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Full Length Article

Oxidative stress with tau hyperphosphorylation in memory impaired 1,2-diacetylbenzene-treated mice

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ABSTRACT

Long-term exposure to organic solvent may be related to the incidence of neuronal diseases, such as, Alzheimer's disease, depression, multiple sclerosis, dementia, Parkinson's disease. Previously, the authors reported 1,2-diacetylbenzene (DAB; a neurotoxic metabolite of 1,2-diethylbenzene) causes central and peripheral neuropathies that lead to motor neuronal deficits. Furthermore, it is known DAB increases oxidative stress and protein adduct levels and impairs hippocampal neurogenesis in mice. The authors examined the relevance of oxidative stress and tau hyperphosphorylation in the hippocampus. Five-week-old male C57BL/6 mice were treated with 1 or 5 mg/kg/day DAB for 2 weeks. Neither overall body weight increases nor behavioral differences were observed after treatment, but kidney and liver weights decreased. Increased ROS production, activated glycogen synthase kinase-3β (GSK-3β) and tau hyperphosphorylation were observed in hippocampal homogenates. To assess memory impairment, the Morris Water Maze was used. Animals in the DAB-treated groups took longer to reach the platform. Movement patterns of DAB treated mice were more complicated and their swimming speeds were lower than those of controls. When SHSY5Y neuroblastoma cells were pretreated with NAC (an antioxidant) or a GSK-3β inhibitor, the expression of active GSK-3β and tau hyperphosphorylation were reduced. These results suggest ROS produced by DAB causes tau hyperphosphorylation via GSK-3β phosphorylation and it might be related to impaired memory deficit.

1. Introduction

Organic solvents are widely used in paints, pharmaceuticals, adhesives, printing inks, pesticides, cosmetics, household cleaners, gasoline, and other products. Exposure to organic solvents by inhalation may adversely affect the central nervous system and cause headaches, dizziness, light-headedness, unconsciousness, seizures, or even death (Dick, 2006). Furthermore, exposure to organic solvents, acutely or chronically, can impair cognitive abilities (Li et al., 2002), and chronic organic solvent exposure is a risk factor of Parkinson's disease (PD) and of Alzheimer's disease (AD) (Smargiassi et al., 1998; McDonnell et al., 2003; Lock et al., 2013; Jiang et al., 2013).

1,2-Diacetylbenezene (DAB) is a neurotoxic metabolite of 1,2-diethylbenzene (DEB) which is a colorless liquid and a minor component of gasoline and fuel oils (Kim et al., 2011). DEB is absorbed dermally or by inhalation and is metabolized in liver to DAB (Payan et al., 1999; Gagnaire and Boucard, 2014). DAB is a chromophore that permeates the blood–brain barrier, reacts with amino acids or proteins in the body, and forms polymers (Kim et al., 2001), and in rodents, has been reported to produce oxidative stress, activate microglia, and impair hippocampal neurogenesis (Kim et al., 2011).

Oxidative stress plays an important role in AD and in PD (Gaki and Papavassiliou, 2014; Garcia-Blanco et al., 2017) and contributes to the development of tauopathy (Yu et al., 2017), which is a pathological condition characterized by abnormal aggregation of hyperphosphorylated tau protein in the form of paired helical filaments (PHFs) or neurofibrillary tangles (NFTs) in AD (Khatoon et al., 1994). Tau is a microtubule-associated protein and stabilizes microtubules by forming assemblies of tubulin subunits. Tau is a phosphoprotein and its degree of phosphorylation regulates microtubule polymerizing activity (Avila et al., 2004). Furthermore, hyperphosphorylated tau dissociates from microtubules and this results in abnormal accumulation of tau protein, neuronal dysfunction and death (Stoothoff and Johnson, 2005).

Glycogen synthase kinase-3 (GSK-3) exists as two isoforms GSK-3α and β. GSK-3β, also called tau protein kinase I, is related to neuronal survival and apoptosis, and plays roles in AD, inflammation, diabetes, PD, and cancer (Jacobs et al., 2012). In particular, GSK-3β plays an important role in oxidative stress-induced cognitive deficit (Wang and

Abbreviations: AD, Alzheimer's Disease; DAB, 1,2-diacetylbenzene; GSK-3β, glycogen synthase kinase-3β; NFTs, neuro fibrillary tangles; PHF, paired helical filament; ROS, reactive oxygen species

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Zhao, 2016). Phosphorylation of tyrosine-216 (Tyr216, active form) of GSK-3β increases its enzymatic activity, whereas phosphorylation of serine-9 (Ser9, inactive form) decreases its activity (Noel et al., 2016).

We hypothesized that ROS elevation by DAB induces tau hyperphosphorylation via GSK-3β activation. This might give us a clue for the mechanism of DAB associated impaired hippocampal neurogenesis and memory deficit.

2. Methods & materials

2.1. Cell culture and chemical treatment

DAB (99% pure), bovine serum albumin, and *N*-acetylcysteine (NAC) were purchased from Sigma-Aldrich Chemical Co. (Madison, WI, USA). 2′-7′-Dichlorofluorescin diacetate (DCFDA) was supplied by Invitrogen (Eugene, OR, USA). GSK-3β inhibitor (Cat. No. SC-24020) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies anti-tau, anti- p-tau(Ser396) from Cell signaling Technology Inc. (MA, USA), anti-p-tau($pSpS^{199/202}$) from Invitrogen, anti-GSK-3β and anti-p- GSK-3β(Ser9, Tyr216) and secondary antibodies from Santa Cruz Biotechnology were obtained. RPMI 1640 medium, fetal bovine serum and PBS were obtained from Gibco Life Technologies (Heidelberg, Germany). Human neuroblastoma SHSY5Y cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and grown in a humidified 95% air/5% CO_2 atmosphere at 37 °C. Cells were treated with NAC (final conc. 500 μM) or GSK-3β inhibitor (final conc. 500 nM) for 1 h before adding DAB (0, 1, 10, or 50 μM).

2.2. Mice and DAB administration

C57BL/6 male mice (6 weeks old) were obtained from Orient Bio Co. (Iksan, Jeonbuk, Korea). Temperature and humidity were maintained at 23–25 °C and 45–55%, respectively, and animals were kept under a 12 h light/12 h dark cycle. Food and water were provided *ad libitum*. Animals were acclimatized for 1 week prior to experiments. Mice were allocated randomly to 4 treatment related groups of 6 animals, as follows; the scopolamine group (animals were treated with scopolamine 1 mg/kg i.p. for 2 weeks), DAB (1 mg/kg or 5 mg/kg) groups (animals were treated with DAB 1 or 5 mg/kg i.p. for 2 weeks), and the saline control group (animals were treated with an equivalent volume of saline i.p. for 2 weeks). Experiments were performed in accordance with the animal care guidelines issued by the Sunchon National University Animal Care.

2.3. Tissue preparation

After the 2-week treatment periods, mice were sacrificed and hippocampi were extracted for biochemical study. Tissues were stored at −80 °C prior to analysis. For Western blotting, tissues were homogenized in lysis buffer; 40 mM Tris buffer (pH 8.0) containing 120 mM NaCl, 0.5% NP-40, 100 μM sodium-orthovanadate, 2 μg/ml aprotinin and 5 μg/ml leupeptin.

2.4. Oxidative stress measurement

To measure ROS production, hippocampal homogenate (100 μg/100 μL) were added to the wells of a 96-well plate and treated with 30 μM of DCFDA. Changes in fluorescence intensity were measured every 5 mins for 30 mins after DCFDA treatment using a Multimode Microplate Reader (Tristar LB 941, Germany) at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

2.5. Western blotting

Ten micrograms of proteins were separated in 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (PROTRAN BA 85; Whatman™). After blocking in 5% nonfat milk for 30 min, membranes were incubated with diluted primary antibodies (anti-tau, anti-p-tau ($pSpS^{199/202}$, Ser396), anti-GSK-3β, anti-p-GSK-3β (Ser9, Tyr216), and actin) in TBS-T buffer (Tris–HCl based buffer containing 0.2% Tween 20, pH 7.5). Proteins were detected by horseradish-conjugated secondary antibody using a Microchemi 4.2 (DNR Bio-Imaging Systems Ltd).

2.6. AGE formation

AGE formation was evaluated as described by Vinson and Howard (Vinson and Howard, 1996). To prepare reaction mixtures, 200 mM fructose and 200 mM dextrose were added to 10 mg/mL bovine serum albumin (BSA) in 50 mM sodium phosphate buffer (pH 7.4) containing 0.02% sodium azide. Reaction mixtures (900 μL) were reacted with various concentrations of samples (100 μL, final conc. In distilled water, DAB 1, 10 or 50 μM). After incubation for 1 day at 37 °C, fluorescence intensities of reaction products were measured using a microplate multimode reader (TriStar LB 941, Berthold Technologies GmbH & Co.) at excitation/emission wavelengths of 355 and 455 nm, respectively.

2.7. Morris Water Maze (MWM) test

A 120 cm diameter circular pool filled with water was used to assess potential spatial memory deficits. Each animal was given five swim trials daily for seven consecutive days. In each trial, three different start positions were used. Animals were placed in the pool in the same order each day and at same times. Mice were given 5 mins to reach the hidden submerged platform. If mice failed to find the platform within 60 s, they were placed on the platform for 5 s before the next test was started. Recorded swimming trajectories, speeds and distances were analyzed using Ethovision XT9 software (Stoelting, USA).

2.8. Statistical analysis

Results are presented as means ± SEMs, and the analysis was performed using Statview software. The significances of differences were determined by analysis of variance (ANOVA) using Fisher's protected least significant difference (PLSD) procedure. Statistical significance was accepted for p-values < 0.05.

3. Results

3.1. Body weight and organ weight changes in DAB-treated mice

To evaluate the effect of DAB, mice (5-week-old, C57BL/6 mice, n = 6/group) were treated intraperitoneally with saline or DAB 1 or 5 mg/kg/day for 2 weeks (Fig. 1A). Scopolamine 1 mg/kg/day was used as a positive control. DAB administered to rats at > 20 mg/kg/day for ≥2 weeks or to mice at > 30 mg/kg/day for 6 weeks induced clear neurological signs, such as, distal forelimb weakness and hindlimb forelimb paresis (Kim et al., 2001; Tshala-Katumbay et al., 2005). Body weights were monitored every three days during the 2 weeks treatment period. The mice were found to gain weight normally, that is, no significant difference in group body weights was observed (Fig. 1B). Furthermore, no noticeable behavioral changes were observed in the two DAB-treated groups. However, mean liver and kidney weights reduced concentration-dependently in DAB-treated mice (Fig. 1C).

3.2. ROS levels in the hippocampal homogenates of DAB-treated mice

ROS plays an important role in neurodegeneration, cell death, motor neuron diseases, and axonal degeneration. ROS levels increased in the hippocampal homogenates of DAB-treated mice versus saline control and these increase were similar to that observed in