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Sex differences in XIAP cleavage after traumatic brain injury in the rat

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Abstract

Sex influences histological and behavioral outcomes following traumatic brain injury (TBI), but the underlying sex-dependent pathomechanisms regulating outcome measures remain poorly defined. Here, we investigated the TBI-induced regulation of the X-linked inhibitor of apoptosis protein (XIAP) that, in addition to suppressing cell death by inhibition of caspases, is involved in signaling cascades, including immune regulation and cell migration. Since estrogen has been shown to have anti-apoptotic properties, we specifically examined sex differences and the influence of estrogen on XIAP processing after TBI. Sprague-Dawley male (TBI-M), female (TBI-F), ovariectomized female (TBI-OVX) and ovariectomized females supplemented with estrogen (TBI-OVX+EST) were subjected to moderate (1.7–2.2 atm) fluid percussion (FP) injury. Animals were sacrificed 24 hrs after FP injury; cortical tissue (ipsilateral and contralateral) was dissected and analyzed for XIAP processing by immunoblot analysis (n=6–7/group) or confocal microscopy (n=2–3/group). Significant differences in XIAP cleavage products in the ipsilateral cortex were found between groups (p<0.03). Post-hoc analysis showed an increase in XIAP processing in both TBI-F and TBI-OVX+EST compared to TBI-M and TBI-OVX (p<0.05), indicating that more XIAP is cleaved following injury in intact females and TBI-OVX+EST than in TBI-M and TBI-OVX groups. Co-localization of XIAP within neurons also demonstrated sex-dependent changes. Based on these data, it appears that the processing of XIAP after injury is different between males and females and may be influenced by exogenous estrogen treatment.

Keywords

traumatic brain injury; X-linked inhibitor of apoptosis; sex differences

Sex differences on outcome measures after central nervous system (CNS) injury have been reported by numerous investigators [for review see ³ and ²⁸]. Many of these studies have demonstrated improvements in function and attenuation of histopathological damage due to the influence of neurohormones such as estrogen and progesterone or a basic sex-dependent

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affect. In contrast, some studies have also shown a negative or no effect on histopathological outcome following brain injury [12, 16]. The mechanisms that may be governing the observed differences due to hormonal affect or sex are for the most part unknown. Apoptotic cell death has been identified as a significant injury mechanism in various model of traumatic brain injury (TBI) [for review see 20, 15]. We have previously reported that the multifunctional IAP family member X-linked inhibitor of apoptosis protein (XIAP) is cleaved and undergoes alterations in cellular expression in male brains subjected to moderate traumatic brain injury (TBI) [15, 17]. Moreover, XIAP is monoubiquitinated after brain trauma and redistributed within neurons [17], which may alter XIAP function after injury. XIAP participates in diverse cellular functions including caspase inhibition, signal transduction, ubiquitination, and copper metabolism. With respect to signal transduction, XIAP has been implicated in regulation of TGF- β and BMP type I receptors and in NF- κ B activation [18] and inhibition of the inflammasome in neurons [5]. IAPs contain one to three copies of the baculovirus IAP repeat (BIR), which drive the anti-apoptotic activity of the IAPs [8] and also interact in signal transduction pathways. The RING domain of IAPs is thought to work by targeting a protein for ubiquitylation resulting in either a modification of protein activity or the cellular localization of a protein via monoubiquitination or the degradation of a protein through the 26S proteasome due to polyubiquitination [23]. Devereaux et al. [6] has reported that in Fas-induced apoptosis, XIAP undergoes cleavage into a BIR1-2 fragment that inhibits Fas-induced apoptosis and also a BIR3-Ring fragment that enhances Fas-induced apoptosis. Whether sex or hormones influence XIAP cleavage after CNS injury remains poorly understood.

Sprague-Dawley rats weighing between 225 and 325 grams were used for these experiments. All animal procedures followed the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Miami's Animal Care and Use Committee. Male and females animals were age-matched for this study. Four groups of animals (male (TBI-M), female (TBI-F) and ovariectomized female (TBI-OVX), and ovariectomized female plus estrogen treatment (TBI-OVX+EST) underwent fluid percussion (FP) brain injury to be used for either western blotting or immunohistochemistry for confocal microscopy. For those female animals undergoing ovariectomy, this procedure was done as previously described [4] 10 days prior to TBI. Both estrogen (Mean $\pm$ SEM; TBI-F: 11.73 $\pm$ 2.84pg/ml; TBI-OVX: 6.79 $\pm$ 2.47pg/ml) and progesterone (TBI-F: 32.21 $\pm$ 3.36 ng/ml; TBI-OVX: 7.48 $\pm$ 0.78 ng/ml) levels were reduced in female animals following this procedure compared to intact females. Following ovariectomy, animals received a subcutaneous implant at the nape of the neck of a continuous release tablet of either high dose 17 β -estradiol (1.19mg/day) or vehicle provided by the manufacturer (Innovative Research of America). A supraphysiological dose was chosen to determine if high levels of estradiol could affect the apoptotic pathway as lower dosages have previously demonstrated [1, 13, 21]. The number of animals for each group for western blotting was n=6–7 and the number for confocal microscopy was n=2–3. Animals were prepared for FP injury as previously described [4, 29]. Briefly, animals were anesthetized (1.0% halothane, nitrous oxide/oxygen (70/30% mix)) and a craniotomy (4.8mm) was preformed over the right parietal cortex 3.8mm posterior to bregma and 2.5 mm lateral to the midline [19]. A modified plastic injury tube was placed over the exposed dura and bonded by adhesive. The injury tube was further fixed to the skull with dental acrylic. The scalp was sutured closed and the animal was returned to their home cage. After fasting overnight, a FP device was used to produce experimental TBI via the injury tube. Intubated anesthetized rats (70% nitrous oxide, 0.5% halothane, and 30% oxygen) were subjected to a pressure pulse of moderate (1.7–2.2 atm) intensity. Prior to TBI, a catheter was placed in the femoral artery to monitor blood gases. Brain and body temperature were maintained at a normothermic (37°C) level throughout the surgical procedure. Prior to sacrifice for western blotting or confocal microscopy, a venous blood sample was taken to determine estrogen and progesterone levels for intact female and ovariectomized animals with or without 17 β -estradiol treatment to confirm hormone levels as

previously described [4]. Male, female and ovariectomized sham control animals were also submitted to all procedures except for the actual FP injury.

For immunofluorescence microscopy, animals were perfused with 4% paraformaldehyde at 24 hours after TBI. Brains were extracted and cut through the neuroaxis, obtaining 35 μ m-thick sections that were systematically distributed and cryopreserved until processing. Sections were incubated for double labeling at 4°C overnight with rabbit polyclonal XIAP antibody, dilution 1:500 (Cell Signaling Technology) and 3 different mouse monoclonal cell markers: anti-glia fibrillary acidic protein (GFAP) antibody (BD Biosciences), anti-CD11b/c antibody (Accurate Chemical & Scientific Corporation) and anti-neuronal nuclei (NeuN) antibody (Chemicon International), dilution 1:1000. Primary antibody binding was detected with fluorophore-labeled antibodies, (Alexa Fluor 594 goat anti-Rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG, 1:200, Molecular Probes). Confocal microscopy images were obtained using a LSM 510 laser scanning confocal microscope (Zeiss, Inc.). For XIAP protein levels, animals were sacrificed at 24 hours after TBI and a 2 mm section of ipsilateral and contralateral cortex was homogenized in cell extraction buffer. Proteins were resolved on 8.5% SDS-PAGE and then transferred to polyvinylidene fluoride membranes overnight. Membranes were placed in blocking buffer and then incubated for 1 hour with anti-XIAP monoclonal antibody (1:250; BD Transduction). Visualization of the signal was enhanced by chemiluminescence. To control for protein loading, the gels were stripped and probed for tubulin using monoclonal β -tubulin (MAB 556321, BD Transduction). Quantification of bands corresponding to changes in protein levels were assessed using NIH Image J software. Immunoblot data were expressed as mean relative density readings of cleavage fragments/full length XIAP (45+30kDa/53kDa). Group differences on density readings were assessed using one-way ANOVA followed by Fisher LSD post hoc analysis. Statistical significance was detected at $p < 0.05$.

Figure 1 shows confocal immunofluorescence images of XIAP expression in rat brains of Sham-M, TBI-M, TBI-F, TBI-OVX and TBI-OVX+EST. In TBI-M and Sham-M, XIAP immunoreactivity was present in neurons and exhibited a punctate staining pattern distributed diffusely in the perinuclear region and in the cell body. All female groups (TBI-F, TBI-OVX and TBI-OVX+ EST) demonstrated a similar XIAP expression pattern to TBI-M, but TBI-F showed more intense XIAP punctate immunoreactivity in the perinuclear region that appeared as an intense nuclear rim of fluorescence. XIAP immunoreactivity did not colocalize with cellular markers for astrocytes or macrophages (data not shown). Thus, XIAP expression in the brain of males and females is different in that females exhibit more intense immunoreactivity in the perinuclear region of neurons.

To study regulation of XIAP after TBI, cortical lysates from the various experimental groups were immunoblotted for XIAP. As shown in Figure 2, levels of full-length XIAP (53 kDa) from sham or animals subjected to moderate TBI are shown. Cortices from traumatized females had higher levels of the 45- and 30-kDa cleaved fragments than traumatized males. In addition, ovariectomized females supplemented with a supraphysiological dose of 17 β -estradiol showed increased levels of XIAP cleavage products than males and TBI-OVX animals. Lysates from sham animals contained low levels of XIAP cleavage products (data not shown).

A determination of the ratio of cleavage/full length XIAP was computed for the ipsilateral and contralateral cerebral cortex of each animal using densitometry. The amount of XIAP cleavage was significant ($p < 0.03$) for group (Figure 3) in the ipsilateral cortex. Posthoc analysis using Fisher LSD indicated that there was a significant ($p < 0.05$) increase in cleavage fragments in the TBI-F and TBI-OVX+EST groups compared to TBI-M or TBI-OVX groups. There were no significant differences between groups within the contralateral cerebral cortex. There appears to be a sex dependent difference in the amount of XIAP cleavage that is also influenced by estrogen levels.

Females and ovariectomized females supplemented with estrogen demonstrate higher levels of XIAP cleavage following TBI compared to males and ovariectomized females. Both the levels of XIAP cleavage and the subcellular distribution of XIAP appear to be influenced by the presence or absence of estrogen. Thus, the regulation of XIAP after injury appears to be not only sex-dependent but also influenced by the neurohormone estrogen. One caveat that requires mentioning is that the female animals had a supraphysiological dosage of estradiol. Therefore, the effects of estrogen should be interpreted under this condition. Physiological levels of estrogen given after TBI may not demonstrate this type of effect on XIAP processing. Although females appear to be protected from either traumatic or ischemic injuries due to innate sex differences or hormone supplementation as assessed by gross morphological damage or apoptotic cell death [1–3, 7, 9, 11, 13, 21, 28, 30], this protection may not be primarily due to the inhibitory effects of XIAP on apoptotic cell death. For example, other components of apoptosis have been shown to be involved in the attenuation of damage following CNS injury including upregulation of Bcl2, decreases in caspase-3 levels, and caspase-mediated spectrin breakdown [1, 7, 13, 21, 31]. Previous studies of IAPs after injury have only been done in male animals. Additionally, XIAP has recently been shown to increase levels of antioxidants in an *in vitro* system indicating other pathomechanism that could be targeted after injury [14]. Furthermore, the interaction of the functional motifs within XIAP, particularly BIR1, with other proteins can result in enhanced function of XIAP through other non-apoptotic signaling mechanisms [27]. Specifically, the BIR1 domain is able to bind to other partners forming complexes that are involved in MAPK signaling. These complex interactions between the different motifs of XIAP and other IAPs are still under investigation and may provide future understanding of this pathway.

The predominant regulator of apoptosis in the female brain or with estrogen supplementation after injury has been an increase in the expression of Bcl-2 which in turn blocks activation of the intrinsic apoptotic pathway. It may be the case that the inhibitory role of XIAP is not as robust in the female animal compared to males because of the increased expression of cleavage fragments after TBI. However, the present study only assessed one time point (i.e. 24 hrs) and it may be the case that males and females respond differently to neurodegenerative processes. Kupina and colleagues [16] recently demonstrated in a weight-drop mouse model of brain injury that males and females have temporal differences in cytoskeletal protein degradation. The apoptotic pathway is extremely complex and different avenues of blocking this pathway may be employed depending on the sex and presence of neurohormones in the animal. However, an alternative explanation is that the cleavage fragments themselves can inhibit caspase activation [6, 10]. Several investigators have shown that the BIR2 domain of XIAP is the binding domain of caspase-3 inactivation and the BIR3 domain effects caspase 9 activation [6, 22, 24–26]. Deveraux and colleagues [6] have reported that the overexpression of the BIR1-2 domains can result in an increase in the inhibition of Fas-induced apoptosis. In a recent article by Fan et al., [10] the BIR3-RING domain was fused to a protein transduction domain and given following focal cerebral ischemia. This treatment resulted in a decrease of TUNEL positive cells and caspase-3 cleavage. Therefore, the administration of a component of XIAP improved histopathological outcome. Although these alternative explanations for the role of XIAP after injury are speculative, there are clearly sex differences after trauma in the amount of XIAP cleavage which also appears to be regulated by estrogen. In summary, the processing of XIAP and cellular distribution in females and ovariectomized females plus estrogen is different compared to males and ovariectomized animals. These sex-dependent differences could be indicative of how neurons survive after injury in males or females by using alternative means of inhibiting apoptotic or other signaling pathways. Supported by NS042133, NS030291 and Eli Lilly & Co.

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Figure 1.

Representative confocal images of cerebral cortical neurons overlying the injured cortex in all groups. Sections were double-stained with XIAP (red) and NeuN (green) and merged to show double labeling. TBI-F, TBI-OVX and TBI-OVX + EST animals exhibit intense XIAP immunoreactivity in the perinuclear region of neurons (arrow, see inset). TBI-M show XIAP less intense and diffuse staining within perinuclear region (arrow head, see inset). Bar = 20 μ m.

Figure 2.

Representative immunoblot of full-length XIAP (53 kDa) and cleavage fragments (45 kDa and 30 kDa) after TBI or sham surgery. Increases in cleavage products are present in the TBI-F and TBI-OVX+EST groups compared to the other groups. The amount of cleavage appears to be regulated by sex of the animal as well as the presence or absence of estrogen.

Cleavage of XIAP in Cerebral Cortex

Figure 3.

Quantification of XIAP cleavage (Mean + SEM) 24 hours post-injury. Ipsilateral cerebral cortical densitometric assessment of the mean cleavage/full-length XIAP ratio was significantly ($p < 0.05$) increased in the TBI-F and TBI-OVX+EST groups compared to TBI-M and TBI-OVX. There were no significant differences in XIAP cleavage in the contralateral cerebral cortex. Sham animals are not presented.