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Progesterone is neuroprotective after transient middle cerebral artery occlusion in male rats

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Abstract

Progesterone (PROG) is a neurosteroid, possessing a variety of functions in the central nervous system. Exogenous PROG has been shown to reduce secondary neuronal loss in conjunction with attenuated brain edema after cerebral contusion and to reduce brain edema after focal cerebral ischemia. In the present study, we assessed the neuroprotective potential of PROG in a model of focal cerebral ischemia in the rat. Forty-eight male Wistar rats were randomly assigned to 4 groups, i.e. pretreatment with water soluble PROG, or dimethyl sulfoxide (DMSO) dissolved PROG, or DMSO as control or delayed treatment with DMSO dissolved PROG. Middle cerebral artery occlusion (MCAO) was induced by insertion of an intraluminal suture and reperfusion was performed by withdrawing the suture. Pretreatments were initiated 30 min before MCAO via intraperitoneal injection. Delayed treatment was initiated upon reperfusion following 2 h of MCAO. Infarct volume, body weight loss, and neurological deficit were measured 48 h after MCAO. Pre- and delayed treatment with DMSO dissolved PROG resulted in a 39% ($P < 0.05$) and 34% ($P < 0.05$) reduction in cerebral infarction, respectively, along with decreased body weight loss and improved neurological function as compared to control animals, whereas no statistically significant reduction in infarct volume by water soluble PROG was found. We demonstrated that administration of PROG to the male rat before or 2 hours after onset of MCAO reduces ischemic cell damage and improves physiological and neurological function 2 days after stroke. These results suggests potential therapeutic properties of PROG in the management of stroke.

Keywords: Progesterone; Cerebral ischemia; Neuronal damage; Rat

1. Introduction

Progesterone (PROG), a female hormone, has drawn increasing attention to its actions in the central nervous system (CNS). PROG receptors are widely distributed in the CNS, including hypothalamus, preoptic area, midbrain, cortex, amygdala, hippocampus, caudate-putamen and cerebellum [35]. Moreover, in addition to its synthesis in endocrine organs (ovary, corpus luteum and adrenal gland) [34], PROG is synthesized locally within central [54,56] and peripheral nervous tissues [25]. PROG and its precursor pregnenolone and metabolites are synthesized de novo from cholesterol or mevalonate by oligodendrocytes in the CNS [2,31,55,57] and by Schwann cells in the peripheral nervous system [25]. As a result of its de novo synthesis in the nervous system and its accumulation within the nervous system being at least in part independent of steroidogenic gland secretion rates [55,57], PROG is a neurosteroid. The actions of PROG in nerve cells are exerted classically through cytosolic/nuclear receptors specific for the steroid [47]; however, PROG has more recently been shown to modify the function of traditional neurotransmitter systems in the CNS, such as the inhibitory γ-aminobutyric acid (GABA) [10,27,28,40,50] and excitatory amino acids (EAA) [64,66] systems. The presence of receptors and sources of PROG within the nervous system as well as its modulation of inhibitory and excitatory amino acids suggest a broader role for PROG than simply as a gestational hormone. PROG and related metabolites are CNS depressants and exert anesthetic [8,38,49], anticonvulsant [1,29,30,33,69] and anxiolytic [7,32] actions by modifying the function of GABA and EAA neurotransmitter systems in the CNS.

Decreased cerebral edema subsequent to brain contusion is associated with a high level of circulating PROG and is independent of estrogen level [61]. Exogenous PROG

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treatment facilitates cognitive recovery and reduces secondary neuronal loss in conjunction with attenuated brain edema following contusion in both male and female rats when treatment is given before or after insult [59,60]. PROG administration also reduces brain edema during the early stages of focal cerebral ischemia [4]. PROG may inhibit active ion transport mediated by Na, K-ATPase, which in turn reduces edema [5]. PROG is 10 times more potent than dexamethasone in inhibiting the active ion transport in isolated brain capillaries [11]. Thus, PROG may be involved in the process of brain damage after ischemic insult and the administration of PROG may be beneficial to the neural tissue. In the present study, we tested the hypothesis that exogenous administration of PROG provides neuroprotection after transient (2 h) focal cerebral ischemia in the male rat.

2. Materials and methods

Male Wistar rats ($n = 48$) weighing 270–300 g were employed in all our experiments. We elected to test the effect of exogenously administered PROG on the male and not the female rat in order to exclude complicating effects on ischemic cell damage of hormonal fluctuations during the estrus cycle. Nonfasted animals were anaesthetized with 3.5% halothane, and maintained with 1.0–2.0% halothane in 70% N_2O and 30% O_2 using a face mask. Rectal temperature was maintained at 37°C throughout the surgical procedure using a feedback regulated water heating system. The right femoral artery was cannulated with medical grade silicone tubing (Technical Products, Inc., Decatur, GA) for monitoring blood pressure, and for sampling of blood for blood gas measurements (pH, pO_2, pCO_2) before and 20 min after initial administration of PROG or vehicle.

Middle cerebral artery (MCA) occlusion (MCAO) was induced, as previously described [12,26]. Briefly, the right common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA) were exposed. A length of 4-0 monofilament nylon suture (18.5–19.5 mm), determined by the animal weight, with its tip rounded by heating near a flame, was advanced from the ECA into the lumen of the ICA until it blocked the origin of the MCA. Two hours after MCAO, animals were reanaesthetized with halothane and reperfusion was performed by withdrawal of the suture until the tip cleared the lumen of the ICA.

Four randomly assigned populations of animals were tested. Group 1 ($n = 12$): water soluble PROG (PROG with balance of 2-hydroxypropyl-β-cyclodextrin) (Sigma Chemical Co., St. Louis, MO) dissolved in saline (4.0 mg/ml) was injected intraperitoneally (4.0 mg/kg) 30 min before MCAO. The remaining injections (all 4.0 mg/kg) were given at 6 and 24 h after MCAO, respectively. Group 2 ($n = 12$): the experimental protocol was identical to that in Group 1, except that PROG (4-pregnene-3,20-dione) (Sigma Chemical Co., St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) (8.0 mg/ml), instead of water soluble PROG, was administered. Group 3 ($n = 12$): the experimental protocol was identical to that in group 2, except that the initial injection was delayed to the onset of reperfusion (2 h after MCAO). Group 4 ($n = 12$): the experimental protocol was the same as that in Group 2, except that the same volume of DMSO without PROG (0.5 ml/kg) as in Group 2 was administered. The dose and the time points of administration of PROG in the present study were adapted from the experiments in which PROG was effective in reducing brain tissue damage after contusion [59,60]. In a preliminary pharmacokinetic study ($n = 2$) using Coat-A-Count PROG Procedure kit (Diagnostic Products Co., Los Angeles, CA), a solid-phase radioimmunoassay, demonstrated that PROG was absorbed rapidly by the intraperitoneal route of administration; the plasma level of PROG increased to 41.9 and 70.7 ng/ml 4 h after administration of DMSO dissolved PROG at the dose of 4.0 mg/kg from the pre-injection level of 7.17 and 5.29 ng/ml, respectively.

All animals were weighed before surgery for MCAO and at 24 and 48 h after MCAO. Neurological abnormali-

Table 1
Physiological parameters ($n = 12$/group)

Group	PROG in saline	PROG pre-MCAO	PROG post-MCAO	DMSO
Pre-injection				
pH	7.44 ± 0.01	7.44 ± 0.01	7.46 ± 0	7.46 ± 0
pCO_2 (mmHg)	41 ± 1	41 ± 1	36 ± 1	39 ± 1
pO_2 (mmHg)	122 ± 5	134 ± 4	133 ± 4	135 ± 3
Mean blood pressure (mmHg)	94 ± 3	94 ± 3	107 ± 3	102 ± 2
20 min post-injection				
pH				
pCO_2 (mmHg)	7.42 ± 0.02	7.43 ± 0.02	77.44 ± 0.01	7.40 ± 0.01
pO_2 (mmHg)	41 ± 1	39 ± 2	36 ± 1	43 ± 1
	123 ± 14	135 ± 5	134 ± 3	131 ± 4
Mean blood pressure (mmHg)	94 ± 3	95 ± 2	103 ± 3	102 ± 3

Values are mean ± S.E.M.

Table 2
The percent infarct volume to the contralateral hemisphere in the 4 experimental groups ($n = 12$/group)

Group	PROG in saline	PROG pre-MCAO	PROG post-MCAO	DMSO
% Infarct volume	28.7 ± 3.8	21.5 ± 2.9 *	23.1 ± 2.3 *	35.1 ± 4.5

Values are mean ± S.E.M.
* $P < 0.05$ versus DMSO group.

ties were also evaluated 24 and 48 h after MCAO using the scale (0–4) described by Zea Longa et al. [70]. Rats were held gently by the tail, suspended one meter above the floor, and observed for forelimb flexion. Normal rats that extend both forelimbs toward the floor and that exhibited no other neurological deficit were assigned grade 0. Rats that consistently flexed the left forelimb (contralateral to the injured hemisphere) accompanying flexion of the wrist toward left without any other abnormality were graded 1. Rats that were allowed to move about freely and circled toward the paretic side consistently were graded 2. Rats that fell down to the left losing the ability to walk were graded 3. Rats that exhibited a depressed level of consciousness were graded 4. Forty-eight hours post MCAO, the animals were reanaesthetized with ketamine (44 mg/kg) and xylazine (13 mg/kg). Transcardiac perfusion with heparinized saline was performed on all animals to remove blood from cerebral vessels. Thereafter, the animals were decapitated, and the brains were quickly removed. Each brain was cut into 2-mm thick coronal sections (7 sections per brain) using a rat brain matrix and was then stained for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C. TTC, a tetrazolium salt, reacts with mitochondrial respiratory enzymes and is reduced to a red formazan-insoluble product by electron acceptance. This reaction is lost in damaged mitochondria or oxidative systems, and the lack of staining demarcates ischemic from normal brain tissue [3]. After TTC staining, the tissues were fixed by immersion in 10% buffered formalin solution. Each TTC stained section was photographed with a 35-mm camera mounted on an operating microscope within 2 days of TTC staining. The unstained area as well as the total right and left hemispheric area for each coronal section were traced using the Global Lab Image analysis system (Data Translation, Malboro, MA). The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated [68]. Infarct, and left and right hemisphere volumes (mm^3) were determined by multiplying the respective corresponding areas by the section interval thickness. The lesion volume is presented as a volume percentage of lesion compared to the contralateral hemisphere.

For parametric variables, a one way ANOVA was applied to determine the statistical significance of differences among groups. If a significant difference was detected, then two sample t-tests with Bonferroni correction were performed to evaluate differences between control and PROG treated groups. Paired t-tests were performed on physiological parameters before and after administration of PROG and DMSO within each group. Values presented in this study are mean ± S.E.M. A probability value less than 0.05 was considered significant.

3. Results

The physiological variables before and after initial PROG/DMSO treatments are shown in Table 1. All values were within the normal range for rats and there was no significant difference in physiological variables before and after injection of either PROG or DMSO.

As shown in Table 2, the percent infarct volume was significantly decreased in both DMSO dissolved PROG pre-treated (39%, $t = 2.5616$, $P = 0.018$) and delayed treated (34%, $t = 2.3690$, $P = 0.027$) groups compared with the DMSO treated control group. The difference of infarct in water soluble PROG treated group in relation to

Table 3
Body weight loss (grams) and the neurological deficit (score) daily after MCAO ($n = 12$/group)

Group	PROG in saline	PROG pre-MCAO	PROG post-MCAO	DMSO
Weight loss				
24 h	36.5 ± 3.3	31.1 ± 2.6 *	33.8 ± 1.9	40.4 ± 3.4
48 h	13.3 ± 3.1	8.8 ± 2.2 **	10.0 ± 3.1 *	19.7 ± 2.9
Neurologic score				
24 h	1.7 ± 0.1	1.4 ± 0.1 *	1.4 ± 0.1 *	1.8 ± 0.1
48 h	1.7 ± 0.1	1.4 ± 0.1 *	1.4 ± 0.1 *	1.8 ± 0.1

Values are mean ± S.E.M.
* $P < 0.05$, ** $P < 0.01$ versus DMSO group.

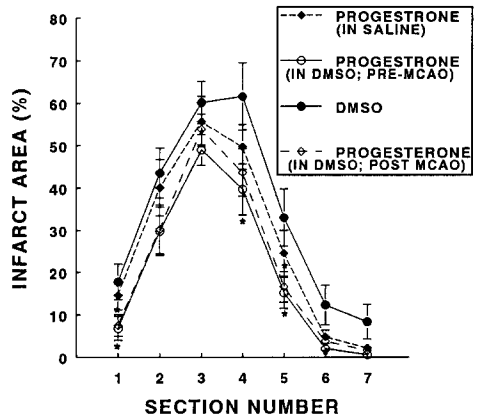

Fig. 1. Line graph shows the percent area of infarction to the area of the contralateral hemisphere in each of seven forebrain sections in PROGs and DMSO treated groups. Values are mean ± S.E.M. * $P < 0.05$ versus DMSO group.

the DMSO treated control group is not statistically significant (18%, $t = 1.0833$, $P = 0.290$). Fig. 1 shows the distribution of percent area of infarct in each of the seven brain sections from all the four groups.

Table 3 presents results of body weight loss and the neurological deficit score 24 and 48 h after onset of ischemia in each group. Both groups of regular PROG treated animals exhibited a significantly improved physiological response as reflected by a reduced body weight loss and improved neurological function (lower score) compared with the DMSO group.

4. Discussion

Our results demonstrate that administration of PROG is neuroprotective in transient focal cerebral ischemia in the rat when treatment is initiated preischemia or 2 h post onset of ischemia. Significant reductions in ischemic cell damage and neurological deficit were observed and physiological function was improved as reflected by a reduction in body weight loss in animals treated with DMSO dissolved PROG, while treatment with water soluble PROG failed to provide statistically significant benefits to the ischemic animal. The finding that PROG offers neuroprotection after an ischemic insult is consistent with reports that PROG treatment facilitates cognitive recovery along with reduction of secondary neuronal loss caused by cortical contusion [60] and that PROG attenuates brain edema after contusion injury [59] and focal cerebral ischemia [4].

Although the mechanisms underlying the neuroprotection against cerebral ischemia by PROG are unknown, influencing brain excitability may be one of them. Potentiating the GABA receptor [19] and/or inhibiting EAA receptors, especially *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor [9,13,17,42,43,63,67], can offer protection against ischemic damage. In vivo studies have established that physiological levels of PROG enhance GABA-mediated inhibition of neuronal activity [65]. PROG rapidly alters the excitability of neurons, in part by potentiating GABA-evoked Cl^- currents and like other GABA potentiating drugs, PROG possesses anticonvulsant activity [27]. PROG's potentiation of the GABA receptor appears to be mediated primarily by its 3α-hydroxy, 5-reduced steroid such as 3α,5α-Tetrahydroprogesterone (3α,5α-THP) which interacts with the γ-aminobutyric acid A ($GABA_A$) receptor. 3α,5α-THP [21,36] prolongs the open time of Cl^- channels during GABA-mediated increases in Cl^- current in cultured hippocampal and spinal cord neurons [16,44]. Similar ionic mechanisms may be responsible for the anesthetic actions of the synthetic progestin alphaxalone [22,62]. Consistent with the concept that PROG acts in a depressant fashion, PROG attenuates EAA responsiveness [64,66]. Locally and systemically applied PROG, at physiological levels, attenuates cerebellar Purkinje cell responses to EAA in a dose-dependent fashion [64]. PROG administration also depresses both kainate and NMDA responses of cerebellar neurons by 55% and 30%, respectively [64]. An in vitro study has shown that PROG protects spinal cord neurons from glutamate neurotoxicity [39]. Furthermore, the suppressant actions of PROG on the EAA system are not secondary to potentiation of $GABA_A$ inhibition [64].

In addition to the potentiating effect on GABA-inhibition and attenuation of EAA responsiveness, there are other possible mechanisms underlying the observed benefit against cerebral ischemia. Endogenous adenosine, a modulator of nerve and glial function, is massively released during ischemia and exerts a protective effect [58]. Exogenous adenosine receptor agonists and the agents that elevate endogenous adenosine level ameliorate ischemic brain injury [37]. PROG, per se, has been shown to amplify adenosine's action of inhibition of cerebral cortical neuronal activity in vivo, an effect which may be due to selective reduction of adenosine uptake by PROG at nM concentration [48]. Reduction of brain edema [4,59,60], a significant causal factor in secondary brain damage and neuronal loss [6], is assumed to be another factor contributing to the observed neuroprotection of PROG. PROG has also been hypothesized to be a free radical scavenger [4,41], and it may act to reduce peroxidative damage.

Circulating PROG is a lipophilic compound and easily passes through the blood-brain barrier (BBB) and enters the CNS at widespread sites [24,45,53]. Once sequestered within the neuronal population, PROG is then metabolized to other more active forms, such as 3α,5α-THP [20,23,24] and the levels of the 3α-hydroxy C21 steroids in CNS parallel cyclic fluctuations of PROG in the circulation [51,52]. The absence of significant protection with treatment with water soluble PROG may be attributed to its poor penetration of the BBB. The solvent used in present study for dissolution of regular PROG is DMSO, rather

than vegetable oil used by other investigators [4,59,60]. Advantages of DMSO over vegetable oil as a solvent for PROG include a higher available saturant concentration of PROG and the ability to be more rapidly absorbed. Though DMSO was reported to be as a free radical scavenger [14] and may be beneficial in cerebral ischemia [18], no beneficial effect was found in our present study at the dose of 0.5 ml/kg.

To assess PROG's clinical potential as a treatment for cerebral ischemia, it is necessary that it be effective in reducing ischemic cell damage when given after the ischemia has occurred. The current study demonstrates that PROG treatments were almost equally effective whether administration was initiated pre MCAO or 2 h post MCAO.

A note of caution in the interpretation of our data is that we evaluated cerebral tissue at 2 days after onset of ischemia. Therefore, we cannot exclude the possibility that intervention with PROG delays the maturation of the lesion and provides only temporary (2 day) improvement in the outcome after stroke, similar to the transient beneficial effect of NMDA antagonist MK-801 on focal cerebral ischemia [15,46]. In addition, although in the normal rat, serum PROG levels increase after administration of PROG measurements of PROG in brain tissue after MCA occlusion are needed. We are therefore developing methods to measure brain PROG levels.

In conclusion, we have demonstrated that administration of PROG to the male rat before or after transient MCAO reduces ischemic cell damage and improves physiological and neurological function 2 days after stroke. Since PROG is a widely and clinically used compound, further investigation into its therapeutic benefit for the treatment of stroke is warranted.

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