Assessment of the role of progesterone in protecting against spinal cord injury via hypoxia-inducible factor-1alpha: An animal model study

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Abstract

Background: A spinal cord ischemia and neural hypoxia models were used to evaluate the neuroprotective effects of progesterone via hypoxia-inducible factor-1α. We aimed to investigate the neuroprotective role of progesterone in spinal cord injury.

Methods: Eighteenfemale New Zealand white rabbits were randomized into three groups. Group 1(control) animals received nothing following reperfusion. Group 2 and 3 (treatment) animals received intraperitoneal progesterone immediately after the onset of reperfusion at a dose of 8 mg/kg. Spinal cords were fixed with 10% formalin and embedded in paraffin wax. The number of intact large motor neuron cells in the ventral grey matter region was counted. Cells expressing HIF1 were counted in high-power microscopic fields (x 400) in spinal cord sections around the areas of developing white matter necrosis.

Results: The number of intact neurons in group 1 was significantly lower than that in both groups 2 and 3 ($p<0.05$).HIF-1α positivity was significantly higher in ischemic animals. HIF-1 α positivity in the non-ischemic arm of group 1 was significantly lower than that in the non-ischemic arm of groups2 and 3.

Conclusions: These data suggest that HIF-1 α plays an important role in hypoxic-ischemic preconditioning. Neuroprotective effects of progesterone may be mediated via the HIF-1α.

Key Words: HIF-1α, spinal cord injury, ischemia, progesterone.

Introduction

Acute spinal cord injury (SCI) is characterized by a progressive course which cannot be entirely explained by its primary mechanical trauma. A series of secondary injuries including ischemia, vascular changes, electrolyte disorders, edema and loss of energy metabolism, which can significantly increase the severity of SCI, have been observed in injured and adjacent segments after the acute post-injury phase [1]. Among all, secondary injuries, ischemia has been demonstrated as the main cause of post-injury pathophysiological changes of acute SCI since it has been believed to aggravate other secondary injuries and arises in parallel with neurological dysfunction.

Currently available treatment options for SCI are limited [2], and the standart drug therapy is aspirin [3]. Diverse therapeutic approaches (including prostaglandins, nimodipine, naloxone, adenosine, magnesium among others) have been used to control the damaging processes that can injure the spinal cord, eventually leading to its repair [4]. Among the limited number of treatments for SCI, currently available, are surgical decompression and the use of methylprednisolone, but these are considered to be ineffective [5] . Vascular endothelial growth factor (VEGF) is a unique neurotrophic factor. As a potential stimulator of angiogenesis, VEGF can improve locomotor function under hypoxic conditions following SCI [6].

It is now well documented that steroid hormones provide neuroprotection after injury of the

central nervous system [7]. The list ofneuroprotective steroids has increased in recent years, and includes progestagens [8], androgens [9], and estrogens [10], which have been shown to decrease the extent of brain injury and to promote neuronal survival. The neuroprotective effects of progesterone after spinal trauma have also been demonstrated in some studies [11, 12].

Many studies have also investigated the mechanisms underlying hypoxic-ischemic brain damage, such as free radical formation [13], excitotoxicity [14], and inflammation [15,16]. Progesterone also reduces edema, necrosis, apopitosis, blood-brain barrier compromise, and the mediators of inflammation [17].

Hypoxia leads to an almost immediate shut down of general protein translation to decrease energy consumption during hypoxic energy starvation [18]. The protective effects of hypoxia may be regulated by improving tissue oxygenation via HIF-1α and the up-regulation of its target genes. HIF-1α is one of the best characterized stimuli for the induction of angiogenic response and of the expression of several genes in a variety of tissues including vascular endothelial growth factor (VEGF) [19,20]. In addition to VEGF, HIF-1α activates genes encoding erythropoietin (EPO), glucose transporters and glycolytic enzymes, cell survival factors, cell surface receptors, extracellular matrix proteins and transcription factors [19,20]. Hypoxia-inducible factor-1α (HIF-1α), which was first identified in 1988 in human hepatoma cells as a key factor mediating the transcription of target genes [21], has been intensively investigated for its role in the modulation of hypoxic-ischemic brain injury since 1995 [22,23]. In our present study, we aimed to investigate whether progesterone demonstrates a neuroprotective effectvia HIF-1α in rabbits.

Materials and Methods

Animals

A total of 18 female New Zealand white rabbits (8-12 months old), each weighing between 2.4 and 3.5 kg, were used in this study. All animals were housed under standard conditions in the Animal Research Laboratory at DokuzEylul University. The study protocol was approved by the animal research committee. The animals were fasted for 12 hours and humanely restrained. Anesthesia was induced with 3% halothane in 100% oxygen and was maintained with 0.5% to 1.5% halothane in a mixture of 50% oxygen and 50% room air. End-tidal concentrations of halothane and CO_2 werecontinuously measured with monitor (Anesthetic Gas Monitor Type 1304; Brüel&Kjaer, Naerum, Denmark) via nasopharyngeal sampling.

The retroauricular vein in the right ear was cannulated, and an infusion of 0.9% NaCl solution was started at a rate of 4 mL/kg per hour. An artery in the left ear was also cannulated to monitor arterial blood pressure and allow for arterial blood gas sampling. To monitor proximal and distal aortic pressures, catheters were placed into the aorta and the femoral arteries. Verification that the appropriate level of sedation had been reached was determined by the lack of a righting reflex and by testing the palpebral and pedal withdrawal reflexes every 10 minutes, as previously described by Wyatt et al. [24]. Allexperiments were performed under the same conditions. Rectal temperatures were maintained at 38.5°C by keeping the animals under a heat lamp until their recovery from anesthesia.

Surgical procedures

The sedated animals continued to breathe spontaneously and were placed in the right lateral decubitus position. The skin was prepared with povidone iodine and anesthetized with bupivacaine (25% solution), and an incision was made in the flank, parallel to the spine at the 12th intercostal level. Following incision and dissection throught the thoracolumbar fascia, the longissimuslumborum, and iliocostalislumborum muscles were retracted. The abdominal aorta was exposed via a left retroperitoneal approach and mobilized just inferior to the left renal artery, where it was clamped, down to the point of the aortic bifurcation. Each rabbit was anticoagulated with 400 U of heparin before aortic occlusion. After 30 minutes of occlusion, the catheters were removed, and the incision was closed. The animals were monitored until they fully recovered and were then returned to their cages.

Experimental design

The animals were randomly divided into three groups, each consisting of six rabbits. Group 1(control) animals received nothing following reperfusi-

on. Group 2and 3 (treatment) received intraperitoneal progesterone (Progynex 50mg/m) immediately after the onset of reperfusion at a dose of 8 mg/kg. This specific dose of progesterone was chosen because it has been shown in multiple studies to prevent neuronal loss after brain injury and ischemia [25,26]. After completion of the surgical procedures, the tube and catheters were removed, and the incision was closed. Two hours after reperfusion, the animals in group 1 were killed. Four and six hours after reperfusion, the animals in groups 2 and 3, respectively, were killed with intraperitoneal sodium thiopental (120 mg/kg). The spinal cords from all animals were removed and fixed in 10% formalin in a phosphate buffer.

Determination of the physiologic parameters and progesterone levels

During the surgical procedure, the heart rate, mean arterial pressure, and rectal temperature were continuously monitored (Biopac MP30 and Biopac BSL pro v.3.6.5; Biopac Systems, Santa Barbara, CA), in addition to the respiration rate and end-tidal CO_2 level. Following surgery, the rabbits were placed in a warming chamber, and their body temperatures were maintained at approximately 37°C until they were completely awake. Blood samples (2 mL) were taken from the peripheral veins of all the animals before surgery and before sacrifice to measure the serum progesterone levels. Once the postsurgical progesterone levels were determined, the animals were killed. Blood samples remained at room temperature for one hour, until they clotted. Samples were then centrifuged for 10 minutes at 4000 rpm to obtain serum specimens. Serum specimens were stored at 4°C and analyzed within 24 hours to determine the progesterone level, which was measured according to thecolorimetric method of Bar-Or et al [27].

Histopathology

Spinal cords were removed and fixed in 10% formalin in a phosphate buffer. After fixation, transverse sections of the spinal cord at the L5 level were embedded in paraffin, cut into 5-µm-thick sections, and stained with hematoxylin and eosin. Neuronal injury was evaluated at x40, x100, x200, and x400 magnifications by a pathologist who was blinded to the treatment groups. Five sections per animal were read. We performed hematoxylin and eosin staining on a set of sections and examined them using light microscopy. The number of intact large motor neuron cells in the ventral gray matter region was counted. The observers, who were blindto the experimental groupings and neurologic outcomes, examined each slide. Following hematoxylin and eosin staining, the cells were considered to be dead if the cytoplasm was diffusely eosinophilic and were considered viable if the cells demonstrated basophilic stippling.

Immunohistochemistry

All specimens were fixed with 10% formalin and embedded in paraffin wax. Paraffin blocks were cut into 4µm sections and stained with hematoxylin and eosin. Immunohistochemical studies were performed on formalin fixed, paraffin-embedded tissue. The slides were stained on a DAKO Autostainer (DAKO Denmark) using the LSAB + System- HRP (Dako) staining reagents. The sections were then incubated with a monoclonal antibody specific for anti HIF-1 alpha (Bioss-USA) at a 1: 100 dilution of the original antibody solution for 60 minutes. Diaminobenzidine (DAB) was used as a chromogen for reaction visualization. Finally the sections were counterstained with Mayer's hematoxylin, dehydrated,cleared with xylene and mounted with coverslips using permanent mounting medium. Nuclear staining was used as a positive counterstain. Non papillary renal cell carcinoma was used as a positive control. Immunostained cell counts were made by two pathologistwho were blind to the subject data. Cells expressing HIF1 were counted in high-power microscopic fields (x 400) in spinal cord sections around areas of developing white matter necrosis.

Statistical analysis

For statistical evaluations, we used the software package SPSS for Windows v.15.0 (SPSS, Inc, Chicago, IL). Data from all groups are expressed as the mean±SD. A probability value of less than 0.05 was accepted as statistically significant. Because the variances were not homogenous (Levene's test statistic $p<.05$), post hoc Dunnett's T3 analysis was performed to determine from which group any significant differences in the findings had arisen.We used non-parametric tests, because

of the small size of groups. The Kruskal–Wallis one-way analysis of variance and the Mann-Whitney U test were used to evaluate values.

Results

The mean value sat baseline (before the surgical procedure), before clamping and before sacrifice (sac) as well as the mean number of non-ischemic neurons and mean progesterone levels for the three groups are listed in Table 1. Statistically significant differences were identified between the baseline mean arterial pressure (MAP) and sac-MAP values of the groups ($p<0.05$). This analysis revealed that the average baseline MAP value for group 1 was significantly higher than that for group 2, and the mean sac MAP value for group 1 was significantly higher than that for group 3 ($p<0.05$). The number of intact neurons in group 1 was significantly lower than the number of intact neurons found in both groups 2 and 3 ($p<0.05$). No other statistically significant differences were found between thegroups, in terms of their mean PROG heart rate (HR) and PROG saturation (SAT) values ($p>0.05$) [Table 2]. No statistically significant correlation was found between the number of live neurons in the ventral grey area and progesterone levels (either pre- or post-surgery) in any of the groups ($p>0.05$) [Table3].

Table 1. Distribution of mean base, clamp, SAC, intact neurons, base progesterone, and after progesterone values of cases among groups

	Group 1 Mean±SD	Group 2 Mean±SD	Group 3 Mean±SD	Total Mean±SD	*P* value
Base MAP	98.5±7.74	79.83±8.82	88.5±9.77	88.94±11.41	.008
Clamp MAP	92.67±14.67	82.17±5.12	80.83±6.43	85.22±10.62	.101
SAC MAP	82.5±17.18	74.33±18.79	55.67±14.08	70.83±19.56	.040
Base HR	288.17±7.6	265.67±22.92	262.83±20.96	272.22±20.9	.061
Clamp HR	239.67±15.62	248.67±36.52	264±17.3	250.78±25.67	.265
SAC HR	238±14.14	240.5±29.04	275.67±34.09	251.39±31.01	.052
Base SAT	99.17±1.6	99.83±0.41	99.67±0.52	99.56±0.98	.774
Clamp SAT	99±1.67	99.17±1.17	99.5±0.55	99.22±1.17	.438
SAC SAT	99.17±0.75	99.17±1.17	99.33±0.82	99.22±0.88	**.001**
Intactneuron	23.17±4.49	37.17±6.91	34.5±4.59	31.61±8.07	.217
Base PROG	0.72±0.59	8.23±14.58	0.24±0.27	3.06±8.77	.217
After PROG	0.74±0.59	98.17±142.95	117.51±179.58	72.14±135.13	.292

Values are the mean ±SD; n=6 in each group. Sat, saturation; PROG, progesterone levels; clamp, clamping time.

Table 2. Distribution of mean progesterone values of the cases in groups 2 and 3

	Group 2 Mean ±SD	Group 3 Mean ±SD	Total Mean ±SD	*P* value
PROG MAP	54.83±11.86	74.17±14.7	64.5±16.25	.031
PROG HR	242.33±35.38	269±22.15	255.67±31.4	.149
PROG SAT	99.5±0.84	99±1.26	99.25±1.06	.938

Values are the mean ±SD; n=6 in each group. PROG, progesterone treatment time.

Table 3. Correlation between number of intact neurons and base progesterone and after progesterone values of the cases in groups 1, 2 and 3

	Group 1		Group 2		Group 3	
	R	P	R	P	r	P
Base PROG	0.294	0.572	-0.062	0.907	-0.556	.252
After PROG	0.309	0.552	-0.348	0.499	0.472	.344

Base PROG, progesterone levels in the blood before progesterone treatment. After PROG, progesterone levels in the blood after progesterone treatment.

Table 4. Comparative analysis of HIF-1α positivity among group

		Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)	*P* value[b]
HIF-1 Positivity	Nonischemic Group (n=6)	0	0	0	.02
		0	1.5	0.5	
		0	1	0	
		0	1.2	0	
		0	1	0.5	
		1	1	0	
	Ischemic Group (n=6)	3	3	1.2	.08
		4	2	2	
		3	1	1	
		3	1.5	1.3	
		1.5	2.2	1.3	
		1	1.5	1.3	
	P value[a]	**.003**	**.027**	**.003**	

[a]:*The difference between ischemic and nonischemic subjects in each group.*
[b]:*The difference between the groups according to the state of ischemia.*

It was limited to evaluate the number of intact large motor neuron cells by light microscopy. Thus, we explored in this study the effectiveness of progesterone as a treatment for SCI. We investigated the neuroprotective effects of progesterone by evaluating HIF-1α positivity. In the experments, HIF-1α positivity was significantly higher in ischemic groups than without ischemic groups. In addition, HIF-1α positivity in the non-ischemic arm of group 1 was significantly lower than in the non-ischemic arm of group 2 and 3. There was no significant difference in the HIF-1-α positivity among the ischemic arms of the patient groups (Table 4).

Discussion

Traumatic SCI causes devastating neurological dysfunction primarily via necrotic damage and following secondary injury events including ischemia, excitotoxicity, altered ionic balance, free radical formation, and inflammatory responses [28].To date, much effort has been expended to elucidate the molecular mechanisms within neurons that mediate neuronal death during ischemia and hypoxia. Studies that will further elucidate the pathophysiological background of ischemia are thus needed to identify novel therapeutical strategies.

A number of potential alternative therapies for SCI have been proposed and tested but they have failed to yield effective improvements.Some studies have reported positive results with certain drugs (including prostaglandins, nimodipine, naloxone, adenosine, and magnesium) [11]. Furthermore, there have been some studies of the efficacy of antihrombotic treatment for SCI [29,30]. Corticosteroid is also one of the alternative therapeutic approaches [31]. In addition, some studies have also reported potential benefits of progesterones [11, 32], androgens [9], and estrogens [10].

Once a destructive process is initiated such as SCI, the release of pro-inflammatory cytokines further stimulates immune cells to become phagocytic. Progesterone decreases the mediators of inflammation [33,34]. In some studies, it was reported that natural progesterone given to both males and females may easily cross the blood-brain barrier and dramatically reduce edema to barely measurable levels in an injured animal brain [35,36]. Progesterone may also reduce lipid peroxidation and the generation of isoprostanes, which in turn contribute to postinjury hypoxic-ischemic conditions [37]. In this context, it will be important to investigate the relationship between hypoxic exposure and therapeutic agents such as progesterone in future studies.

HIF-1α may act as a critical regulatory factor for those of its target genes associated with the modulation of glycolysis and re-establishment of microcirculation in SCI. HIF-1α isalso involved in ischemia [38]. Kalesnykas et al. have reported that HIF-1α increased in rat neurons after unilat-

eral occlusion of a common carotid artery [39].The authors of that study suggest that decreased blood flow and ischemia resulted in cellular hypoxia during the common carotid artery occlusion, leading to stabilization HIF-1α. Other studies have shown that HIF-1α protein levels increase immediately after the hypoxic exposure, peak at 3-4 hours after hypoxic-ischemic injury, and persist at elevated levels for up to 24 hours after the insult [22].As mentioned earlier, hypoxic ischemia is an important cause of spinal injury. However, a sublethalhypoxic/ischemic exposure can improve the tolerance of tissue or of cells to a subsequent lethal hypoxic/ischemic insult. This phenomenon is called hypoxic/ischemic preconditioning (H/IPC) [40]. Some studies support the hypothesis that HIF-1α plays an important role in H/IPC, and that the protective effects of H/IPC may be partially mediated by improving tissue oxygenation via HIF-1α and upregulation of its target genes. In our present study, we aimed to prove that progesterone has neuroprotective effects via HIF-1α.

Our current results are consistent with reported findings in the literature. HIF-1α positivity has been reported previously to be significantly higher in ischemic groups than non- ischemic groups [22, 38]. Thus,HIF-1α possibly participates in the ischemic and hypoxic pathways that operate after SCI, and may mediate the traumatic process involved.In our current study, HIF-1 α positivity in the non-ischemic arm of group 1 was found to be significantly lower than in the non-ischemic arm of groups 2 and 3.We thus concluded that progesterone increases HIF-1α and induces neuronal improvement. This result is consistent with other studyfindings in terms of neuroprotective effects [11]. Ultimately,our present data supportthe neuroprotective effects of progesteroneagainst SCI.

Limitations

The rapid acceptance of immunohistology as an invaluable adjunct to morphologic diagnosis has been possible because of the development of new and more sensitive antibodies and detection systems that allow its application to formalin-fixed, paraffin-embedded tissue (FFPT. While it was not a major issue when the technique was employed in a qualitative manner, the numerous variables in the preanalytical and analytical phases of the test procedure that influence the immunoexpression of proteins in FFPT become critical to standardization. Tissue fixation is pivotal to antigen preservation but exposure to fixative prior to accessioning by the laboratory is not controlled. There is great variation in reagents, methodology, and duration of tissue processing and immunostaining procedure, and the detection systems employed are not standardized between laboratories. While many of these variables are offset by the application of antigen retrieval, which enables the detection of a wide range of antigens in FFPT, the method itself is not standardized. Failure to recognize false-positive and false-negative stains leads to further errors of quantitative measurement.

Conclusion

Our present data suggest that progesterone administration facilitates neuronal protection through a hypoxic inducible system in SCI. The involvement of HIF-1α after spinal injury brings new insights into the role of progesterone in neuroprotection.

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