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Neurosteroids reduce inflammation after TBI through CD55 induction

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Abstract

The inflammatory cascade that follows traumatic brain injury may lead to secondary cell death and can impede recovery of function. Complement factors and their convertases are increased in glia after brain injury and lead to the production of inflammatory products that kill vulnerable neurons. Progesterone and its metabolite allopregnanolone (5α-pregnan-3β-ol-20-one) have been shown to reduce the expression of inflammatory cytokines in the acute stages of brain injury, although how they do this is not completely understood. In this study we show that both progesterone and allopregnanolone treatments enhance the production of CD55 following contusion injuries of the cerebral cortex in rats. CD55, a single-chain type 1 cell surface protein, is a potent inhibitor of the complement convertases which are activators of the inflammatory cascade. The increased expression of CD55 could be an important mechanism by which steroids help to reduce the cerebral damage caused by inflammation.

Keywords: Traumatic brain injury; Progesterone; Allopregnanolone; CD55; Inflammation

Traumatic brain injury (TBI) produces a significant inflammatory reaction that is generally accompanied by heavy gliosis and apoptosis in brain areas proximal and distal to the locus of injury [6,33]. The insult triggers an invasion of macrophages and neutrophils into the impact area, producing much of the inflammation and swelling associated with CNS damage. These potentially cytotoxic events can directly affect patient outcome after TBI, which can be further worsened by uncontrolled intracranial pressure (ICP) [17] caused by a rise in brain water content (cerebral edema) [18]. Uncontrolled ICP can produce greater secondary injury through ischemia [39] and an increase in mortality caused by herniation of the brain [23]. Other than mannitol, which has limited effects on edema, there are no substantially effective therapeutic agents which can rapidly and safely reduce both marked swelling and inflammation after brain injury.

Over the last decade, we have focused on the use of progesterone (PROG) as a therapeutic agent for TBI. In rats with bilateral medial frontal cortex (MFC) contusions, post-injury injections of PROG reduced cerebral edema, blood–brain barrier disruption, and the expression of inflammatory cytokines [7,31,32]. We have also shown that PROG's metabolite, allopregnanolone (ALLO; 5α-pregnan-3β-ol-20-one), a GABA-A agonist, can reduce the mediators of inflammation after TBI at a dose that is 50% lower than PROG [14]. The effectiveness of PROG in the context of TBI was most recently demonstrated by a Phase IIa clinical trial showing that 3 days of intravenous injections of PROG post-TBI can reduce mortality by over 50% in severely-injured human patients and improve cognitive functions in moderately-injured patients tested 30 days after their injuries [42].

Exactly where PROG and ALLO interact with the inflammatory cascade to reduce its intensity is unknown, but complement factors seem to play one of the major roles [29]. Sewell et al. [35] recently demonstrated that mice deficient in complement factor C3 expressed significantly lower levels of inflammatory cytokines than did wild-type mice, and complement inhibition has been shown to attenuate CNS injury, whether induced by external agents or genetic deficiency [1,25,43].

Complement C3 is a key component in the activation of the complement system. The C3 precursor is a 185 kD protein that is

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cleaved into an alpha (120 kD) and a beta chain (75 kD) linked by a disulfide bond. This mature C3 is further cleaved by C3 convertase to release C3a and C3b [28]. C3a, one of the anaphylatoxins of the complement system, is a potent vasoconstrictor and immune cell activator and has been shown to be a promoter of inflammatory cytokines in human disease processes as diverse as cerebral ischemia [26] and Alzheimer's disease [9]. C3b participates in cellular adherence and enhances phagocytosis in addition to its crucial role in forming cellular intermediates that perpetuate the complement activation process in both the classical and alternative pathways. Clearly such a self-promoting inflammatory system can be detrimental if left unchecked. Disrupting the activity of convertase could short-circuit this injury cascade, and PROG and ALLO may play a role in this process.

The evidence for this comes from a recent study in our laboratory [29], which reported that post-TBI administration of PROG reduces the expression of both the C3a and C3b fragments (9 and 75 kD, respectively) of the pro-inflammatory complement factor C3 without influencing its overall expression, suggesting an effect on C3 convertase. In particular, the reduction of C3a indicates that PROG treatment reduces complement-mediated cytotoxicity [4]. More importantly, a reduction in C3b [3] can inhibit further activation of the complement system and the resultant amplification of the inflammatory process.

The specific mechanism by which PROG and ALLO reduce the C3 fragments after injury is not yet known. However, based on the literature in reproductive biology [41] and our previous results, we hypothesized that acute treatment with PROG after TBI may block the C3 cascade by increasing the expression of CD55 [22]. CD55, also known as decay-accelerating factor (DAF), is a 70 kD protein that disrupts C3 convertase activity both by inhibiting formation of the C3 convertase complex and by accelerating its decay [20]. Early inactivation of the complement cascade by CD55 effectively halts the progression of inflammatory processes and prevents subsequent cell injury [38].

In the present study, we report that post-injury treatment with either PROG or ALLO sustains the synthesis of CD55 in the injured brain. Combined with our previous data showing that a decrease in neuroinflammation is correlated with improved outcomes after contusion injuries to the cortex [14,29], these findings can be taken to suggest that CD55 activation represents an important mechanism by which PROG and ALLO exert their anti-inflammatory effects after TBI.

Sixty male Sprague–Dawley rats weighing 300–330 g were used in this study. Fifteen animals were assigned to each of four treatment groups (including shams). Five rats from each group were used for gene expression analysis and ten rats were used for protein expression assays. All experiments were performed in accordance with Emory IACUC guidelines and the approved protocol #146-2005.

Rats were anesthetized with 2.5% isoflurane prior to and during surgery. Body temperature was monitored and maintained between 36 and 37 °C with a homeothermic blanket system (Harvard Apparatus, Holliston, MA). Using a pulse oximeter (SurgiVet^{TM} V3304; Waukesha, WI), heart rate and blood oxygen were also monitored and maintained above 300 beats/min and 92%, respectively. Animals were placed in a stereotaxic frame and a midline incision was made to expose the skull. A mid-sagittal 6-mm diameter craniotomy was made using a trephine, 3 mm rostral to bregma. Cortical contusion impact (CCI) injury to the MFC was induced by a computer-controlled, pneumatically driven 5-mm diameter steel impactor tip at a velocity of 2.25 m/s that penetrated the exposed cortex to a depth of 2 mm. The injury was sutured closed after all bleeding had stopped and the animals were placed into heated recovery boxes and allowed to recover from anesthesia before being returned to their home cages [15]. Sham rats underwent the same pre-injury procedures but without the craniotomy and CCI.

PROG (16 mg/kg), ALLO (8 mg/kg) and vehicle (22.5% 2-hydroxypropyl-β-cyclodextrin) were given intraperitoneally at 1 h and then subcutaneously at 6 h and every 24 h after injury until survival endpoints were reached. Both dose and injection schedules were determined from previous studies [7,8,10,12,36]. Sham groups received no injury or injections. Brains were harvested at 48 h for mRNA analysis, and at 24 and 72 h post-injury for protein analysis.

Brain tissue from the penumbral region of the injury was extracted after decapitation and placed on an ice-chilled glass plate. Tissue samples were dissected and collected. Equivalent regions were collected for sham animals. Samples for protein analysis were snap-frozen in chilled 2-methylbutane and stored at −20 °C. Samples for RNA analysis were placed in RNAlater (Qiagen, Valencia, CA) and stored at −20 °C.

Total RNA was extracted from the MFC dissections taken 48 h post-TBI (RNAeasy kit, Qiagen). Spectrophotometric measures were taken to determine RNA concentration, and Invitrogen SuperScript First-Strand Synthesis System for RT-PCR (11904-018, Invitrogen, Carlsbad, CA) was used to synthesize cDNA from total RNA using random primers.

Real-time PCR was performed in a 50-μl reaction using 5 μl of the diluted first-strand cDNA template (800 ng), an optimized quantity of primers, water, and 25 μl of the 2X SYBR Green I dye PCR Master Mix (Applied Biosystems, Foster City, CA). PCR reactions were performed at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. 18S ribosomal RNA abundance was also amplified using real-time PCR to normalize for nucleic acid loading (Ambion, Austin, TX). Primers were designed using Primer Express software (Applied Biosystems) and synthesized by the Microchemical Facility at Emory University. The primer sequences were; forward: CAA ACT CGG GTA AAC ACA and reverse: CAT TTT CAA GGC AGA CCA CT.

MFC dissections collected at 24 and 72 h after injury were homogenized in T-per (Pierce, Rockford, IL) and protease inhibitor cocktail (Sigma, St. Louis, MO). A Bicinchoninic Acid protein assay (Pierce, 23225) was performed to ensure that all samples had equal amounts of protein.

Twenty microliters of each sample were added to individual wells of 12.5% acrylamide Criterion gel (BioRad, Hercules, CA). A protein standard (BioRad) was loaded to detect protein size. The gel was run at 200 V for 1 h. Proteins were transferred to a polyvinylidene difluoride membrane at 100 V for 30 min. Ponceau S was added to membranes to show protein bands.