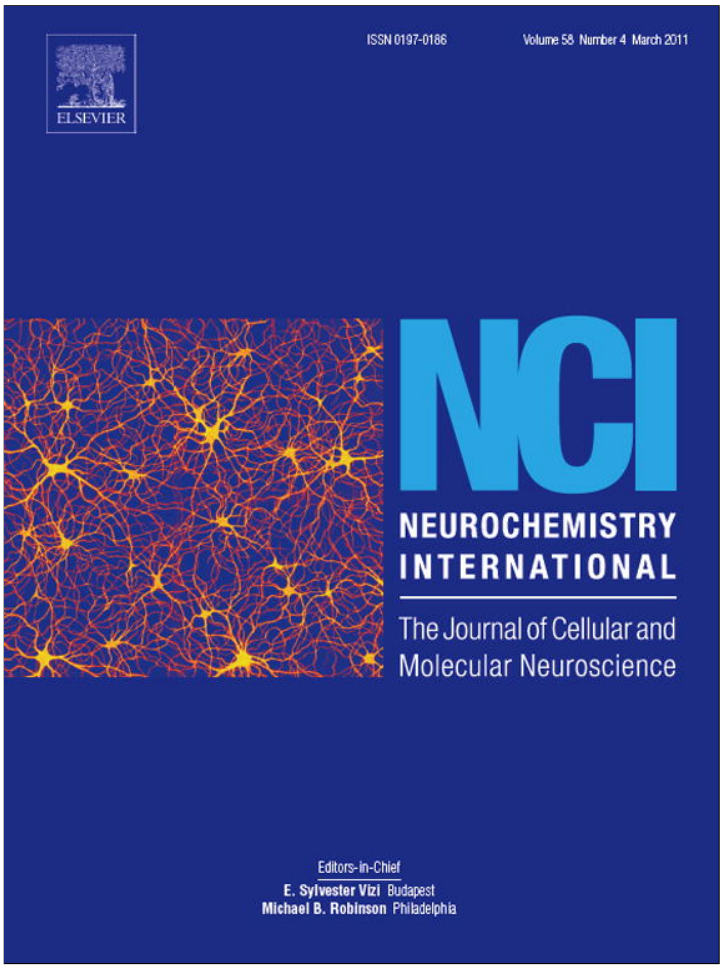

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Rapid communication

Does IGF-1 administration after a mild traumatic brain injury in mice activate the adaptive arm of ER stress?

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ABSTRACT

Mild traumatic brain injury (mTBI) induces along with cognitive impairments, both cell death and survival pathways. Previously, IGF-1 (Insulin like growth factor 1) administration prevented TBI-induced damage. This study was aimed at testing the effect of mTBI on ER (endoplasmic reticulum) stress activation and looking for a possible interaction between IGF-1 and ER stress pathways. Mice were subjected to a weight drop closed head injury. Western blot analysis revealed that mTBI induced activation of ATF6 (activating transcription factor 6), but not of CHOP or grp78. IGF-1 administration following mTBI did not change ATF6 or grp78 levels, but significantly elevated CHOP. These results suggest that IGF-1 may exert its neuroprotection via PERK/CHOP, the adaptive arm of the unfolded protein response.

1. Introduction

Traumatic brain injury (TBI) is characterized by a broad range of neurological and other impairments (Slemmer et al., 2008). We have previously reported that a non-invasive closed-head mild TBI in mice induced behavioral and biochemical changes in the brain (Rubovitch et al., 2010; Tweedie et al., 2007). These results were similar in nature to reports regarding moderate and severe TBI (Slemmer et al., 2008). mTBI induced elevations in the levels of various pro-apoptotic proteins in different brain regions (Tweedie et al., 2007). In addition, data have been published indicating parallel, TBI-induced anti-apoptotic pathways, pointing to the involvement of the pro-survival, IGF-1R pathway and its target proteins AKT/PKB and ERK in this process (Madathil et al., 2010; Rubovitch et al., 2010).

The IGF-1 system is involved in growth, differentiation and survival signaling in the central nervous system [For a review see (Aleman and Torres-Aleman, 2009). Consistent with these findings, IGF-1 has been implicated as a neuroprotective agent in brain injuries (Rubovitch et al., 2010). IGF-1R was also found to be up-regulated following TBI (Madathil et al., 2010). However, reports have been published suggesting a paradoxical effect of this axis: for example, reduced IGF-1 in a mouse model of Alzheimer disease (AD) protected the animals from the AD symptoms (Cohen et al., 2009).

ER is an important cell organelle that is responsible for the correct folding and sorting of proteins (Boyce and Yuan, 2006). Disturbed ER functions induce expression of chaperones, attenuate protein translation, and activate ER-associated degradation (Boyce and Yuan, 2006). This occurs by the activation of ER sensor proteins controlled by the chaperone Bip/Grp78 (Bip), which is localized in the ER. ER stress leads to the activation of ATF6 (activating transcription factor-6), PRK (RNA-dependent protein kinase)-like ER protein kinase (PERK)/pancreatic eukaryotic translation initiation factor 2 subunit_ (eIF2_) kinase, and the inositol-requiring enzyme-1 (IRE1), which in turn activate distinct signaling cascades mediating the ER stress response (Lee et al., 2002). Apart from the UPR that is mainly adaptive and restorative in function, prolonged ER stress can trigger cell death (Hetz et al., 2006).

A linkage between the ER stress system and the IGF-1 signaling pathway has been suggested in recent years. Reduced IGF-1 signaling was found to enhance the UPR activity and extend life span in *C. elegans* (Tang, 2006). ER stress-induced apoptosis was blocked by the administration of IGF-1 (Guan et al., 2003). XBP-1, a key regulator of unfolded protein response, activates transcription of the IGF-1 and Akt phosphorylation in zebrafish embryonic cell line (Hu et al., 2007).

The main goal of the present study was to evaluate the involvement of the ER stress system in mTBI-induced events. In addition, this study was aimed at assessing possible relations between the IGF-1 pathway and the ER stress system following mTBI.

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2. Materials and methods

2.1. Mice

Male ICR mice weighing 25–30 g were kept five per cage under a constant 12-h light/dark cycle, at room temperature (23 °C). Food (Purina rodent chow) and water were available *ad libitum*. Each mouse was used for one experiment and for one time point only. The Ethics Committee of the Sackler Faculty of Medicine approved the experimental protocol (M-04-063), in compliance with the guidelines for animal experimentation of the National Institutes of Health (DHEW publication 85-23, revised, 1995). The minimal possible number of animals was used and all efforts were made to minimize their suffering.

2.2. Brain injury

Experimental mTBI was induced using the concussive head trauma device described previously (Tweedie et al., 2007) Briefly, mice were slightly anesthetized by inhalation of 0.5 ml of isoflurane in a closed glass chamber. Mice were placed under a device consisting of a metal tube (inner diameter 13 mm), placed vertically over their head. The animals were held in a way that the impact on the skull (closed scalp) was anteriolaterally, just anterior to the right ear. A sponge immobilization board was employed allowing small head movements during the injury, analogous to those that occur during closed head injury in car accidents. The injury was induced by dropping a metal weight (30 g), from 80 cm height down the metal tube, striking the skull. Immediately after the injury, mice were placed back in their cages for recovery. Injured mice did not exhibit any apnea after the injury. The effect of the injury was studied at 7 and 30 days following the trauma, using different groups of mice at each time point (at least 10 mice per group).

2.3. Drug administration

IGF-1 (Cytolab, Rehovot, Israel) (4 μg/kg) was injected (ip) 24 and 48 h post injury. This paradigm was chosen due to the poor permeability of IGF-1 through the blood brain barrier (BBB). mTBI was found to induce a transient rupture of the BB around 24 h post injury. Thus we injected IGF-1 at 2 time points: 24 and 48 h post injury as was suggested by Guan et al. (2003). The selective IGF-1R inhibitor AG1024 (Calbiochem, Germany) was injected, ip, (50 μg/kg) 1 h before the injury, and 24 and 48 h post TBI. These dosages and administration routes were chosen after reviewing the relevant literature and consulting with the developers of the drug (Drs. A. Gazit and A. Levitzki, Hebrew University, Jerusalem, Israel). The ER stressor Tunicamycin (either 80 or 40 μg/kg) was ip injected 48 h prior to brain removal.Western blots

Whole brains were removed at 1, 24, and 72 h, and 1, 2 and 3 weeks post-mTBI. Cortex samples were immediately frozen in liquid nitrogen and homogenized with T-PER Tissue Protein extraction Reagent (Pierce, Rockford, IL), with appropriate protease inhibitors (Halt Protease Inhibitor Cocktail; Sigma–Aldrich). Separate samples (cortex and hippocampus) were run on 10% Bis-Tris gels (Bio-Rad) and transferred to nitrocellulose membranes. Blots were blocked for 1 h with Tris-buffered saline containing 0.01% Tween-20 and 5% powdered milk. Membranes were incubated with antibodies against ATF6 (2 h at room temperature), GRP78 AND CHOP (over night at 4 °C, Santa Cruz Biotechnologies, Santa Cruz, CA) diluted 1:1000 and incubated with secondary horseradish peroxidase-linked antibodies (Jackson Immunoresearch, West Grove, PA) at room temperature for 1 h. Bands were visualized by enhanced chemiluminescence (Pierce Rockford, IL) and exposed to an X-ray film. Protein band intensities were quantified by using the TINA software. Uniform loading was verified by stripping and re-probing with antibodies against tubulin (1:1000; Santa Cruz Biotechnologies).

2.5. Data analysis

All results are given as mean ± SEM and data were analyzed using one way ANOVA; statistical significance was set at $^{*}p$, ^{y}p, $^{z}p < 0.05$; $^{**}p$, ^{yy}p, $^{zz}p < <0.01$; $^{***}p$, ^{yyy}p, $^{zzz}p < <0.001$. p Values of post hoc tests were adjusted using the Fisher LSD test and a nominal significance level of 0.05 was used.

3. Results

3.1. mTBI induced ATF6 activation in the mouse cortex

To evaluate the involvement of the ER stress system in the events following mTBI, we initially examined the effect of mTBI on ATF6 activation. Results indicate that mTBI induced a significant activation of ATF6 on both ipsi and contra-lateral cortex. In the injured hemisphere elevation was found from 1 h post mTBI and remained high even 2 weeks after injury. Maximal effect was found 24 h post mTBI (2.61 ± 0.08 fold over control for the right, injured cortex; $n = 5$, $p < 0.0001$; Fig. 1A). In the left (contra-lateral) cortex a

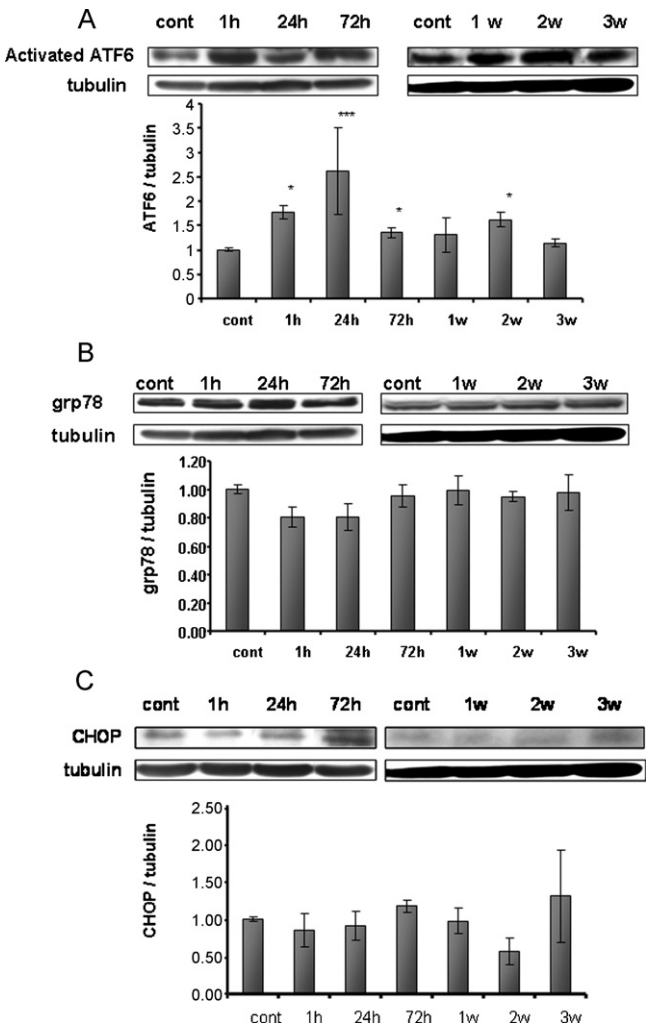

Fig. 1. The effect of mTBI on ER stress markers. (A) Cortex and hippocampus protein extracts (ipsi-lateral injured hemisphere; separate samples) prepared from sham or mTBI-animals at the indicated time points were subjected to gel electrophoresis, followed by immunoblot analysis using antibodies against ATF6, grp78 or CHOP. The level of activation/induction was evaluated by densitometry analysis. The control values were set to 1, and the relative values of respective treatment were calculated accordingly. Results indicate that mTBI induced a significant activation of ATF6 from 1 h up to 2 weeks following mTBI. Maximal effect was found after 24 h ($n = 5$, $^{***}p < 0.0001$ compared with control). mTBI had no effect on grp78 (B) or on CHOP (C) expression.

significant activation of ATF6 was found only at 72 h post mTBI (3.07 ± 0.23 fold over control; $n = 8$, $p < 0.03$). Tunicamycin (TM, the ER stressor) administration served as a positive control for ATF6 activation (see methods section; data not shown). *Hippocampi* tested showed almost no activation of ATF6, although initial amounts of the inactivated form of this factor were similar to those in the cortex (data not shown).

In order to gain a broader picture of the ER stress processes triggered by mTBI, we assessed the changes in the expression of additional ER stress participants. Bip/grp78 and CHOP (GADD153) were tested at the same time points as ATF6 following mTBI. Neither of these factors was affected by mTBI (Fig. 1B and C).

3.2. The effect of IGF-1 administration on ER stress markers following mTBI

The next set of experiments was aimed at revealing the underlying mechanism of exogenous IGF-1 protection of brain

injuries. Thus, mice were subjected to mTBI and IGF-1 (4 μg/kg) was injected 24 and 48 h post injury. Brains were removed 72 h after injury. Results indicate that IGF-1 administration had no effect neither on mTBI-induced ATF6 activation nor on grp78 expression (both in control and mTBI mice); Fig. 2A and B). However, a significant elevation in CHOP expression was found in the IGF-1 treated mTBI mice: 1.8 ± 0.08 fold over control; $n = 9$ (Fig. 2C).

Blocking the IGF-1R activation following mTBI, by the selective inhibitor AG1024, did not change the activation and expression of ATF6, grp78 or CHOP.

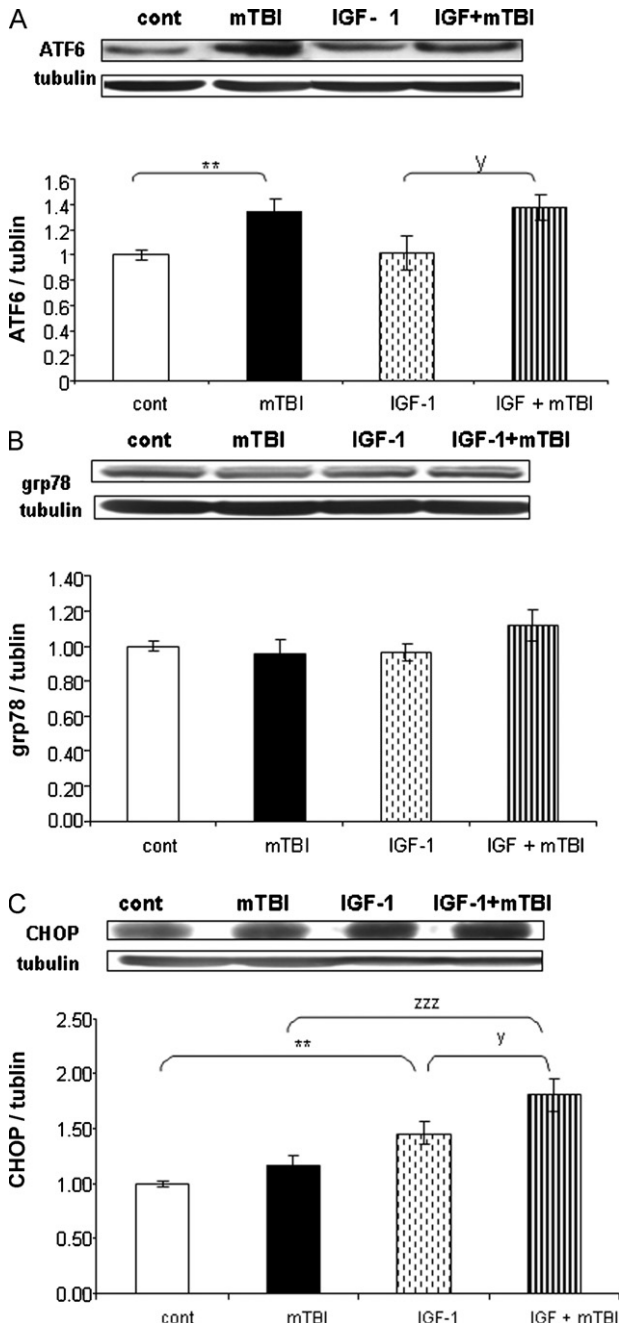

Fig. 2. The effect of IGF-1 administration on ER stress markers following mild traumatic brain injury. Cortex and hippocampus protein extracts prepared from sham or mTBI-animals (ipsi-lateral injured hemisphere; separate samples) at the indicated time points were subjected to gel electrophoresis, followed by immunoblot analysis using antibodies against ATF6 (A), grp78 (B) or CHOP (C). The level of activation/induction was evaluated by densitometry analysis. The control values were set to 1, and the relative values of respective treatment were calculated accordingly. IGF-1 administration did not change ATF6 or grp78 expression as seen in western blot analysis, either in control or mTBI mice (A and B). (C) CHOP expression was significantly elevated by IGF-1 compared with control mice ($n = 8$, $^{**}p < 0.01$). IGF-1 had a pronounced effect on CHOP expression in mTBI mice compared with mTBI mice ($n = 9$; $^{zzz}p < 0.001$) and a significant elevation compared with IGF-1 treated mice ($^{y}p < 0.05$).

4. Discussion

The results of the present study show for the first time that the ER stress can even be activated after a minimal traumatic brain injury. Moreover, we suggest that the effect of IGF-1 on CHOP expression in mTBI mice may reveal the mechanism underlying its neuroprotective effect.

ATF6 (activating transcription factor-6), is one of the three sensor proteins of ER stress (Benjamin, 2006). We found that ATF6 was activated in our model of mTBI which is in agreement with previous reports regarding the activation of the unfolded protein response by moderate or severe TBI for review see (Larner et al., 2006). While in the ipsilateral cortex the activation of ATF6 occurred as soon as 1 h post mTBI and kept high even two weeks later, on the contra-lateral hemisphere ATF6 was significantly activated only 72 h post injury (data not shown). This could represent a time-dependent diffused injury, meaning that the damage occurs over a more widespread area than in focal brain injury or time post injury. It occurs in about half of all cases of severe head trauma and also occurs in moderate and mild brain injury.

ATF6 was activated in the cortex of all the mice that were subjected to mTBI. However, in the hippocampi of the same mice, mTBI did not activate ATF6 although the amounts of its non-activated form were identical in these two regions. This could be a result of the superficial location of the cortex in comparison to the deep location of the hippocampus.

Bip/grp78 expression was not altered by mTBI (Fig. 1B). It is possible that we did not see a change since western blot analysis can detect free grp78 molecules, and due to the denaturative nature of the SDS-PAGE, grp78 molecules are all free, thus we cannot detect a significant change of this ER stress protein.

Surprisingly, although IGF-1 administration did not change ATF6 or grp78 activation, it significantly changed CHOP expression (Fig. 2A–C). CHOP can be induced either by the ATF6 arm and hence was regarded as a pro-apoptotic factor in the ER stress cascade. Recently, however, an additional, anti-apoptotic role for this molecule was suggested (Gow and Wrabetz, 2009). In this pathway CHOP can be induced by the PERK/elf2a arm, leading to adaptation to the ER stress. The fact that IGF-1-administration affected only CHOP but not ATF6 suggests that IGF-1 exerted its neuroprotective effect via PERK, which activates the survival/anti-apoptotic arm of the ER-stress machinery. We also tested the impact of IGF-1R inhibition (by the selective inhibitor AG1024) and found that it did not alter the effect of mTBI on ER stress markers (data not shown). In our previous study we found that while IGF-1 administration improved mTBI induced-damage to spatial memory, inhibiting the IGF-1R by AG1024 had no effect on this cognitive performance (Rubovitch et al., 2010). Together these results suggest that the intrinsic IGF-1R signaling pathway is different from the exogenous administration of IGF-1. The latter may mediate its neuroprotective effect via the adaptive arm of the ER stress machinery, while the endogenous pathway does not.

In conclusion, our results show that the ATF6 arm of the UPR is activated even after a mild traumatic brain injury. Moreover, we show for the first time that the neuroprotective effect of IGF-1 may be mediated via the PERK/CHOP arm of ER-stress. These results enlighten our understanding of the mechanism underlying brain injuries and might identify a possible target for this prevalent medical problem.

Conflict of interest

The authors declare no conflict of interest.

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