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Progesterone Protects Against Lipid Peroxidation Following Traumatic Brain Injury In Rats

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ABSTRACT

The gonadal hormone, progesterone, has been shown to have neuroprotective effects in injured nervous system, including the severity of postinjury cerebral edema. Progesterone's attenuation of edema is accompanied by a sparing of neurons from secondary neuronal death and with improvements in cognitive outcome. In addition, we recently reported that postinjury blood–brain barrier (BBB) leakage, as measured by albumin immunostaining, was significantly lower in progesterone-treated than in nontreated rats, supporting a possible protective action of progesterone on the BBB. Because lipid membrane peroxidation is a major contributor to BBB breakdown, we hypothesized that progesterone limits this free radical-induced damage. An antioxidant action, neuroprotective in itself, would also account for progesterone's effects on the BBB, edema, and cell survival after traumatic brain injury. To test progesterone's possible antiperoxidation ef-fect, we compared brain levels of 8-isoprostaglandin $F_{2\alpha}$, (8-isoPGF$_{2\alpha}$), a marker of lipid peroxidation, 24, 48, and 72 h after cortical contusion in male rats treated with either progesterone or the oil vehicle. The brains of progesterone-treated rats contained approximately one-third of the 8-isoPGF$_{2\alpha}$ found

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in oil-treated rats. These data suggest progesterone has antioxidant effects and support its potential as a treatment for brain injury.

Index Entries: 8-isoPGF$_{2\alpha}$; free radicals; antioxidant; contusion; steroid; neuroprotection; blood-brain barrier; immunocytochemistry.

INTRODUCTION

Over the last several years, we have published a series of reports describing progesterone's neuroprotective effects in the injured central nervous system. Our initial observation of a sex difference in severity of postinjury edema led to the discovery of progesterone's ability to reduce cerebral swelling (Roof et al., 1992, 1993, 1996a). We have since found that progesterone is equally effective at treating postinjury cerebral edema in males and females (Roof et al., 1992), and when given either before the injury (Roof et al., 1993) or as late as 24 h afterward (Roof et al., 1996a). These findings are clinically relevant, because postinjury cerebral edema can cause substantial cell loss (Cervos-Navarro and Lafuente, 1991; Smith et al., 1994). In addition, because of the interactive and self-perpetuating nature of the secondary injury cascade, rapid amelioration of edema is likely to result not only in the sparing of these cells, but also those that would have been damaged as a result of edema's exacerbation of other secondary injury processes (Cervos-Navarro and Lafuente, 1991; McIntosh, 1994; Stein and Roof, 1996).

Although progesterone's ability to reduce edema is now well established (Roof et al., 1992, 1993, 1996a), the mechanism by which it does so is not yet clear. Since a casual factor in the formation of edema is disruption of the blood–brain barrier (BBB) (Betz et al., 1989; Wahl et al., 1993), we hypothesized that progesterone may have a protective or reparative effect on it. A comparison of BBB function in progesterone and nontreated rats during the week after contusion provided support for this hypothesis. Three and five days after contusion we found significantly less albumin immunostaining, a measure of BBB leakage, in the brains of progesterone-treated than in oil-treated rats (Roof et al., 1996b).

A significant cause of postinjury BBB damage, and a possible mechanism for the apparent protection associated with progesterone, is the peroxidation of lipid membranes by arachidonate metabolites and oxygen radicals (Demopoulos et al., 1982; Smith et al., 1994; Kukreja et al., 1986). These reactive molecules cause their damage by attacking and removing allylic hydrogens from the polyunsaturated fatty acids in the phospholipid bilayer of cell membranes (Hall and Braughler, 1993). If not controlled, lipid peroxidation will spread geometrically, both over the cell's membrane and into adjoining areas, resulting in substantial damage. The BBB is particularly susceptible to this process (Demopoulos et al., 1980, 1982; Kontos et al., 1980; Wei et al., 1981; Hall et al.,

1993), which can result in its abnormal and prolonged opening (Chan et al., 1985; Hall et al., 1988; Greenwood, 1991; Wahl et al., 1993).

We suggest that progesterone's observed protection of the BBB may be due to, at least in part, blocking the damage caused by lipid peroxidation. Compounds such as vitamin E (Stein et al., 1991) and methylprednisolone (Hall et al., 1994) have been shown to act in this way, conferring their protection either by scavenging and neutralizing free radicals (Braughler et al., 1989) or by restricting their movement and propagation in the membrane (Audrus et al., 1991; Hall, 1993). Progesterone has already been demonstrated to limit peroxidative damage in the uterus (Walsh, 1994) and corpus luteum (Shimamura et al., 1995). A similar effect in brain tissue has been demonstrated in vitro (Goodman et al., 1996), although this finding was not replicated in a similar study (Behl et al., 1995). Progesterone is capable of membrane stabilization (Whiting et al., 1995) and has been hypothesized to scavenge free radicals (Seligman et al., 1979; Betz and Coester, 1990; Walsh, 1994), either of which would limit the damage caused by lipid peroxidation.

To determine whether progesterone can limit lipid peroxidative damage, we used an enzyme immunoassay to measure postinjury levels of 8-isoprostaglandin $F_{2\alpha}$ (8-isoPGF$_{2\alpha}$). This nonenzymatically produced prostaglandin is a product of oxidation of arachidonate by oxygen radicals (Morrow et al., 1990) and an indirect marker of lipid peroxidation. We have previously found this to be a reliable and sensitive assay, and have used it to demonstrate that high levels of 8-isoPGF$_{2\alpha}$ are found in the brain following contusion of the medial frontal cortex (Hoffman et al., 1996). This marker has been used in other studies of CNS injury, including a demonstration of the antioxidant effects of methylprednisolone (Yonkers et al., 1993). We hypothesize that lower brain levels of this marker, indicative of reduced lipid peroxidation, will be found in progesterone-treated rats compared to those treated only with the oil vehicle.

METHODS

Subjects

Subjects for this experiment were 66 male Sprague-Dawley rats, approx 90 d of age. The rats were housed in group cages (4–5/p cage) with a 12-h light/12-h dark, reverse light cycle. Food and water were provided ad libitum throughout the experiment.

Surgery and Progesterone Treatment

Medial frontal cortex contusions were produced using a pneumatically driven piston device that impacts the brain through a 6-mm

diameter craniotomy at a velocity of 2.25 m/s, compressing the cortex to a depth of 2 mm. The impactor device consists of a 9/16" dual-stroke air cylinder containing a 3-mm diameter piston with a 5-mm stainless-steel tip.

Forty-eight rats were anesthetized (10 mg/kg Xylazine, ip + 50 mg/kg Ketamine, ip) and mounted in a stereotaxic device. The scalp was incised at the midline to expose the skull, and a 6-mm craniotomy was made immediately anterior to bregma at the midline. After impact, and once all bleeding was stopped, the fascia and scalp were sutured closed. An additional 18 rats served as noncontused controls.

Following surgery, the rats were randomly assigned to the progesterone (n = 33) or oil vehicle (n = 33) group. Progesterone was dissolved in peanut oil (4 mg/mL), and the initial injection (4 mg/kg, 5 min postinjury) was given ip to ensure rapid absorption. The remaining injections (also 4 mg/kg) were given subcutaneously for more gradual absorption at 6 h postinjury and again once each 24-h period postinjury until the rats were killed at either 24, 48, or 72 h after contusion for 8-isoPGF$_{2\alpha}$ assessment. The oil-treated group received equivalent injections of peanut oil.

Purification Protocol

The rats were anesthetized with pentobarbital (Nembutal, 75 mg/kg) and then killed by decapitation at 24, 48, or 72 h postinjury (8 progesterone-treated, 8 oil-treated, and 6 nonsurgical control rats at each time-point). The brains were quickly removed, and the frontal lobes (all tissue anterior to the pituitary stalk) were dissected out on ice and weighed. Two milliliters of ice-cold, absolute ethanol were added, and the frontal lobes were homogenized for 20 s with a biohomogenizer. Homogenates stood for 5 min at 4°C, and then were centrifuged at 1500 rpm for 10 min to remove precipitated proteins. The supernatants were diluted with 8 mL of 0.1M phosphate buffer (prepared with deionized dH_2O; pH 7.2) and vortexed. Each sample was passed through a C-18 Sep-Pak actived with 5 mL absolute ethanol and 5 mL deionized dH_2O and the cartridge rinsed with deionized dH_2O and 5 mL of HPLC-grade hexane. The target eicosanoid was eluted with 5 mL of HPLC-grade ethyl acetate containing 1% HPLC-grade methanol, and then divided into five 1.5-mL Eppendorf snap-cap centrifuge tubes. The samples were placed in a vacuum centrifuge and spun until lyophilized (1 h) to remove all traces of solvent. Samples were reconstituted with enzyme immunoassay buffer and added to a 96-well plate for EIA analysis using an 8-isoPGF$_{2\alpha}$ EIA kit from Cayman (Ann Arbor, MI). The plate was incubated for 18 h and then developed under darkness with Ellman's reagent on a plate shaker.

Plate readings were made beginning 15 min after the addition of Ellman's reagent, using a computer-interfaced Techan SLT Spectra™ microplate reader. Concentration of 8-isoPGF$_{2\alpha}$ in each well was calculated using DeltaSOFT II™ Microplate Analysis software for the Macintosh™ (Biometallics, Inc., Princeton, NJ). A four-parameter standard curve calculation was used to interpolate the concentration (pg/mL) of the samples. The estimated amount of 8-isoPGF$_{2\alpha}$ in the tissue was then calculated by the following equation:

$$[\text{Conc. (pg/mL)} \times \text{vol reconstituted sample (1 mL)/frontal lobe wt (g)}] = \text{8-isoprostane levels (pg)/brain wt (g)} \quad (1)$$

RESULTS

Assay Parameters

The typical accuracy of the assay as determined by the standard curve was very high (r = 0.995; R = 0.990; RMS = 0.157). The range of "r" for all of the assay runs in this study fell between 0.950 and 0.998.

Brain levels of 8-isoPGF$_{2\alpha}$ from 45 contused rats and 18 nonsurgical control rats were included in the analysis. Data from three rats could not be used because of death (n = 1) or errors in procedure (n = 2). An analysis of variance of 8-isoPGF$_{2\alpha}$ levels, measured in pg/g of brain tissue, resulted in significant main effects of treatment, $F(2, 54)$ = 20.199, $p < 0.001$, and time after injury, $F(2, 54)$ = 16.57, $p < 0.001$; as well as an interaction between treatment and time, $F(4, 54)$ = 8.091, $p < 0.001$. Post hoc t-tests showed that oil-treated contused rats had higher levels of 8-isoPGF$_{2\alpha}$ compared to nonsurgical controls at 24 h ($t[12]$ = 7.718, $p < 0.001$) and 48 h ($t[12]$ = 2.194, $p < 0.05$) after injury. By 72 h after contusion, 8-isoPGF$_{2\alpha}$ levels had dropped to the control baseline in all groups. The rise in levels of 8-isoPGF$_{2\alpha}$ for progesterone-treated rats was much less than that of the oil-treated rats at 24 h after injury ($t[13]$ = 5.941, $p < 0.001$) and, in fact, was not significantly different from that of nonsurgical controls at this time ($t[11]$ = 1.965, p = 0.0545), or when measured 48 and 72 h after injury. These means are shown in Fig. 1.

To determine whether any possible heterogeneity of variance in the data influenced these results, the data were transformed using the square root, and reanalyzed. The transformation did not produce changes in any of the statistical results. Therefore, the untransformed data are presented.

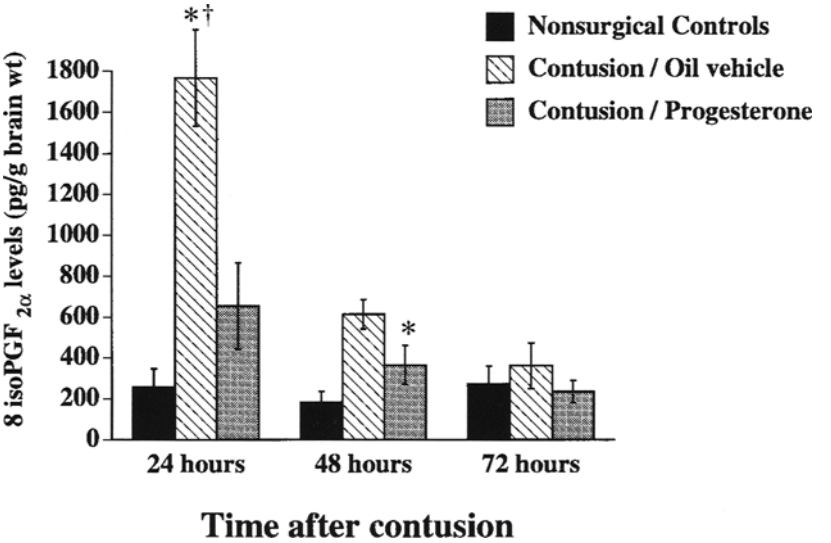

Fig. 1. Brain levels of 8-isoPGF$_{2\alpha}$ (pg/g tissue) in male rats at 24, 48, or 72 h after medial frontal cortical contusion as well as from nonsurgical controls. Asterisks indicate significant difference ($p < 0.05$) from nonsurgical controls. Dagger indicates significant difference ($p < 0.05$) from progesterone-treated rats.

DISCUSSION

The results of this study suggest an antioxidant action by progesterone and provide further support for its potential as a treatment for brain injury. The comparatively low levels of 8-isoPGF$_{2\alpha}$ found in the brains of rats given progesterone treatment after a contusion injury indicate they were spared from much of the damage normally caused by lipid peroxidation (Hall, 1989; Hoffman et al., 1996), which is substantial enough to result in significant functional impairment (Hall and Braughler, 1989; Hall, 1995).

Although the current data are not proof of a causal relationship, progesterone's effects on the BBB and edema may be secondary to inhibition of lipid peroxidation. The pattern of BBB breakdown and repair in our injury model is consistent with this hypothesis. Leakage of the BBB, as measured by brain levels of Evan's blue and albumin, is evident within 2 h of contusion, probably as a direct result of the impact. This leakage increases over subsequent days, as shown by high levels of the two markers 1–7 d postinjury (Duvdevani et al., 1995). Although progesterone treatment did not eliminate the initial disruption, significantly reduced brain levels of albumin extravasation found 3 and 5 d after contusion in progesterone-treated rats suggest the barrier was spared from much of the secondary deterioration (Roof et al., 1996b). Since lipid peroxidation is known to contribute to secondary BBB damage (Demopou-

los et al., 1982; Kukreja et al., 1986; Smith et al., 1994), progesterone's effects on the BBB may be accounted for with this action.

Sparing of the BBB from postinjury lipid peroxidative damage would, in addition, be expected to impact on other secondary injury processes, including the formation of cerebral edema (Chan et al., 1985). Although interactions between these secondary injury processes are complex and not limited to a linear sequence of events, the reduced edema associated with progesterone (Roof et al., 1992, 1996a) may be a result of reduced lipid peroxidative damage of the BBB. The rapid initiation of lipid peroxidation is consistent with this scenario, which in our injury model, occurs at least within 2 h of contusion (Hoffman et al., 1996), the earliest we have seen so far. Our current data do not allow us to claim that the initiation of lipid peroxidation precedes that of BBB leakage and edema, which are also detectable 2 h after injury. Other studies, however, in which very early assessment of lipid peroxidation was made suggest that it begins within minutes of injury (e.g., Hall et al., 1993). We have demonstrated that peak BBB leakage and edema levels lag behind that of lipid peroxidation by 24–48 h (Duvdevani et al., 1995; Hoffman et al., 1996). Although it is quite possible that multiple mechanisms are involved, an antioxidant action may be at the heart of progesterone's general neuroprotective capacity.

We are actively investigating, but have not yet determined, the specific mechanism for progesterone's antioxidant effect. As discussed earlier, both radical scavenging and membrane stabilization actions are likely possibilities. The antioxidant effects of other steroids, including tirilizad (Hall and Travis, 1988; Hall et al., 1988, 1994; Zuccarello and Anderson, 1989) and methylprednisolone (Hall, 1985), have been attributed to one or both of these actions. Progesterone, also a steroid, is thought to be capable of both actions (Seligman et al., 1979; Olson et al., 1988; Betz and Coester, 1990; Walsh, 1994), either of which could account for its observed neuroprotective effects.

An alternate explanation for progesterone's protective effects, and one we are currently examining, is through its interaction with GABA and/or glutamate receptors. Progesterone and some of its metabolites are known to bind to and potentiate activity at the $GABA_A$ receptor (Majewska et al., 1986; Harrison et al., 1987; Gee et al., 1988), and to contribute to GABA-related anticonvulsant, antianxiety, and anesthetic effects (Hogskilde et al., 1988; Belelli et al., 1989; Kokate et al., 1994; Frye, 1995). There is also evidence that progesterone can act as antagonist at glutamate receptors (Smith, 1991). GABA potentiation and glutamate antagonism can both contribute to the reduction of secondary excitotoxic damage following brain injury (Faden et al., 1989; McIntosh et al., 1990; Panter and Faden, 1992). Such an action by progesterone is supported by the results of an in vitro study in which it was shown to reduce the calcium influx associated with excitotoxic injury (Goodman et al., 1996). This last action was observed to occur in the presence

of RNA and protein synthesis inhibitors, suggesting a nonreceptor mechanism.

This demonstration of an apparent antioxidant action by progesterone has significant implications for functional outcome after brain injury, because oxygen radical-induced lipid peroxidation is a cause of considerable secondary damage after brain trauma (see reviews by Kontos and Povlishock, 1986; Braughler and Hall, 1989; Hall and Braughler, 1989, 1993). The current data bring us one step closer to understanding progesterone's ability to reduce postinjury cerebral edema and improve functional outcome (Roof et al., 1994) after traumatic brain injury. Because it is effective in relatively moderate doses and throughout a window of at least 24 h postinjury (Roof et al., 1996a), progesterone has considerable potential as a treatment for head injury in humans.

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