ORIGINAL ARTICLES

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The role of 8-OH-DPAT on the rat neuronal apoptosis after diffuse brain injury coupled with secondary brain injury

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Received August 28, 2014, accepted October 27, 2014

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Pharmazie 70: 251–255 (2015) *doi: 10.1691/ph.2015.4768*

The potential role of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) on rat neuronal apoptosis after diffuse brain injury (DBI) coupled with secondary brain injury (SBI) was investigated. One hundred and twelve adult male Wister rats weighing 305-355 g were randomly divided into four groups and received an intraperitoneal injection of 8-OH-DPAT (0.5 mg/kg) or an equal volume of normal saline. Neurological severity score (NSS) was recorded and the injured extent was observed after hematoxylin-eosin (HE) staining. The neuronal cell apoptosis index and the expression of Bax and Bcl-2 were detected by TUNEL method and immunohistochemistry respectively. We found a higher NSS value for rats in the DBI + SBI groups compared with those in normal control and sham-operated control groups ($P < 0.01$). HE staining showed that 8-OH-DPAT treatment could alleviate the occurrence of injury in rats CA3 hippocampus and PFC. The neuronal apoptosis index decreased in the 8-OH-DPAT treatment group compared with the NS group ($P < 0.05$) and gradually increased at 6 h, reached the peak level at 72 h and still had a high performance at 168 h in not only CA3 hippocampus but also PFC. Expression of Bax and Bcl-2 increased after DBI + SBI, however, with 8-OH-DPAT treatment Bcl-2 expression increased while Bax expression decreased. 8-OH-DPAT had an inhibitory effect on the rat neuronal apoptosis in CA3 hippocampus and PFC after DBI coupled with SBI.

1. Introduction

Diffuse brain injury (DBI) is a consequence of traumatic brain injury (TBI) typically induced by a rapid acceleration or deceleration of the head (Huisman et al. 2003). DBI could cause direct neuronal somatic injury and neurodegeneration in the somatosensory cortex (Singleton and Povlishock 2004; Lifshitz and Lisembee 2012). Secondary brain injury (SBI) is another important cause of damage to the brain. After severe DBI, medical and surgical therapies are performed to minimize SBI. Increased intracranial pressure, which is typically caused by cerebral edema is an important secondary insult (Bullock et al. 2006).

5-Hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptors are abundantly expressed in brain regions such as the prefrontal cortex (PFC) and hippocampal CA3 area that are susceptible to neuronal damage induced by TBI (McAllister-Williams et al. 2014; Limón-Morales et al. 2014; Watry and Lu 2013). Our previous studies have shown that the serotonin 5-HT$_{1A}$ receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) could reduce the cerebral temperature of TBI in rats and inhibit neuronal apoptosis (Mao et al. 2013). Recent reports show that the 5-HT$_{1A}$ receptor agonist has the ability to confer neuroprotection and improve the neurobehavioral performance in a variety of brain injury models and could promote sport, cognitive function recovery and reduce the brain pathological performance (Mao et al. 2013; Cheng et al. 2008; Kline et al. 2010). However, the knowledge about molecular mechanisms of 8-OH-DPAT on neuronal apoptosis after brain injury is not sufficient, and the impact of 8-OH-DPAT on DBI coupled with SBI has been rarely reported.

In order to clarify the role of 8-OH-DPAT on neuronal apoptosis in CA3 hippocampus and PFC of rats, we detected the development of neuronal apoptosis and expression of Bcl-2 and Bax in adult rats after the closed head with DBI and SBI. The findings could identify potential therapeutic targets for anti-apoptotic and neuroprotective therapy for DBI coupled with SBI.

2. Investigations and results

2.1. Neurological severity scoring

Basically, there was no difference between normal control and sham-operated control groups ($P > 0.05$); therefore they were combined into one group (designated as control group). As shown in Fig. 1, there were significant differences between control and experimental brain injured groups ($P < 0.01$). Besides, after treatment with 8-OH-DPAT, the NSS value was significantly decreased ($P < 0.05$).

2.2. HE staining

HE staining showed that control groups had no damage performance while injured groups had clear tissue edema, capillary

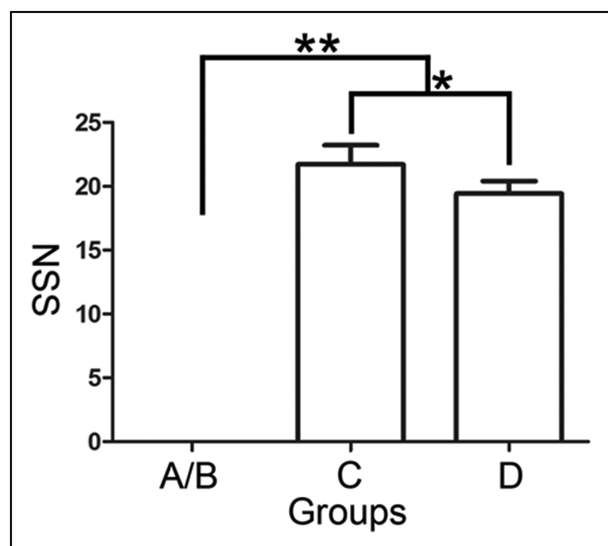

Fig. 1: Rat neurological severity score in different groups. A, control group; B, sham-operated group; C, DBI + SBI + NS group; D, DBI + SBI + 8-OH-DPAT group. A/B: A or B group. The bar graph shows that there were significant differences in the NSS of rats among normal, sham-operated control and experimental brain injured groups ($P < 0.01$). C *vs.* D, $^{*}P < 0.05$; C or D *vs.* A/B, $^{**}P < 0.01$.

congestion, neuronal edema as well as disorganization and decrease in quantity. Compared with DBI + SBI + NS group, the injured extent was alleviated in 8-OH-DPAT treatment group (Fig. 2).

2.3. *Cell apoptosis in CA3 hippocampus and PFC*

The analysis of cell apoptosis revealed that the control group had fewer apoptotic cells in hippoeampus CA3 area and PFC, with the apoptotic index of 1.94 ± 0.44% and 1.36 ± 0.14%, respectively. The apoptotic cells index in brain injured group gradually increased from 6 h, reached the peak level at 72 h and still had a high performance at 168 h (Table 1). At 72 h the experimental brain injured group led to a significant increase in cell apoptosis (33.41 ± 3.47 % in CA3 hippocampus and 75.57 ± 8.54 % in PFC) and the apoptosis index decreased in the 8-OH-DPAT treatment group compared with the NS group (8-OH-DPAT treatment *vs*. control: 25.34 ± 3.01 % *vs*. 1.92 ± 0.73 % in CA3 hippocampus and 66.85 ± 6.57 % *vs*. 1.98 ± 4.27 % in PFC, $P < 0.05$).

2.4. *Immunohistochemistry assay for expression of Bax and Bcl-2*

Immunohistochemistry staining of Bax and Bcl-2 revealed that the brown granules were clearly visible, particularly gathering in the cytoplasm of nuclear membrane surrounding, which demonstrated the expression of Bax and Bcl-2 in CA3 hippocampus and PFC. Besides, with 8-OH-DPAT treatment, the brown granules decreased compared with the experimental group. As no brown granule in the cytoplasm was observed, the immunohistochemical staining of the control groups was not given (Fig. 3). In CA3 hippocampus, the expression levels of Bax and Bcl-2 in normal control groups were very low (5.44 ± 0.90 and 3.59 ± 0.91 respectively) while in injured groups they were significantly increased at all indicated time compared to sham-operated groups (for example: at 72 h, injured *vs*. sham-operated: 50.46 ± 4.13 % *vs*. 8.12 ± 0.85 % and 14.66 ± 1.54 % *vs*. 6.09 ± 0.54, $P < 0.01$). The expression levels reached a peak at 72 h and decreased after 168 h. Besides, Bax expression in 8-OH-DPAT treatment group was lower than DBI + SBI + NS group ($P < 0.05$ or $P < 0.01$), while Bcl-2 expression in 8-OH-DPAT treatment group was higher than DBI + SBI + NS group ($P < 0.05$ or $P < 0.01$). In short, 8-OH-DPAT could increase Bcl-2 expression while decrease Bax expression (Table 2). The expression variation of Bax and Bcl-2 in PFC was similar to CA3 hippocampus (Table 3).

3. Discussion

Cell apoptosis is documented to occur following TBI in both humans and animals (Raghupathi 2004). Studies have found that the occurrence of neuronal apoptosis is associated with the intracellular Ca^{2+} (zipfel et al. 2000) and may contribute to secondary brain damage after DBI (Marmarou et al. 1994). The TUNEL technique is, a powerful method for rapid detection and quantitative analysis of apoptosis with high sensitivity and specificity (Kelly et al. 2003). In this study, the TUNEL technique was used for quantitative evaluation of neuronal apoptosis in the CA3 hippocampus and PFC of rats after DBI coupled with SBI. Consistent with previous studies, our findings manifested that neuronal apoptosis occurred after experimental barin injury (Chen et al. 2013; Palzur et al. 2004).

8-OH-DPAT, a serotonin 5-HT_{1A} receptor agonist, has been demonstrated to have neuroprotective activity against apoptosis induced by glutamate and serum deprivation as well as traumatic brain injury in rats (Kline et al. 2002; Mauler et al. 2001). The neuroprotective effect of 8-OH-DPAT is associated with an increase in H_2O_2-induced Ca^{2+} concentration (Lee et al. 2005), G-protein-induced opening k^+ channels (Penington et al. 1991) and blocking voltage-sensitive Na^+ channels (Melena et al. 2000). In this study, we found that, under 8-OH-DPAT treatment neuron cells apoptosis and the apoptotic index decreased in CA3 hippocampus and PFC after brain injury. While it seems clear that 8-OH-DPAT can play an important role in neuroprotection against apoptosis through affecting synaptic physiology, the signaling mechanisms mediating the effect of 8-OH-DPAT on Bax

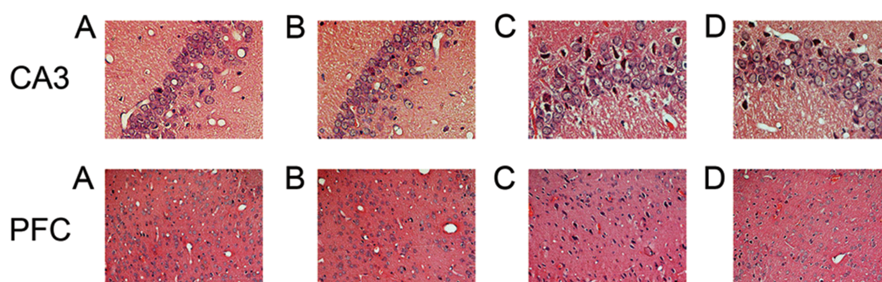

Fig. 2: The injured extent of 72 h subgroups in 4 groups of CA3 hippocampus and PFC (Hematoxylin-Eosin staining, × 400). A, control group; B, sham-operated group; C, DBI + SBI + NS group; D, DBI + SBI + 8-OH-DPAT group.

Table 1: Apoptotic index in CA3 hippocampus and PFC

Groups	6 h	12 h	24 h	72 h	168 h
CA3 hippocampus (%)					
B	1.43 ± 0.40	1.52 ± 0.51	2.04 ± 0.72	1.92 ± 0.73	1.33 ± 0.46
C	5.21 ± 1.42[a]	7.92 ± 1.81[a]	19.42 ± 2.95[a]	33.41 ± 3.47[a]	23.36 ± 2.69[a]
D	4.76 ± 1.10[a]	5.62 ± 1.14[ab]	13.50 ± 1.75[ac]	25.34 ± 3.01[ac]	16.72 ± 2.15[ac]
PFC (%)					
B	1.64 ± 0.62	2.15 ± 1.28	2. 83 ± 2.16	1.98 ± 4.27	1.59 ± 2.32
C	21.84 ± 3.48[a]	34.78 ± 5.07[a]	51.3 ± 5.96[a]	75.57 ± 8.54[a]	41.02 ± 5.28[a]
D	13.87 ± 2.56[ab]	24.64 ± 3.09[ab]	35.38 ± 4.01[ab]	66.85 ± 6.57[ab]	31.91 ± 4.57[ab]

B, sham-operated control group; C, DBI + SBI + NS group; D, DBI + SBI + 8-OH-DPAT group. a, compared with normal control group, $P < 0.01$; b, compared with sham-operated control group, $P < 0.05$; c, compared with DBI + SBI + NS group, $^{c}P < 0.01$.

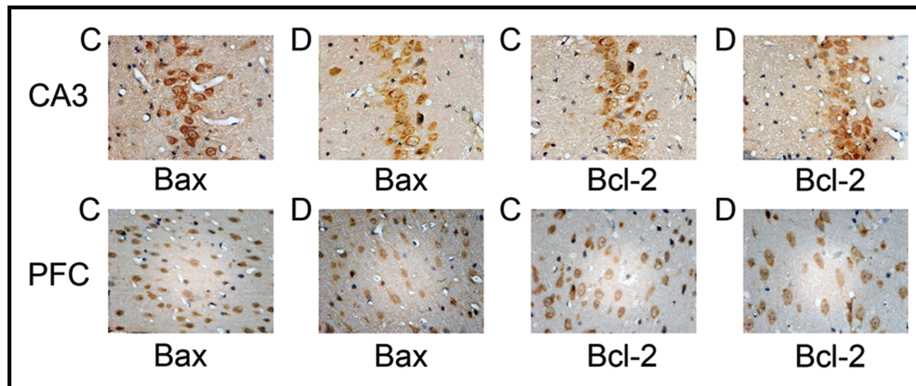

Fig. 3: Immunohistochemistry analysis of Bax (72 h subgroup) and Bcl-2 (24 h subgroup) in CA3 hippocampus and PFC Of rat in DBI + SBI + NS group (C) and DBI + SBI + 8-OH-DPAT group (D). The brown granules are clearly visible particularly gathering in the cytoplasm of nuclear membrane surrounding. Original magnifications: × 400.

Table 2: Expression of Bax and Bcl-2 in each injured group at different time in CA3 hippocampus

Groups	6 h	12 h	24 h	72 h	168 h
Bax					
B	5.94 ± 0.85	6.27 ± 0.91	7.88 ± 0.76	8.12 ± 0.85	6.95 ± 0.64
C	13.32 ± 1.51[a]	25.48 ± 2.31[a]	46.17 ± 3.61[a]	50.46 ± 4.13[a]	33.31 ± 3.19[a]
D	12.61 ± 1.53[ab]	20.08 ± 1.81[ac]	41.29 ± 2.14[ac]	44.39 ± 3.57[ac]	28.45 ± 3.14[ac]
Bcl-2					
B	4.81 ± 0.98	5.22 ± 0.75	6.71 ± 0.81	6.09 ± 0.54	5.91 ± 0.28
C	5.28 ± 0.73[a]	9.45 ± 1.08[a]	17.70 ± 1.57[a]	14.66 ± 1.54[a]	11.26 ± 1.26[a]
D	6.83 ± 1.16[ab]	11.44 ± 1.21[ac]	23.54 ± 1.88[ac]	18.63 ± 1.55[ac]	14.62 ± 1.73[ac]

B, sham-operated control group; C, DBI + SBI + NS group; D, DBI + SBI + 8-OH-DPAT group. a, compared with normal control group, $^{a}P < 0.01$; b, compared with sham-operated control group, $^{b}P < 0.05$; c, compared with DBI + SBI + NS group, $^{c}P < 0.01$.

Table 3: Expression of Bax and Bcl-2 in each group at different time in PFC

groups	6 h	12 h	24 h	72 h	168 h
Bax					
B	8.426 ± 1.162	9.092 ± 1.662	8.738 ± 1.146	9.618 ± 1.360	8.635 ± 0.886
C	28.618 ± 2.679[a]	44.603 ± 3.800[a]	55.980 ± 4.096[a]	79.007 ± 6.810[a]	48.258 ± 3.758[a]
D	26.147 ± 2,248[ab]	39.675 ± 2.539[ab]	49.538 ± 4.394[ab]	68.299 ± 4.801[ac]	42.284 ± 3.015[ac]
Bcl-2					
B	6.896 ± 1.213	7.683 ± 1.170	6.423 ± 1.154	7.287 ± 1.224	6.806 ± 1.006
C	16.576 ± 1.462[a]	25.616 ± 2.158[a]	34.876 ± 2.614[a]	24.293 ± 2.498[a]	17.504 ± 1.327[a]
D	21.128 ± 1.829[ac]	33.534 ± 3.301[ac]	42.128 ± 3.838[ac]	27.830 ± 2.520[ab]	22.277 ± 2.699[ac]

B, sham-operated control group; C, DBI + SBI + NS group; D, DBI + SBI + 8-OH-DPAT group. a, compared with normal control group, $^{a}P < 0.01$; b, compared with sham-operated control group, $^{b}P < 0.05$; c, compared with DBI + SBI + NS group, $^{c}P < 0.01$.

and Bcl-2 of neuronal apoptosis are not completely understood and remain to be further elucidated.

Bax, a pro-apoptotic protein, usually presents with elevated levels during apoptosis (Gross et al. 1998). Recent research has found that the mechanism of Bax in inducing apoptosis is associated with its crystal structures, which reveal the process of BH3 domains activate Bax and nucleate its oligomerization thereby induce apoptosis (Czabotar et al. 2013). In neonatal hypoxic-

ischemic brain injury, down-regulated Bax could reduce cell apoptosis and protect neurons (Kumral et al. 2006). Bcl-2 inhibits apoptosis and plays an important role in neuroprotection after TBI. Its inhibition is performed by preventing productive oligomerization of Bax needed to become active (Dlugosz et al. 2006). In mouse brain, down-regulation of Bcl-2 could promote cell survival and up-regulation of Bax could promote apoptotic cell death (Gillardon et al. 1995). Recent research documented expression of Bcl-2 and Bax as well as occurrence of apoptosis in rat brains after experimental DBI (Chen et al. 2013). However, there were no more studies in depth on the roles of these proteins in apoptosis after SBI. Here, we studied expression of Bcl-2 and Bax in CA3 hippocampus and PFC of rat brain at different time points after DBI coupled with SBI by immunohistochemistry techniques. Along with quantitative analysis of neural cell apoptosis, interactions of 8-OH-DPAT in regulation of apoptosis were also examined. Our results revealed that after treatment with 8-OH-DPAT, the expression of Bcl-2 increased while expression of Bax decreased in both CA3 hippocampus and PFC after brain injury.

In summary, we confirmed that 8-OH-DPAT could inhibit neuronal cells apoptosis in CA3 hippocampus and PFC of rats after DBI coupled with SBI, which provides a breades experimental basis for clinical drug development. However, there are still some shortcomings in the study, such as the administration mode, time and dosages as well as its difficulty of giving medication immediately after 15 min of TBI in clinical practice. In addition, the inhibition of 8-OH-DPAT for apoptosis is multifaceted and further exploration is needed.

4. Experimental

All human studies have been approved by the General Hospital of Shenyang Military Region Ethics Committee and performed in accordance with their ethical standards.

4.1. Subjects and surgery

One hundred and twelve adult healthy male Wister rats weighing 330 ± 25 g were housed in standard steel-wire mesh cages and maintained at a temperature of $21 \pm 1\,^{\circ}$C and light-controlled (7:00 am to 7:00 pm) environment with free access to food and water. After one week of acclimatization, the rats were randomly divided into the following 4 groups: normal control (n = 7), sham-operated control (n = 35), DBI + SBI + Normal saline (NS, n = 35) and DBI + SBI + 8-OH-DPAT (n = 35). Except for the normal control group, other groups were further subdivided into subgroups, each comprising 7 animals. The subgroups were differentiated according to the studied time points (i.e., 6, 12, 24, 72 and 168 h) after experimental brain injury.

The widely used diffuse brain injury model reported by Marmarou et al. (1994) was applied in the current study. After DBI, rats were stabilized for 15 min and then their bilateral common carotid artery was ligated for 30 min to construct the secondary ischemic brain damage model. Sham-operated control rats underwent all surgical manipulations the same as brain injured groups with the exception of the impact and bilateral common carotid artery ligation while normal control rats without performing any surgery. 8-OH-DPAT which was purchased from Sigma-Aldrich (Sigma-Aldrich, Inc., St. Louis, MO) and prepared daily by dissolving in sterile physiological saline was administered intraperitoneally at 0.5 mg/kg 15 min after DBI. As a control, DBI + SBI + NS group received an intraperitoneal injection of an equal volume of normal saline while the normal control and sham-operated control groups remained without any injection. At the indicated time, the brain was collected by a conventional method. Paraffin-embedding and sectioning were performed subsequently.

4.2. Outcome measures

The rats in each 24 h subgroups were subjected to NSS assay according to previous studies (Björkman et al. 2010; Zakzanis et al. 2013; Sears et al. 2014). At 6, 12, 24, 72 and 168 h after DBI + SBI, the rats were anesthetized with an intraperitoneal injection of 10 % chloral hydrate (2 ml/kg) and perfused with 250 ml of ice-cold heparinized saline through the aorta at a flow rate of 80-100 ml/min. After decapitation, 2 mg of brain tissues from the right side of CA3 hippocampus and PFC were collected and resuspended in phosphate buffered saline (PBS; pH 7.4). Following that, 4 slices were extracted randomly in CA3 hippocampus and prefrontal cortex (PFC) and 5 random fields of each slice were observed under magnification of 400 times. The slices was fixed in neutral buffered 10% formalin, embeded in paraffin and cut into 6 μm, which were stained by hematoxylin-eosin (HE) staining and mounted. HE staining of CA3 hippocampus and PFC was used to observe the injured extent.

Cell apoptosis was detected by the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) method (Negoescu et al. 1996) and the apoptotic index was calculated as apoptotic cells/total cells × 100%. Brain tissue samples were collected and eight of them were used to analyze expression of Bcl-2 and Bax while the remaining four samples served as negative controls. Samples were stained with rat anti-serum polyclonal primary antibodies and rabbit polyclonal antibodies against rat Bcl-2 or Bax (Santa Cruz Biotechnology, Santa Cruz, CA), followed by staining with the avidin-biotin-peroxidase staining kit (Wuhan Boster Bio-Engineering Ltd., Wuhan, China). The expression of Bax at 72 h subgroup and Bcl-2 at 24 h subgroup in CA3 hippocampus and PFC in experimental injured group was randomly selected and detected by immunohistochemical staining (magnification of 400 times). Integral optical density (IOD) values of immunohistochemistry stain were evaluated using Image-Pro Plus 6.0 Software (Media Cybernetics, CA, USA).

4.3. Statistical analysis

All statistical analyses were performed using linear polynomial ANOVA with LSD post hoc examination by SPSS 18.0 software (SPSS, Chicago, IL, USA). P value < 0.05 was considered statistically significant.

Acknowledgements: The research was funded by Key Science and Technology Program of Liaoning Province (2009225018).

Conflict of Interest: The authors declare that they have no conflict of interest.

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