a reduction in lipid peroxidation and improvement in cell membrane stabilization (Roof et al., 1997; Shohami et al., 1999; Kuebler et al., 2003). Our findings suggest that

PROG, ALLO and *ent*-PROG improve GSH antioxidant status in the injured brain. Further work to characterize other antioxidant systems is needed as progestin treatments for TBI are developed.

4.5. Anti-apoptotic effects of ent-PROG

Studies have shown that PROG and ALLO reduce excitotoxic cell death mediated through NMDA receptors and excessive release of post-synaptic glutamate (Ciriza et al., 2004; Lockhart et al., 2002). Furthermore, progestins have been shown to reduce the expression of the cell death mediator, caspase-3, in acute stages of brain injury (Djebaili et al., 2004; Cutler et al., 2005). p53-dependent apoptosis is associated with mitochondrial release of cytochrome *c* and subsequent activation of caspase-3 (Miller et al., 2000). Our studies confirm that treatment with PROG and ALLO decreases expression of p53 acutely at 6 h compared to vehicle-injury. The same pro-survival effects on p53 expression were found after treatment with *ent*-PROG. These findings suggest that PROG, ALLO and *ent*-PROG can improve neuronal survival by reducing the p53-mediated apoptotic cascade. However, these findings are not seen at 72 h post-TBI.

5. Summary

Treatment with *ent*-PROG is effective at the same treatment (1, 6, 24 and 48 h) and analysis (72 h) times as previously studied for PROG and ALLO (Grossman et al., 2004; Pettus et al., 2005; Shear et al., 2002). The fact that *ent*-PROG, PROG and ALLO have comparable molecular effects suggests they could act through the same mechanism. Our findings show that *ent*-PROG inhibits PROG binding to the PR and does not activate PR-mediated transcription. This work further substantiates the finding that the protective effects of PROG on TBI can be mediated either independently of the PR or via transcription-independent PR-regulated signaling. This hypothesis is worth more consideration in light of the fact that treatment with *ent*-PROG, though neuroprotective, may inhibit *de novo* PROG binding to the neuronal PR. Although ALLO does not bind to the PR, it is hypothesized to mediate the molecular expression of neuroprotective components through GABAergic actions. This may be the same mechanism used by PROG following its conversion to ALLO. At present, we cannot rule out the possibility that ent-PROG may also work through a GABAergic mechanism. However, currently there is neither evidence for the binding of *ent*-PROG to the $GABA_A$ receptor nor evidence that *ent*-PROG can be metabolized to ALLO. We also cannot ignore the possibility that *ent*-PROG may be acting through a novel receptor mechanism not yet defined. This novel mechanism may be through the activation of the pregnane X receptor (PXR), given its promiscuity and subsequent ability to produce both an anti-inflammatory and anti-apoptotic effects (Toell et al., 2002; Zucchini et al., 2005). Another possibility is that *ent*-PROG could act through the membrane-associated progesterone binding protein 25 Dx (Meffre et al., 2005). Furthermore, though *ent*-PROG is protective despite antagonizing PROG's binding to the PR, we cannot dismiss the unlikely possibility that *ent*-PROG may still mediate some of its protective effects as an antagonist at the PR. In conclusion, the mechanism of *ent*-PROG-mediated neuroprotection and the use of *ent*-PROG treatment for TBI warrant further attention. Future work will analyze the effects of *ent*-PROG on functional outcomes including; spatial learning and memory, sensory neglect and stress response. These studies will confirm if our molecular findings reported here are transferred into behavioral improvements.

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