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The intricate involvement of the Insulin-like growth factor receptor signaling in mild traumatic brain injury in mice

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ABSTRACT

Insulin-like growth factor-1 (IGF-1) was suggested as a potential neuroprotective treatment for traumatic brain injury (TBI) induced damage (cognitive as well as cellular). The main goal of the present study was to evaluate the role of the IGF-1R activation in spatial memory outcome following mild traumatic brain injury. mTBI-induced phosphorylation of IGF-1R, AKT and ERK1/2, in mice hippocampus, which was inhibited when mice were pretreated with the selective IGF-1R inhibitor AG1024. IGF-1 administration prevented spatial memory deficits following mTBI. Surprisingly, blocking the IGF-1R signaling in mTBI mice did not augment the spatial memory deficit. In addition, this data imply an intriguing and complex role of the IGF-1 signaling axis in the cellular and behavioral events following mTBI.

Introduction

Traumatic brain injury (TBI) is one of the most common neurological disorders. The diagnosis of TBI includes a broad range of short- and long-term physical, cognitive, and emotional impairments, depending on the severity of the injury (Albensi, 2001; Waxweiler et al., 1995). In contrast to moderate and severe TBI in which brain morphological alteration is detectable (Graham et al., 2000), mild traumatic brain injury (mTBI) is commonly presented as a number of imprecise perceptual cognitive symptoms without diagnosable objective structural brain damage (the so-called "post-concussion syndrome") (Kibby and Long, 1996). We have previously reported that a non-invasive closed-head mTBI in mice induced short- and long-term deficits in a number of cognitive tests including impaired spatial memory and learning (Baratz et al., in press; Edut et al., 2008; Zohar et al., 2003). Nevertheless, no structural alterations were detected either by MRI or TTC staining (Tweedie et al., 2007; Zohar et al., 2003). Spatial memory deficits were also found in other mTBI animal models (Thompson et al., 2006; Wei et al., 2009).

Various cellular and biochemical changes in the brain, especially neuronal death (apoptosis, necrosis and gliosis), were found not only in moderate or severe TBI (Ferrer and Planas, 2003; Slemmer et al., 2008), but also in our mild TBI model (Tashlykov et al., 2007, 2009; Tweedie et al., 2007). mTBI-induced elevations in the levels of various pro-apoptotic proteins in different cortical and hippocampal regions of both injured and contralateral hemispheres (Tashlykov et al., 2007, 2009; Tweedie et al., 2007). However, additional, opposing data have been accumulating indicating a parallel, survival and anti-apoptotic pathway, pointing to the involvement of the serine–threonine kinase Akt/PKB (Noshita et al., 2002; Zhang et al., 2006). One of the major cascade that was found to activate Akt via phosphoinositide 3-kinase (PI3K) was the insulin-like growth factor-1 receptor (IGF-1R) (Povsic et al., 2003). Autophosphorylation of the IGF-IR also leads to the activation of the Ras— extracellular signal-regulated kinase (ERK) pathway (Franke, 2008; Vincent and Feldman, 2002). The serine–threonine protein kinase ERK plays distinct roles in the regulation of apoptosis in different cell types (Sawatzky et al., 2006). ERK1/2 have been implicated both as protective and damaging molecules signaling to define cell fate (Scheid et al., 1999).

The IGF-1 system is involved in growth, differentiation and survival signaling. [For a review see (Vincent and Feldman, 2002)]. It leads to the phosphorylation and inactivation of several pro- and anti-apoptotic proteins and transcription factors (Brami-Cherrier et al., 2002; Datta et al., 1997). Accumulating evidence indicates a major role for IGF-1 in central nervous system development and survival [for a review see (Aleman and Torres-Aleman, 2009)]. IGF-1 and its receptor are expressed in brain from the embryonic to the adult stage. Hence, IGF-1 has been implicated as a potential neuroprotective agent in brain injuries (Saatman et al., 1997) and in hypoxia–ischemia induced damage (Guan et al., 2003; Lin et al., 2009). In addition, the IGF-1R was found to be up-regulated following

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TBI (Madathil et al., 2009). In contrast to this protective role, reports have been published regarding a paradoxical effect of the IGF-1R signaling axis. Specifically, blockade of IGF-1 signaling has been reported to extend lifespan from invertebrates to mammals (Carter et al., 2002; Tang, 2006).

The main goal of the present study was to evaluate the involvement of the brain IGF-1R in minimal traumatic brain injury, and to assess its activation (phosphorylation) during this process. This study was aimed at revealing the unique and complex involvement of this pathway both at the biochemical and behavioral levels.

Materials and methods

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.

Brain injury

Experimental mTBI was induced using the concussive head trauma device described previously (Milman et al., 2005; Pan et al., 2003; Zohar et al., 2003). 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).

Drug administration

IGF-1 (Cytolab, Rehovot, Israel) (4 μg/kg) was injected (i.p.) 24 and 48 h post injury. AG1024 (Calbiochem, Germany) was injected, i.p., (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).

Western blots

Whole brains were removed 1, 24, and 72 h post mTBI, and hippocampi 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). Samples were run in duplicate on precast 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, 5% powered milk or 5% bovine albumin (Sigma-Aldrich). Membranes were incubated for 2 h at room temperature with antibodies against phospho-IGF-1R, phospho-Akt and phospho-ERK1/2 (Cell Signaling Technology) 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 reprobing with antibodies against total IGF-1R, Akt or ERK1/2 (1:1000; Cell Signaling Technology).

Behavioral performance

Y-maze test

Spatial memory was assessed by using the Y-maze, which was first described by Dellu et al. (1992) and then subsequently validated as a task—requiring hippocampal function and spatial memory (Conrad et al., 1996). The Y-maze was constructed of black Plexiglas with three identical arms (30 × 8 × 15 cm). Overt cues were attached inside of the Y-maze. The test included two trials separated by two minute intervals. The first trial was 5 min with only two arms open (the start arm and the arm called "old" arm), and the third arm was blocked by a door (the novel arm). The mouse was put in the start arm in the part most distant from the other two arms. After the first (familiarization) run the mouse was put back into the cage for 2 min. The second run lasted 2 min, and all three arms were open. Time spent in each of the arms was measured. Between each run and between each mouse the maze was cleaned with 70% ethanol. The new arm preference index was calculated as follows: (time in the new arm − time in the old arm) / (time in the new arm + time in the old arm).

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, $^{zz}p \ll 0.01$; $^{***}p$, ^{yyy}p, $^{zzz}p \ll 0.001$. p values of *post hoc* tests were adjusted using the Fisher LSD test and a nominal significance level of 0.05 was used.

Results

The main goal of our study was to evaluate the IGF-1R pathways activated following minimal traumatic brain injury. The rationale for our focus on the IGF-1R pathway was its confirmed pro-survival and anti-apoptotic activities, and the fact that the IGF-1R axis is involved in a number of neuronal processes.

mTBI-induced IGF-1R phosphorylation

Initially we examined the effect of mTBI on IGF-1R phosphorylation. For this purpose, mice were subjected to mTBI, and brains were removed after 1, 24 and 72 h. Hippocampi extracts prepared from brain tissues were subjected to immunoblot analyses. Results indicated that mTBI induced a significant elevation in IGF-1R phosphorylation 24 h post mTBI (1.87 ± 0.18 fold over control for the right, injured hippocampus; $n = 5–7$, $p<0.0001$; Fig. 1A). Similar changes were found in the left hippocampus (data not shown).

mTBI-induced Akt phosphorylation

Since the PI3-kinase/AKT is one of the main downstream pathways activated by the IGF-1R, we next tested whether Akt was also activated in response to mTBI. As shown in Fig. 2A, mTBI induced a significant phosphorylation of hippocampal Akt in injured mice as compared to control mice. However, while the major phosphorylation of the IGF-1R was found 24 h post injury, significant Akt phosphorylation was found already after 1 h, and remained constant up to 72 h

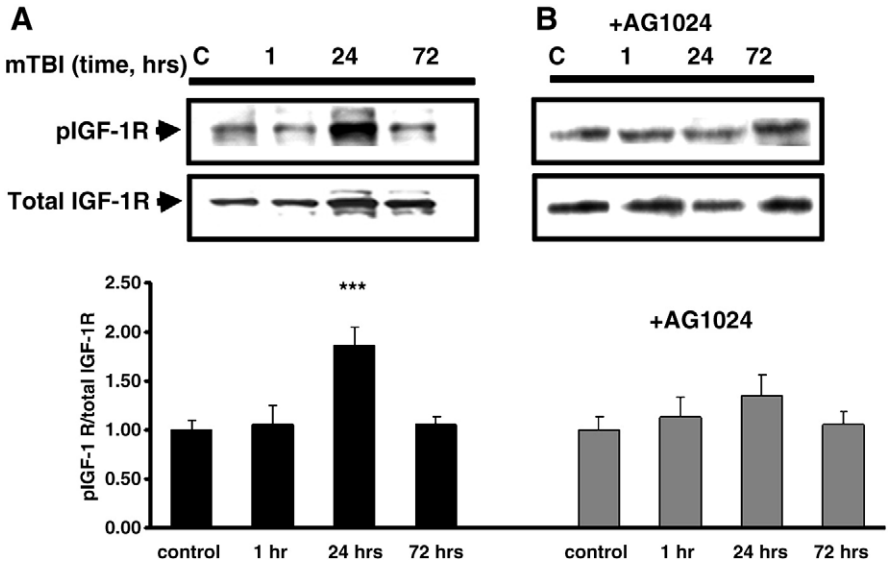

Fig. 1. IGF-1R phosphorylation is significantly elevated after mTBI. (A) Hippocampus protein extracts prepared from sham or mTBI-animals at the indicated time points were subjected to gel electrophoresis, followed by immunoblot analysis using antibodies against pIGF-1R and IGF-1R. The level of phosphorylation was evaluated by densitometry analysis. The control values were set as 1, and the relative values of respective treatment were calculated accordingly. Results indicate that mTBI induced a significant elevation in IGF-1R phosphorylation 24 h post mTBI (1.87 ± 0.18 fold over control for the injured hippocampus; $n = 5–7$, $p < 0.0001$; LSD *post hoc* test). One way ANOVA revealed a significant effect of group: [$F(9,41) = 5.78$; $p < 0.0001$). The selective IGF-1R inhibitor AG1024 abolished the mTBI-induced phosphorylation (B).

post mTBI (2.05 ± 0.13; 1.87 ± 0.21 and 2.06 ± 0.1 fold over control at 1, 24, and 72 h post mTBI, respectively; $n = 5–7$, $p < 0.0001$; Fig. 2A).

mTBI-induced ERK1/2 phosphorylation

The IGF-1R is also known to phosphorylate the ERK1/2 pathway. Similar to the mTBI effect on AKT, maximal effect of mTBI on ERK1/2 phosphorylation was observed 1 h post injury (2.3 ± 0.1 fold over control; $n = 5–7$, $p < 0.001$). This elevation declined by 24 h (Fig. 3A).

The effect of the selective IGF-1R inhibitor, AG1024, on mTBI-induced AKT and ERK activation

Since the time scale of AKT and ERK activation was different from that of IGF-1R phosphorylation, our next goal was to elucidate whether the injury-induced activation of AKT and ERK was mediated via the IGF-1R. Mice were injected i.p. with 50 μg/kg AG1024 1 h before the injury and every 24 h for 2 consecutive days. Indeed, mTBI failed to induce IGF-1R phosphorylation in AG1024-treated mice (Fig. 1B).

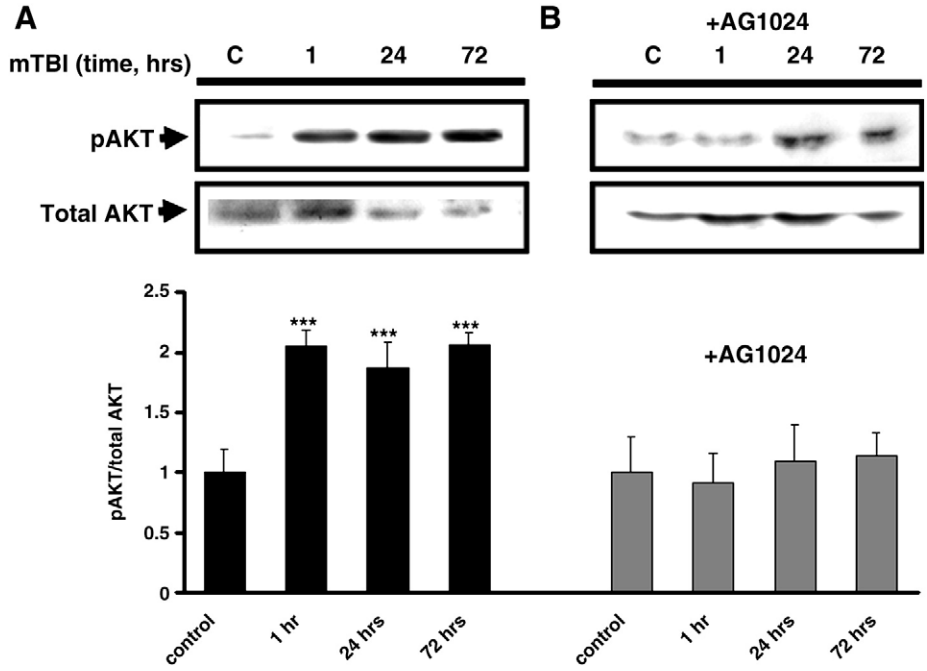

Fig. 2. mTBI induced a significant enhancement of AKT phosphorylation. (A) The selective IGF-1R inhibitor AG1024 significantly reduced the mTBI-induced phosphorylation (B). Hippocampus protein extracts prepared from sham or mTBI-animals at the indicated time points were subjected to gel electrophoresis followed by immunoblot analysis using antibodies against pAKT and AKT. The level of phosphorylation was evaluated by densitometry analysis. The control values were set as 1, and the relative values of respective treatments were calculated accordingly. Results are mean ± SEM of 5–7 animals. mTBI induced a significant phosphorylation of hippocampus-AKT. This effect was found already after 1 h, and remained constant up to 72 h post mTBI. One way ANOVA revealed a significant effect of group: [$F(9,38) = 7.04$; $p < 0.0001$; LSD *post hoc* test). The selective IGF-1R inhibitor, AG1024, abolished this mTBI-induced phosphorylation (B).

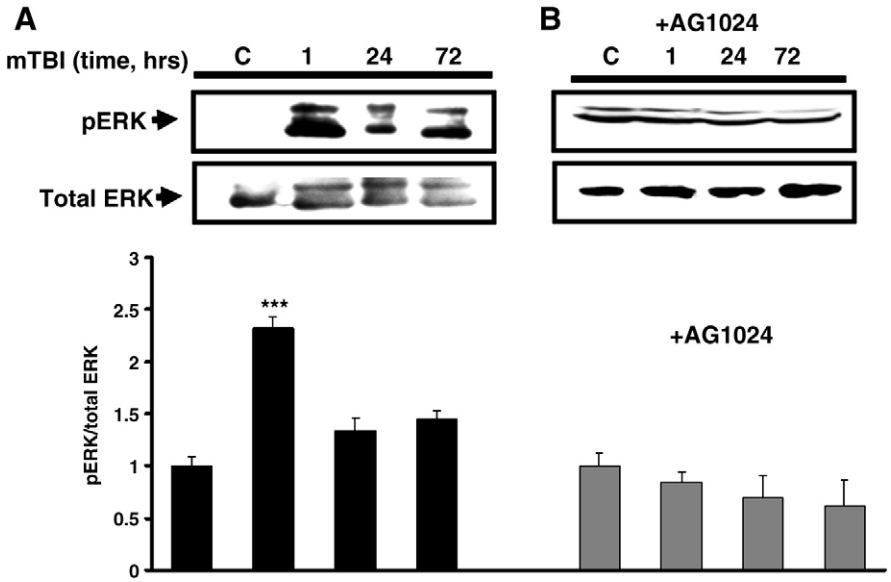

Fig. 3. mTBI induced a significant elevation of ERK1/2 phosphorylation. (A) The selective IGF-1R inhibitor, AG1024, significantly reduced this mTBI-induced phosphorylation (B). Hippocampus protein extracts prepared from sham or mTBI-animals at the indicated time points were subjected to gel electrophoresis followed by immunoblot analysis using antibodies against pERK1/2 and total ERK1/2. The level of phosphorylation was evaluated by densitometry analysis. The control values were set as 1, and the relative values of respective treatment were calculated accordingly. A significant effect of mTBI on ERK1/2 phosphorylation was found 24 h post injury. One way ANOVA revealed a significant effect of group: [$F(10,37) = 11.3$; $p<0.0001$; LSD *post hoc* test). Results are mean ± SEM of 5–7 animals.

Furthermore, mTBI-induced AKT and ERK phosphorylation were almost abolished (Figs. 2B and 3B). These results suggest that mTBI-induced AKT and ERK activation via IGF-1R phosphorylation.

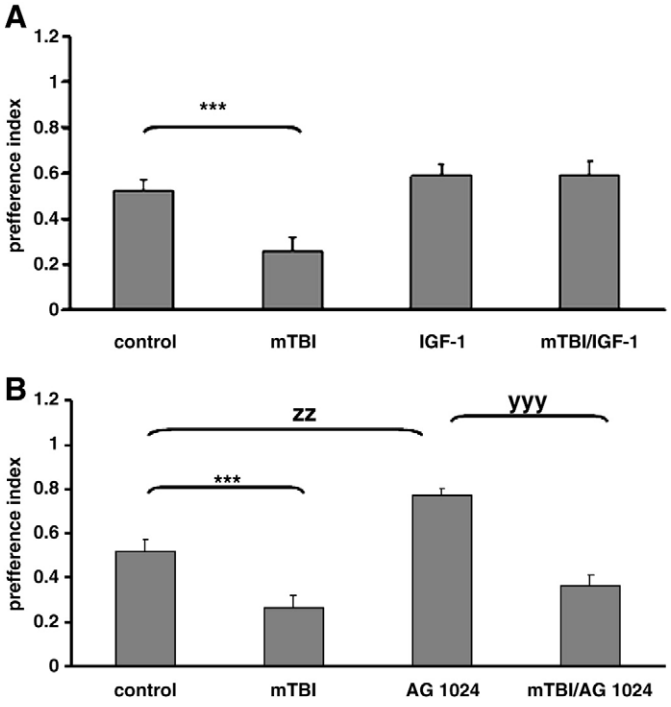

Fig. 4. The effect of IGF-1R signaling pathway on mTBI-induced damage to spatial memory. Spatial memory was assessed in the Y-maze by calculating the relative time that the mice spent in a novel arm compared to an old, familiar arm ("preference index", see Materials and methods). One way ANOVA revealed a significant effect of group: [$F(7,77) = 100.3$; $p<0.0001$; LSD *post hoc* test). (A) The significant decrease of preference index in mTBI mice was completely abolished by the administration of IGF-1 (4 μg/kg, i.p.). (B) Naïve mice that were treated with the selective IGF-1R inhibitor AG1024 (50 μg/kg, i.p.) had a significant better memory than control mice. mTBI-induced damage, however, was not altered by AG1024 pretreatment.

The effect of IGF-1 signaling on mTBI-induced damage to spatial memory performance

The next set of experiments was aimed at evaluating the role of the IGF-1R signaling pathway on the cognitive outcome after brain injury. In order to test the spatial memory we used the Y-maze test (see Materials and methods). Seven days post mTBI mice had a much lower preference to the new arm in the maze compared to control mice (0.26 ± 0.06 and 0.51 ± 0.05 respectively, $p<0.05$) indicating an impaired spatial memory (Fig. 4). IGF-1 administration (4 μg/kg, i.p. injection) significantly abolished this deficit (0.65 ± 0.06) (Fig. 4A). Surprisingly, IGF-1R blockade significantly improved the performance of the uninjured mice: AG1024-treated mice had a much better performance compared to control mice (0.7 ± 0.1 and 0.47 ± 0.08, respectively; $n = 5–7$; $p<0.05$). AG1024 had no effect on the spatial memory performance in mTBI mice (0.36 for AG1024-mTBI mice and 0.27 for mTBI mice) (Fig. 4B).

Discussion

IGF-1 is regarded as a neurotrophic factor due to its pro-survival and anti-apoptotic properties (Vincent and Feldman, 2002). The IGF system of ligands, receptors and binding proteins plays an important role in somatic growth by promoting cellular proliferation and differentiation. In addition, signaling through the IGF system plays a major role in cell survival and prevention of programmed cell death. Ligand-induced activation of the IGF-IR is a particularly important survival-promoting signal during development. The best defined pathways by which IGF-IR signaling can prevent apoptosis are mediated by PI3K (Povsic et al., 2003). Hence, IGF-1 has been suggested for use as a neuroprotective drug in hypoxia–ischemia brain damage models (Guan et al., 2003); its main target molecules are the serine–threonine kinases PKB/AKT and ERK1/2, both of which were reported to be involved in the molecular events following TBI (Noshita et al., 2002; Zhang et al., 2006). Hence, the main goal of the present study was to investigate the potential involvement of the IGF-1 pathway in the biochemical as well as behavioral events following mTBI. Little is known about the activation and involvement of the IGF-1R signaling axis following TBI. In the present study we provide evidence showing that IGF-1R was phosphorylated following mTBI as

assessed by immunoblot analysis (Fig. 1). IGF-1R activation was time-dependent (maximum at 24 h) with no difference between the two hemispheres. These results conclude that activation of the IGF-1R anti-apoptotic pathway by mild traumatic brain injury could be part of a neuroprotective and neurogenic process aimed at maintaining normal brain function following a relatively stressful event.

In addition to IGF-1R activation, its target molecules AKT and ERK1/2 were also phosphorylated following mTBI (Fig. 2). Since the phosphorylation of these proteins was elevated at earlier time points compared to that of the IGF-1R (1 h and 24 h, respectively) one could suggest that AKT and ERK were phosphorylated by a different activator. However, blocking IGF-1R activation with the selective inhibitor AG1024 not only blocked the mTBI-induced IGF-1R phosphorylation (Fig. 1), but also inhibited the mTBI-induced phosphorylation of AKT and ERK (Fig. 2), suggesting that the IGF-1R is responsible (at least partially) for the phosphorylation of AKT and ERK following mild brain injury.

The outcome signs of TBI include headaches, dizziness, fatigue, irritability, memory problems, concentration problems and emotional lability (Kibby and Long, 1996; Levin et al., 1987). The clinical signs and symptoms presented by patients with mTBI include memory disorders and affective lability (Finset et al., 1999; Hamm et al., 1993; Margulies, 2000). We have previously reported that mTBI exerted a number of cognitive and emotional short- and long-term deficits which were characterized in a number of behavioral and cognitive tests (Milman et al., 2005; Zohar et al., 2003). In these studies we showed that this mTBI model induced impaired spatial memory and learning as assessed by the Morris Water Maze, the Dry-Maze and the Y-maze (Baratz et al., in press; Edut et al., 2008; Zohar et al., 2003) Hence, since IGF-1 was found to be protective in other brain damage models (Guan et al., 2003; Lin et al., 2009), and since in the present study we found that the IGF-1R was phosphorylated following mTBI, the next set of experiments was aimed at evaluating the role of IGF-1R signaling in the spatial memory deficit following mTBI. As expected, exogenous IGF-1 administration 24 and 48 h post mTBI completely abolished the injury-induced spatial memory deficit (Fig. 4A). If, indeed, IGF-1R is activated post mTBI as a neuroprotective mechanism, blocking its activity, prior, during, or after the TBI, should amplify the mTBI-induced damage to memory. Surprisingly, spatial memory performance in AG1024/mTBI treated mice (as assessed by the Y-maze test) was not altered compared to mTBI mice (Fig. 4B). Our results could imply that mTBI activates additional neuroprotective mechanisms, and blocking IGF-1R signaling might enhance another compensatory pathway, for example the insulin receptor signaling. Insulin is widely reported to be neuroprotective and act via Akt (similar to IGF-1R). Both IGF-1 and AG1024 also bind the insulin receptor (although with significantly lower IC50s) due to the 60% homology of these two receptors. The fact that AG1024 treated mice (not injured) had a significantly better spatial memory compared to control mice (Fig. 4B), and studies reporting paradoxical effect of IGF-1R pathway in other models (Carter et al., 2002; Tang, 2006), raises questions regarding its neuroprotectivity as the only effect underlying its mechanism of action (similar results have been obtained in a visual memory test, the novel object recognition; data not shown). Thus, it is possible that the nature of the intrinsic mTBI-induced activation of the IGF-1R pathway is different from the one activated by the exogenous administration of IGF-1. Future studies will further address the physiological role of the IGF1-R in traumatic brain injury and other pathological conditions.

Acknowledgments

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