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PAR MEDIATION OF THROMBIN-INDUCED EFFECTS ON MOTONEURONS

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ABSTRACT

Past studies have suggested that activation of the proteolytically activated receptor (PAR-1) by thrombin or the peptide SFLLRNP results in decreased motoneuron survival. Although PAR-1 appears to be the primary receptor for thrombin activation, recent studies suggest that thrombin may activate other PARs in the nervous system. The fact that thrombin has the ability to activate other receptors in a similar manner raises the question as to whether thrombin's effects are mediated via PAR-1 alone or in combination with additional PARs. Our results suggest that while activation of PARs 2, 3 and 4 do not affect cell survival, PARs 3 and 4 may alter morphology, thus strengthening the previous finding that thrombin-induced programmed cell death in the motoneuron column is mediated via PAR-1, but they also suggest alternative roles for PARs 3 and 4.

INTRODUCTION

Throughout embryonic development, various neuronal cell populations, including motoneurons, undergo a period of naturally occurring programmed cell death (PCD). During development of the chick embryo, 40-70% of postmitotic lumbar motoneurons undergo PCD between embryonic days (E)6 and E10 (Hamburger, 1975; Oppenheim, 1991; Clarke and Oppenheim, 1995), which corresponds to gestation weeks 11 to 18 in humans (Hamburger and Oppenheim, 1982; Forger and Breedlove, 1987). Programmed cell death is thought to remove aberrant neurons, correct synaptic errors, and regulate cell numbers (see Houenou and Oppenheim, 1994). The motoneurons that die during PCD appear to die by apoptosis (Kerr et al., 1972), which is characterized by cleavage at nucleosome linker regions, producing 180-200 base pair fragments (see Lo et al., 1995).

Furthermore, there are several motoneuron diseases marked by apoptotic-like cell death in the brain or spinal cord (Swash and Schwartz 1992). Such diseases include amyotrophic lateral sclerosis, X-linked spinal and bulbar muscular atrophies, and infantile and juvenile spinal muscular atrophies. Each of these diseases is characterized by muscle weakness, atrophy, and motoneuron loss. Furthermore, both the human protease-activated receptor-1 (PAR-1) and PAR-2 genes are tightly linked on chromosome 5q13, near the spinal muscular atrophy locus (Schmidt et al., 1997), suggesting that these receptors and their serine protease ligands may be involved in neurodegeneration.

Recent studies have localized serine proteases, such as thrombin, and their receptors to different cellular populations within the nervous system (see Festoff et al., 1996; Turgeon and Houenou, 1997; Turgeon et al., 2000). While thrombin is well known for its important role in

coagulation, it also appears to play an important role in the nervous system. Exogenous thrombin has been shown to alter cell morphology and differentiation in astrocyte, neuroblastoma, and motoneuron cultures (Gurwitz and Cunningham, 1988, 1990; Zurn et al., 1988; Grabham et al., 1989; Suidan et al., 1992; Turgeon et al., 1998). Thrombin appears to exert its effects through activation of PAR-1. Activation of this receptor requires specific cleavage of the extracellular domain to generate a new amino terminus capable of undergoing a conformational change allowing it to bind to and activate the receptor. The newly generated amino terminus is SFLLRNP (serine-phenylalanine-leucine-leucine-arginine-asparagine-proline; Vu et al., 1991). Using this knowledge, we have previously shown that *in ovo* treatment with this peptide decreases motoneuron cell survival during the naturally occurring PCD (Turgeon et al., 1999).

During the initial investigation of thrombin's role in programmed cell death, PAR-1 was thought to be the only receptor activated by thrombin. Recent studies indicate the presence of other proteolytically activated receptors (PARs), which may also be activated by thrombin (Cupit et al., 1999). These newly found receptors seem to have many similarities to PAR-1 (see Turgeon et al., 2000), particularly PARs 3 and 4. Furthermore, in addition to PAR-1, PAR-3 and PAR-4 appear to be important in thrombin-induced platelet activation (Kahn et al., 1998). All members of the PAR family are seven transmembrane G protein-coupled receptors; each with its own unique tethered ligand responsible for its receptor's activation (Smith-Swintowsky et al., 1997; Kahn et al., 1998). Using these unique sequences, we can examine the individual activation of each PAR. While PAR-2 is not specifically categorized as a thrombin receptor, it can be activated by the PAR-1 tethered ligand, SFLLRNP. Thus, in addition to examining the effects of PAR-3 and 4 we also examined PAR-2 activation in our models. The purpose of this study was to investigate whether or not PARs besides PAR-1 play a role in thrombin-induced motoneuron cell death.

METHODS AND MATERIALS

***In Ovo* Treatment of Chick Embryos.** Eggs were obtained from the Charles E. Morgan Poultry Center at Clemson University (Clemson, SC) and were incubated at 37°C and 90% humidity. On E4, a small circle was carefully cut from the top of the egg. Those eggs that contained viable embryos with blood vessels in the chorioallantoic membrane and visible heartbeats were then resealed with clear tape. Embryonic age was determined by the staging criteria established by Hamburger and Hamilton (1951).

Beginning on E5, embryos were treated once daily with 200 µl of various stock concentrations of SFLLRNP (PAR-1 peptide; Bachem, King of Prussia, PA), SLIGRL (PAR-2 peptide; Bachem), SLIGVK (PAR-3 peptide; Bachem), or GYPGKF (PAR-4 peptide; Bachem) to achieve final concentrations of 10 µM, 20 µM, and 40 µM. The control embryos received 200 µl of 1X phosphate-buffered saline (PBS) or 200 µl of inactive or stock antagonist peptides FSLLRNP (inactive PAR-1 peptide, Bachem), LSIGRL (inactive PAR-3 peptide, Bachem), LSIGVK (inactive PAR-3 peptide, Bachem), GAPGK (inactive PAR-4 peptide, Bachem) to achieve a final concentration of 40 µM for each treatment.

Histological preparation and analysis. Embryos were sacrificed on E9, eviscerated, and trimmed to expose the intact spinal cords. The tissues were preserved overnight in Carnoy's fixative (150 ml glacial acetic acid, 900 ml ethanol, 450 ml chloroform) and then transferred to 70% ethanol. These tissues were then embedded in paraffin and serially sectioned at 10 µm

using a rotary microtome. Finally, the sections were stained with 1% thionin and examined. Using criteria described by Clarke and Oppenheim (1995), lumbar motoneurons were counted. Motoneuron counts were performed blind on every tenth section in the lateral motor column throughout the entire lumbar spinal cord. Cells with a large (> 10 μm) nucleus, containing at least one distinct nucleolus, and a large distinctly stained cytoplasm were counted. Using these rather stringent criteria for cell counting, it has been shown that less than 1% of the cells appear on two successive sections and, therefore, only an insignificant number of cells would be counted twice (Oppenheim et al., 1989).

To measure the nuclear area, a microscope equipped with a camera (Nikon Coolpix990; 3.34 megapixels; 3X 200 zoom) captured images at 400X total magnification. These pictures were saved to a PowerMac computer and analyzed with NIH image 1.61.

Motoneuron Cultures. Motoneurons were isolated from E5 chick embryos and cultured using modified methods from Dohrmann et al., (1986) and Arakawa et al., (1990). Briefly, the ventral portions of the lumbar spinal cords of chick embryos were removed using tungsten needles and kept in ice-chilled sterile-filtered PBS until dissections were complete. The ventral lumbar spinal cords, containing primarily motoneurons, were dissociated in 30 mM subermidate (Sigma; St. Louis, MO) for 15 minutes. The partially dissociated cells were then added to Lebowitz-15 (L-15; Gibco, Grand Island, NY), defined serum-free medium and further dissociated by running the mixture through a 1 ml pipette, followed by centrifugation (400 x g for 15 min; Beckman Centrifuge) over a layer of 6.8% metrizamide (Sigma). Motoneurons remained in the top half of the metrizamide fractions, forming a visible white band, which was collected and added to 5 ml of L-15 medium. A 4% bovine serum albumin cushion was then gently added beneath the cells and centrifuged at 200 x g for 10 min (Beckman). The supernatant was discarded and the pellet resuspended in 1 ml of L-15 and filtered through a 50-mm nylon filter. A portion of this preparation was loaded onto a hemocytometer for an initial cell count. From this initial count, the cells were diluted appropriately in L-15 medium, supplemented with glutamine, glucose, sodium bicarbonate, penicillin-streptomycin, conalbumin, putrescine, insulin, progesterone, and sodium selenite (Sigma), and plated at a density of 2000 motoneurons/well in 35 mm^3 petri dishes, each with four wells that were 10 mm^3 in diameter (Greiner dishes; USA Scientific, Atlanta, GA). The dishes were incubated with laminin (Sigma) and poly-D-ornithine (Sigma). Cells were incubated in a CO^2 water-jacketed incubator at 37°C. Motoneurons were identified by immunostaining the cultures with SC-1 (Microbial Strain Data Network; ECACC) a specific marker for early developing motoneurons (Henderson et al., 1994). After plating, the motoneurons were allowed to attach to the plate for two hours prior to co-treatments with 14 μg/ml chicken muscle extract (CMX) and 1-1000nM of SFLLRNP, FSLLRNP, SLIGRL, LSIGRL, SLIGVK, LSIGVK, GYPGKF, or GAPGKF. Control cultures were grown in either L-15 medium (negative control) or CMX supplemented L-15 medium (positive control; Milligan et al., 1994).

Cell numbers were obtained by counting the viable cells across two diameters of each culture well at 200X total magnification on a phase-contrast miscroscope (Unitron TCM-BR; Martin Microscopes, Easley, SC). To examine motoneuron survival, the criteria used included the presence of two or more neurites per neuron, with the length of at least one of those neurites being greater than twice the diameter of the cell soma, and the absence of cytoplasmic vacuoles and/or degenerating neurites (Milligan et al., 1994).

cAMP Assay. To determine cAMP concentrations following PAR activating peptide treatments, a cAMP immunoassay kit (Sigma) was used. Motoneurons were cultured as stated above. Twenty-four hours following culture and appropriate treatment, the media were removed and cells were treated with 0.1 M HCl for 10 min. Following the HCl incubation, the cells were visually examined to verify lysis. The cells were centrifuged at 1000 x g (Beckman) at room temperature. The supernatant was collected and used in the remainder of the assay. The cAMP standards (20 pmol/ml, 5 pmol/ml, 1.25 pmol/ml, 0.312 pmol/ml and 0.078 pmol/ml cAMP) and the samples were incubated at room temperature with primary and secondary antibodies. The excess reagents were removed and the enzyme substrate was added. Following incubation, the standards and samples were read on a microplate reader (MRX Microplate Reader, Dynatech Laboratories, Chantilly, VA) at 450 nm. The intensity of the yellow color was inversely proportional to the concentration of cAMP. The measure of optical density was then used to calculate the cAMP concentration.

Statistical analysis. All data were analyzed using one-way ANOVA followed by the Tukey-Kramer multiple comparison post hoc test, if $p \leq 0.05$.

RESULTS

As shown in Figure 1, administration of the PAR-2, -3, or -4 synthetic peptides (10, 20, and 40 μM) during the period of PCD had no significant effect on the number of surviving lumbar spinal motoneurons in comparison to PBS treated controls ($p>0.05$), whereas treatments of 10-40 μM SFLLRNP, the PAR-1 peptide, decreased the number of surviving neurons ($p<0.001$) as previously found by Turgeon et al. (1998).

Furthermore, treatments with the requisite inactive peptides did not alter motoneuron numbers (data not shown).

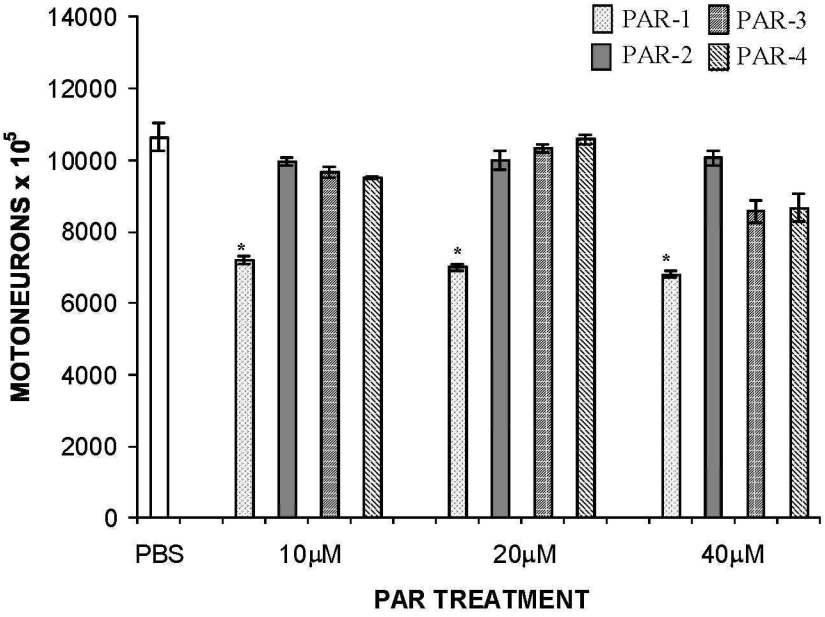

Figure 1. Motoneuron numbers (means ± SEM) in the lumbar lateral motor column of E9 embryos following once daily treatments with 200 μl 1X PBS (control), 10-40 μM PAR-1, PAR-2, PAR-3 or PAR-4 activating peptide from E5-E8. Only treatments with the PAR-1 activating peptide decreased motoneuron cell survival in comparison to the control ($p < 0.001$; $n = 8$ for each group). Embryos treated with 200 μl of 10-40 μM of the inactive peptides for PAR-1, -2, -3, and -4 resulted in no significant difference in the mean number of motoneurons in comparison to the control ($p > 0.05$; data not shown).

Concentrations greater than 40 μM of SLIGRL and 100 μM of SFLLRNP, SLIGKV or GYPGKF resulted in degeneration of the chorioallantoic vasculature and subsequent death of the embryos within two days of treatment (data not shown).

Motoneuron cultures treated with SLIGRL, SLIGKV, or GYPGKF (1-1000 nM) did not show any signs of decreased survival in comparison to control cultures ($p>0.05$; Figure 2); whereas, identical concentrations of SFLLRNP decreased motoneuron cell survival ($p<0.05$) (Figure 2).

To further examine the potential effects of PAR ligand administration on motoneuron viability, we measured cAMP concentrations *in vitro* and the nuclear area of surviving motoneurons *in vivo*. As shown in Table 1, treatment with various concentrations of SLIGRL, SLIGKV, or GYPGKF *in vitro* did not alter cAMP production, whereas SFLLRNP treatments decreased cAMP production ($p<0.05$).

Furthermore, the addition of exogenous SLIGRL concentrations (10-40 μM) did not alter the mean nuclear area in comparison to the PBS treated controls ($p>0.05$; Figure 3); whereas identical concentrations of exogenous SFLLRNP or SLIGKV decreased mean nuclear area ($p<0.05$) and GYPGKF increased nuclear area ($p<0.05$).

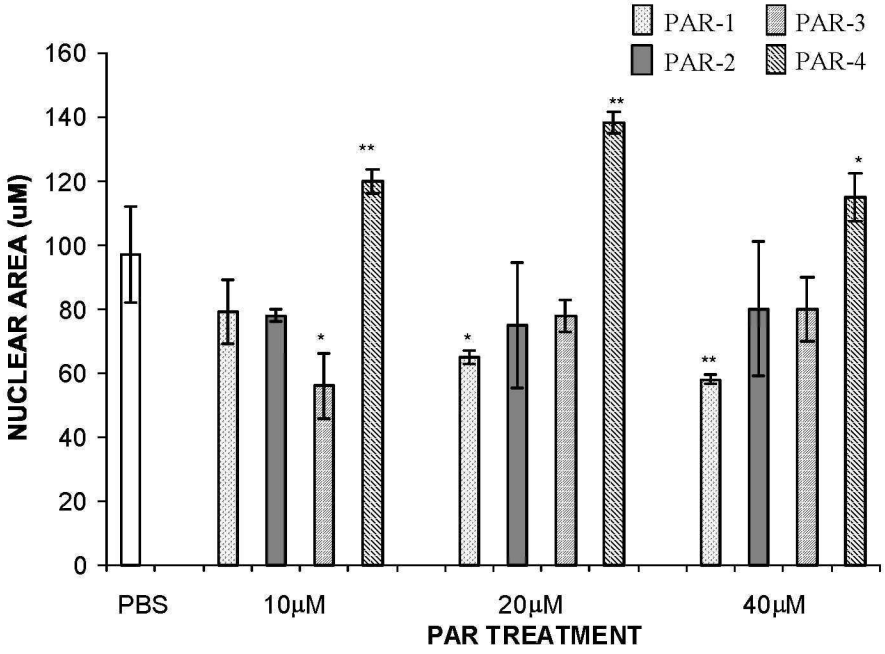

Figure 2. Motoneuron survival (mean percentages ± SEM) in 48-h cultures maintained in 1.5 ml of Lebowitz-15 (L-15) medium containing 1-1000nM of the PAR-1, PAR-2, PAR-3 or PAR-4 activating peptide. Control cultures were grown L-15 medium and assessed at the 48-h time point. Only treatments with 10, 100, and 1000 nM of the PAR-1 activating peptide resulted in decreased motoneuron cell survival (*$p< 0.05$; ** $p< 0.01$) in comparison to the control. Cultures maintained in 1.5 ml of L-15 containing 1000nM of the inactive peptides for PAR-1, -2, -3, and -4 resulted in no significant difference in the mean number of motoneurons in comparison to the control ($p >0.05$; data not shown). n = 3 trials performed in triplicate.

Table 1. cAMP concentrations following treatment with various PAR activating peptides

Treatment	cAMP (pmol/ml)
Negative control*	0.030 ± 0.001
Positive control†	0.14 ± 0.002
SFLLRNP‡	
10nM	0.020# ± 0.001
1000nM	0.021# ± 0.001
SLIGRL§	
10nM	0.030 ± 0.003
1000nM	0.029 ± 0.001
SLIGVK‖	
10nM	0.030 ± 0.002
1000nM	0.029 ± 0.002
GYPGKF¶	
10nM	0.030 ± 0.001
1000nM	0.030 ± 0.002

*Negative controls were grown in Leobowitz-15 medium.

†Positive controls were grown in Leobowitz-15 medium supplemented with 14μg/ml of chicken muscle extract.

‡SFLLRNP – serine-phenylalanine-leucine-leucine-arginine-asparagine-proline

§SLIGRL – serine-leucine-isoleucine-glycine-arginine-leucine

‖SLIGVK –serine-leucine-isoleucine-glycine-valine-lysine

¶GYPGKF –glycine-tyrosine-proline-glycine-lysine-phenylalanine

#$p<0.05$ in comparison to the negative control.

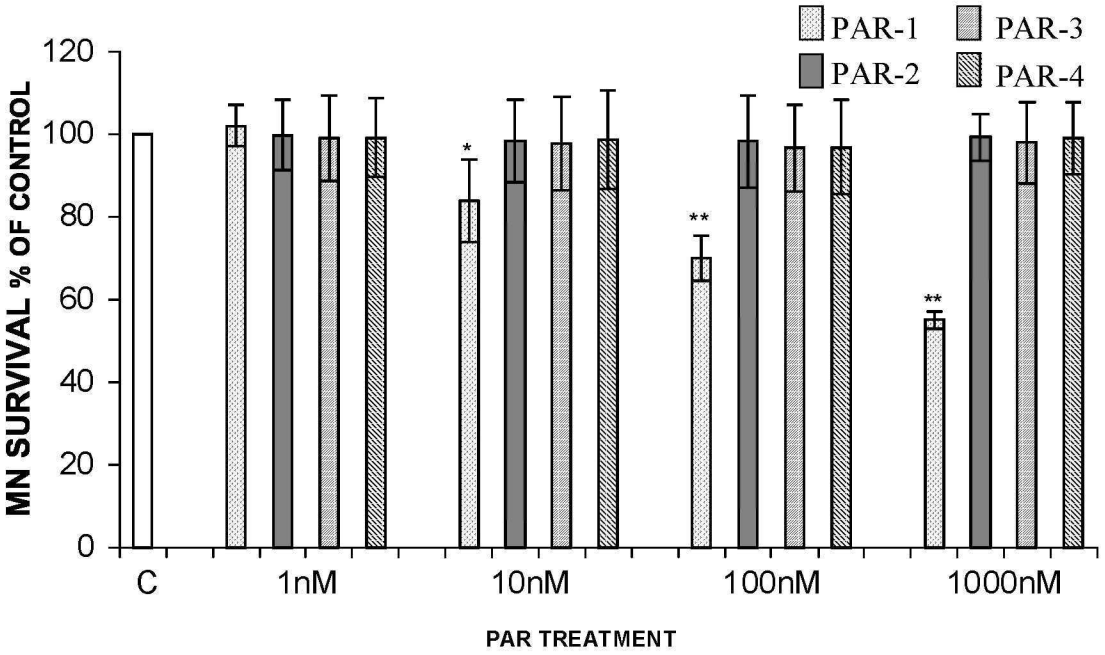

Figure 3. Nuclear area (means ± SEM) of surviving motoneurons in the lumbar lateral motor column of E9 embryos following once daily treatments with 200 μl 1X PBS (control), 10-40 μM PAR-1, PAR-2, PAR-3 or PAR-4 activating peptide from E5-E8. Treatment with 10 μM of the PAR-3 activating peptide decreased nuclear area in comparison to the control ($p<0.05$) as did treatments with 20 and 40 μM of the PAR-1 activating peptide ($p<0.05$). However, treatment with 10, 20 or 40 μM of the PAR-4 activating peptide increased nuclear area in comparison to control ($p<0.05$). For each treatment, 500 motoneurons from 5 separate embryos were measured.

DISCUSSION

The main goal of this experiment was to examine the effects of PAR-2, -3, and -4 activation in embryonic chick spinal motoneurons *in vivo* during PCD, and *in vitro* and to compare these results to previously published studies involving PAR-1. Our results indicate that activation of PAR-2, -3, or -4 does not significantly affect the viability of these neurons *in vivo* or *in vitro*. However, this does not rule out the presence or functionality of these receptors on motoneurons. While, no direct effects on motoneuron viability were seen, our results did show that the ligands for PARs 3 and 4 altered motoneuron nuclear area. Like SFLLRNP (Turgeon et al., 1998), SLIGKV decreased motoneuron nuclear area, while GYPGKF increased nuclear area. These results suggest that a delicate balance of serine proteases and their receptors may exist in the spinal cord to aid in the coordination of growth and development. This study also supports the conclusion from previously published papers that thrombin-induced motoneuron cell death is mediated via the PAR-1 receptor, since treatments with the other PAR agonists did not alter the survival of these cells.

Studies conducted by Turgeon et al. (1998, 1999) suggest that the serine protease, thrombin, or the synthetic peptide, SFLLRNP, induce degeneration and death of avian lumbar motoneurons. These studies, however, did not rule out the fact that additional PARs could play a role in these processes. Although PARs have been initially identified and shown to play major roles in hemostasis and fibrinogenesis (Kahn et al., 1998), there is increasing evidence suggesting that these receptors have different functions in the nervous system (see Festoff et al., 1996; Turgeon and Houenou, 1997; Turgeon et al., 2000). PAR-1 and PAR-2 are coexpressed by hippocampal neurons, and their activation leads to neurodegeneration (Smith-Swintowsky et al., 1997). In addition, it has been reported that primary mouse spinal motoneurons and a motoneuron cell line express PAR-1 and activation of this receptor induces cell death (Smirnova et al., 1998). Due to the specificity of SFLLRNP for PAR-1 (Ishihara et al., 1997), it is unlikely that other PARs are involved in the thrombin/SFLLRNP-induced motoneuron cell death.

Together these results support the hypothesis that thrombin/SFLLRNP-induced motoneuron cell death is achieved through activation of PAR-1. Information concerning the possible role(s) of PARs in different areas of the nervous system may help to elucidate the mechanisms involved in certain neurodegenerative diseases. The developmental expression of PARs, their respective ligands, and their effects upon motoneuron survival may be related to neuronal cell death following injury and disease.

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