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α-Pinene Attenuates Methamphetamine-Induced Conditioned Place Preference in C57BL/6 Mice

Chan Lee[1], Jung-Hee Jang[1,*] and Gyu Hwan Park[2,*]

[1]Department of Pharmacology, School of Medicine, Keimyung University, Daegu 42601,
[2]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 41566, Republic of Korea

Abstract

Methamphetamine (METH) is a powerful neurotoxic psychostimulant affecting dopamine transporter (DAT) activity and leading to continuous excess extracellular dopamine levels. Despite recent advances in the knowledge on neurobiological mechanisms underlying METH abuse, there are few effective pharmacotherapies to prevent METH abuse leading to brain damage and neuropsychiatric deficits. α-Pinene (APN) is one of the major monoterpenes derived from pine essential oils and has diverse biological properties including anti-nociceptive, anti-anxiolytic, antioxidant, and anti-inflammatory actions. In the present study, we investigated the therapeutic potential of APN in a METH abuse mice model. METH (1 mg/kg/day, i.p.) was injected into C57BL/6 mice for four alternative days, and a conditioned place preference (CPP) test was performed. The METH-administered group exhibited increased sensitivity to place preference and significantly decreased levels of dopamine-related markers such as dopamine 2 receptor (D2R) and tyrosine hydroxylase in the striatum of the mice. Moreover, METH caused apoptotic cell death by induction of inflammation and oxidative stress. Conversely, APN treatment (3 and 10 mg/kg, i.p.) significantly reduced METH-mediated place preference and restored the levels of D2R and tyrosine hydroxylase in the striatum. APN increased the anti-apoptotic Bcl-2 to pro-apoptotic Bax ratio and decreased the expression of inflammatory protein Iba-1. METH-induced lipid peroxidation was effectively mitigated by APN by up-regulation of antioxidant enzymes such as manganese-superoxide dismutase and glutamylcysteine synthase via activation of nuclear factor-erythroid 2-related factor 2. These results suggest that APN may have protective potential and be considered as a promising therapeutic agent for METH-induced drug addiction and neuronal damage.

Key Words: α-Pinene, Methamphetamine, Conditioned place preference, Apoptosis, Inflammation, Oxidative Stress

INTRODUCTION

Methamphetamine (METH) is a highly abused neurotoxic psychostimulant. METH abuse has recently become a significant public health concern throughout the world (Chomchai and Chomchai, 2015; Jayanthi *et al*., 2021). Because of the rapid increasing use of METH especially among young individuals, METH is predicted to be one of the most widely used addictive drugs. After long abstinence periods, diverse cues associated with the METH rewarding properties can elicit drug craving and seeking; therefore, METH addiction becomes an issue that requires global attention for pharmacological as well as non-pharmacological treatment interventions.

The mesolimbic dopamine (DA) pathway referred to rewarding system plays an important role in acquisition, expression, and extinction of METH-induced conditioned place preference (CPP) as positive and negative reinforcement. DA release is involved in the incentive learning, acquisition and expression of motivation as positive reinforcement. However, DA must be reduced gradually in a long-term intake of METH, so that the function of the whole reward system is not compromised as a result of the lower concentration of DA and fewer number of DA receptors. This may lead to negative reinforcement, the state of quicker, stronger, and longer compulsive consumption of drugs despite of the evident negative consequences.

METH rewarding effects are mediated by activation of mesolimbic DA transmission. METH inhibits DA reuptake into vesicles via type 2 vesicular monoamine transporter (VMAT2), increases cytoplasmic DA, and facilitates DA release into the synaptic cleft (Riddle *et al*., 2006; Kim *et al*., 2020). METH-

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***Corresponding Authors**
E-mail: park014@knu.ac.kr (Park GH), pamy202@kmu.ac.kr (Jang JH)
Tel: +82-53-950-8576 (Park GH), +82-53-258-7453 (Jang JH)
Fax: +82-53-950-8557 (Park GH), +82-53-258-7448 (Jang JH)

induced massive release of DA leads to the oxidative stress through generation of reactive oxygen species (ROS; superoxide radicals and dopamine quinones) and reactive nitrogen species (RNS; nitric oxide and peroxynitrite), which ultimately causes long-lasting neuronal cell death and damages. The neurotoxic damages to dopaminergic system caused by repeated METH exposures led to a reduction in DA, dopamine transporter (DAT), and tyrosine hydroxylase (TH) levels and the loss of DA terminals and cell bodies (Volz *et al*., 2007a; Nguyen *et al*., 2019). Both postmortem and neuroimaging studies have reported the decreased levels of DA, DAT, and TH in the brains of chronic METH abusers (Volkow *et al*., 2001a; Volz *et al*., 2007b). In addition, positron emission tomography imaging studies have reported that METH appears to induce persistent decreases in dopamine D2 receptors (D2R) as well as striatal DAT (Volkow *et al*., 2001b). This reduction lasts as long as 3 years after METH withdrawal. These neurobiological changes in the dopaminergic system are believed to contribute to the challenges in the abstinence treatment and the high relapse potential. In addition to these toxic effects, METH also causes reactive astrocytosis and microglial activation in the striatum (LaVoie *et al*., 2004; Thomas *et al*., 2004; Loftis and Janowsky, 2014). METH abusers exhibited elevated peripheral benzodiazepine receptor binding levels, a marker for microglial activation and inflammatory glial response (Veerasakul *et al*., 2016). Therefore, medications targeting dopaminergic pathways, oxidative stress, and inflammation could be presumed to have a potential for METH abuse treatment.

In spite of the recent advances in the knowledge of the neurobiological mechanisms underlying METH abuse, there are no FDA-approved pharmacotherapies for METH abuse and few effective options to prevent and/or ameliorate METH-induced brain damage and neuropsychiatric deficits. Therefore, there is still an urgent need in searching for more effective agents specifically designed to attenuate and/or protect METH-associated neuronal injuries which may be critical for a successful recovery that are imperative. In this line, we investigated a therapeutic potential of α-Pinene [APN, 2,6,6,-trimethylbicyclo(3.1.1)-2-hept-2-ene], in a METH abuse mice model using METH-injected C57BL/6 mice (Fig. 1A). APN has been widely used as a food flavoring ingredient approved as a generally safe food additive by the FDA (Aydin *et al*., 2013; Yang *et al*., 2016). In addition, previous researchers have reported diverse biological properties of APN including antimicrobial (Rivera-Yañez *et al*., 2017), hypertensive (Menezes *et al*., 2010), anti-nociceptive (Aydin *et al*., 2013), anti-anxiolytic (Satou *et al*., 2014), antioxidant (Aydin *et al*., 2013), and anti-inflammatory effects (Kim *et al*., 2015). APN exerts effects on multiple signaling pathways such as noradrenergic, GABAergic, serotoninergic, dopaminergic neurotransmitter systems. In the present study, we tested whether APN administration significantly alters the METH-induced CPP and to elucidate the APN protective and restorative mechanisms on METH-induced dopaminergic impairment and neurotoxicity.

MATERIALS AND METHODS

Animals

C57BL/6 male mice at 6 weeks old were supplied from Dae-Han Bio-Link Co., Ltd (Eumsung, Korea) and maintained in a humidity- and temperature-controlled environment (50 ± 5 %, 22 ± 1°C) under a 12 h light/dark cycle with free access to water and standard rodent food. Experimental procedures concerning animals were approved by the Ethics Committee of School of Medicine, Keimyung University (Daegu, Korea). All animal care and experimental procedures were accomplished with the National Institutes of Health guideline for laboratory animal care and use.

Reagents

METH was supplied from Ministry of Food and Drug Safety (Cheongju, Korea) and was dissolved in 0.9% sterile saline. Anti-actin antibody and other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-TH antibody was obtained from Millipore Co. (Temecula, CA, USA). Anti-D2R antibody was supplied by Biorbyt LLC. (Wobum, MA, USA) and Bioss Antibodies Inc. (Woburn, MA, USA), respectively. Anti-phospho-nuclear factor-erythroid 2- related factor 2 (Nrf2), 4-hydoxynonenal (4-HNE), and Iba-1 antibodies were obtained from Abcam (Waltham, MA, USA). Anti-Nrf2, manganese superoxide dismutase (MnSOD), glutamylcysteine synthase (GCS), Bax, and Bcl-2 antibodies were the products from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Procedures of drug treatment

After the adaptation for a week, mice were randomly divided into four groups (5 mice per group) as follows: group 1 (control), group 2 (METH), group 3 (METH+3 mg/kg of APN), and group 4 (METH+10 mg/kg of APN). All groups except for control were administered METH (1 mg/kg, i.p.) daily for 8 days. The control group received an equivoluminal 0.9% normal saline solution. In the APN group, APN (3 and 10 mg/kg, i.p.) was also administered for 8 days before METH injections; on the other hand, mice in the groups (control and METH only) received the same amount of normal saline.

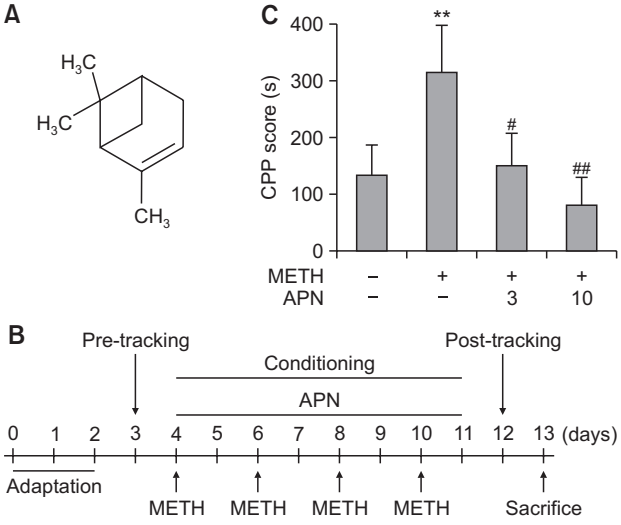

Fig. 1. Effect of APN on METH-induced CPP in C57BL/6 mice. (A) Chemical structure of (+)-α-pinene (APN). (B) Experimental schedule for METH-induced CPP in C57BL/6 mice. (C) Mice were pretreated with 3 and 10 mg/kg of APN for 30 min before METH (1 mg/kg, i.p.) injection and performed CPP test. Data were presented as mean ± SEM. Significantly different between groups: $^{**}p<0.01$ saline-treated control mice vs METH-treated mice, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ METH-treated mice vs APN-treated mice (n=5).

CPP apparatus

Conventional CPP apparatus with two compartments of equal size was utilized. One compartment was black-colored, with stainless steel grids in the floor consisting with perpendicularly placed rods, and the other compartment was white-colored, with stainless steel mesh floor. The time spent in each compartment and movement of the animals were recorded using a computerized video tracking system (Smart 3.0 software, Panlab, Barcelona, Spain).

Measurement of METH-induced CPP

The CPP paradigm consisted of three phases as follows: pre-tracking, conditioning, and post-tracking (Fig. 1B). In the pre-tracking phase (day 3), the mice were allowed to freely access the two compartments for 20 min daily. The time spent in each compartment was recorded during this habituation period to exclude biased mice that spent more than 800 s in either compartment. In the conditioning phase (day 4-11), the mice received METH (1 mg/kg, i.p.) or saline alternately on every other day for 8 days (4 METH sessions and 4 saline session). Each animal received saline injections in their initially preferred compartment (saline-pared side) and METH injections in their initially non-preferred compartment (drug-paired side). The mice were then immediately confined in the appropriate drug-paired or saline-paired compartments for 60 min. To assess the effects of APN on the acquisition of METH-induced CPP, naive animals received a vehicle or APN (3 and 10 mg/kg, i.p., n=5 per group) 30 min prior to each METH or saline injection during the METH conditioning phase. In the post-tracking phase (day 12), the CPP test was conducted 24 h after the last injection, and there was no drug treatment on the test day. A preference score was calculated as the difference between the relative amount of time spent in the drug-paired, non-preferred side of the apparatus and the time spent in the preferred side during the preconditioning phase.

Western blot analysis

After the treatments, cortical and striatal regions from the mice were rapidly isolated. Brain tissues were homogenized and lysed with ice-cold RIPA buffer [150 mM NaCl, 1.0% Triton-X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Trizma base, pH 8.0]. Lysates were centrifuged at 14,000 g for 30 min, and supernatants obtained were used for western blot analysis. Protein concentration was determined by using a BCA protein assay reagent (Thermo Scientific, Rockford, IL, USA). Total protein lysates were separated by 10-12% SDS-polyacrylamide gels, were transferred into the polyvinylidene fluoride membranes, and were blocked in PBS buffer containing a 5% non-fat dry milk for 1 h. The membranes were further incubated with primary antibodies at 4°C overnight. The membranes were then washed with PBS and then reacted with secondary anti-rabbit antibody for 60 min at room temperature. After the second incubation, the blots were washed in PBS for 10 min 3 times and developed with the enhanced chemiluminescence system (Pierce, IL, USA) for visualization. Relative intensities of the bands were quantified by using an ImageQuant LAS 4000 Multi Gauge software (Fujifilm, Tokyo, Japan) and were then normalized to the intensity of Actin.

Statistical analysis

The data were expressed as the means ± SEM (standard error of the mean). Differences between groups were compared by using a one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test for multiple comparisons. All of the statistical analyses were performed by using SPSS ver. 20.0 (IBM, Chicago, IL, USA). *p* values less than 0.05 were considered to be statistically significant.

RESULTS

Effect of APN on the development of METH-induced CPP

CPP was used to assess the behavioral effects of APN against repeated administration of METH. CPP test was performed 24 h after the last injection of METH. Conditioning mice with METH for 8 days markedly increased the time spent in the METH-paired compartment, indicating that METH has a significant rewarding effect. APN treatment before METH injection significantly attenuated METH-induced CPP in a dose-dependent manner. Specifically, the group pretreated with APN (10 mg/kg) showed a marked inhibition of METH-induced CPP (Fig. 1C).

Effects of APN on the expression of TH and D2R in the striatum of METH-dependent Mice

It is known that METH decreases TH, a key enzyme in DA synthesis and generally used as a marker for dopaminergic neurons (Gibb and Kogan, 1979). METH is also reported to produce neurotoxicity via D2R and elicited a reduction of D2R (Ares-Santos *et al*., 2013). Western blot analysis was used to examine the effect of APN treatment on the expression of TH and D2R in the striatum. Repetitive injection of METH caused a significant decrease in striatal TH protein level. Protein expression of TH in the METH group was decreased 74.4 ±3.06% compared to that of the control group. On the contrary, APN administration prevented and restored the METH-decreased TH protein levels up to 89.8 ± 3.44% (Fig. 2A). In the METH group, METH administration led to a significantly reduction in D2R levels to 43.3 ± 5.01% of the control group (Fig. 2B). APN treatment suppressed this decrease and main-

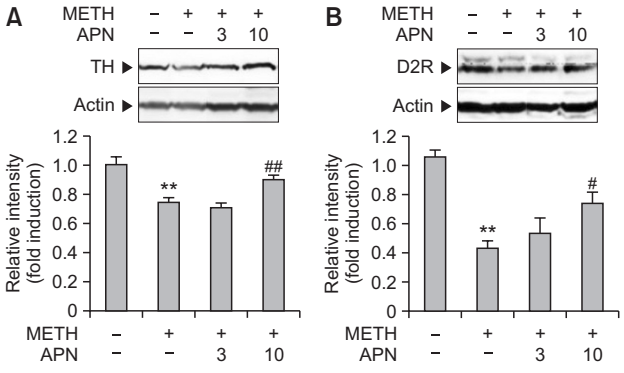

Fig. 2. Effect of APN on METH-decreased TH and D2R in striatum of C57BL/6 mice. Expression of TH (A) and D2R (B) in METH-treated mice was compared with saline- or APN-treated mice. The relative intensity of TH/Actin and D2R/Actin was represented in the right panel. Data were expressed as mean ± SEM. Significantly different between groups: $^{**}p<0.01$ saline-treated control mice vs METH-treated mice, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ METH-treated mice vs APN-treated mice (n=5).

tained D2R up to 74.3 ± 7.37% of the control group, which were significantly different from the levels observed in the METH group (Fig. 2B).

Effect of APN on METH-induced apoptosis in the striatum

To understand the mechanisms underlying APN-mediated attenuation of METH-induced neurotoxicity, we explored the effects of APN on METH-induced alteration of Bcl-2/Bax ratio. Proapoptotic cell death has been reported to play an important role in METH-mediated neurotoxicity (Jayanthi *et al*., 2004). METH treatment increased Bax and decreased Bcl-2 expression in the striatum (Fig. 3A). Conversely, these changes were prevented by APN treatment. APN significantly restored METH-reduced Bcl-2/Bax ratio more than control levels, suggesting the protective effect of APN might be mediated via the anti- and pro-apoptotic factors regulation.

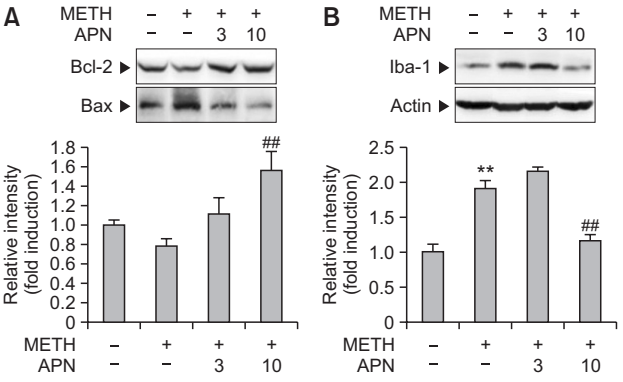

Fig. 3. Effect of APN on the expression of apoptosis and/or inflammation mediating proteins. (A) The expression of Bcl-2 family proteins was measured by the relative ratio of Bcl-2/Bax. (B) Microglial activation associated with METH neurotoxicity was measured by the expression of Iba-1. Protein levels are expressed as fold change over corresponding saline-treated control values. Values are expressed as mean ± SEM. Significantly different between groups: $^{**}p<0.01$ saline-treated control mice vs METH-treated mice, $^{\#\#}p<0.01$ METH-treated mice vs APN-treated mice (n=5).

Microglia is reported to participate in neuroinflammation-associated METH intoxication (Kitamura *et al*., 2010). We examined a change in Iba-1 expression, a marker of microglial activation and inflammation after METH exposure. METH treatment caused significant increases in striatal Iba-1 protein level. Iba-1 protein content had increased 1.91 fold over that in respective saline-treated controls (Fig. 3B). However, the METH-elevated Iba-1 levels were effectively decreased in the striatum of C57BL/6 mice administered with APN (10 mg/kg).

Effect of APN on METH-augmented oxidative stress in the cortex

Accumulating evidence suggests that oxidative stress links inflammation to neuronal death. Induction of oxidative stress and impairment of antioxidant system might mediate dopaminergic degeneration induced by multiple doses of METH. To examine the extent of oxidative stress, we measured 4-HNE protein level as a marker of oxidative stress-induced lipid peroxidation using western blot analysis. The level of 4-HNE protein was increased in METH-treated mice compared with saline-treated control group, whereas, APN pretreatment reduced METH-elevated 4-HNE protein expression (Fig. 4A). To further investigate the impairment of antioxidant system, we examined the expression of antioxidant enzymes as well as Nrf2 which is a representative redox-sensitive transcription factor regulating antioxidant and detoxifying enzymes. Mice treated with METH decreased the activation of Nrf2 via phosphorylation at Ser 40 and reduced the levels of antioxidant enzymes such as MnSOD and GCS when compared with saline-treated control mice. However, APN treatment restored the Nrf2 phosphorylation and subsequent expression of MnSOD and GCS (Fig. 4B, 4C).

DISCUSSION

In the present study, we found that APN had neuroprotective effects on the METH-induced behavior and neuronal cell death in C57BL/6 mice. METH (1 mg/kg) increased the time spent on CPP experiment compared with saline-treated con-

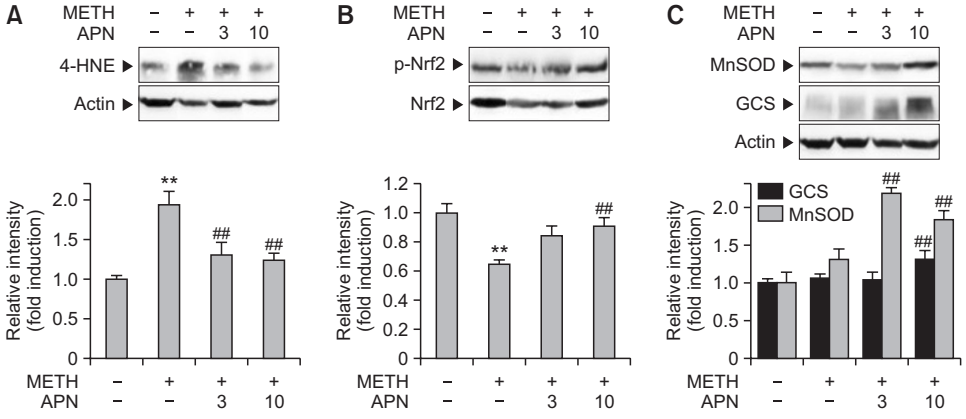

Fig. 4. Protective effect of APN against METH-induced oxidative stress. (A) The level of lipid peroxidation was determined by western blot using a 4-HNE-specific antibody in striatum of C57BL/6 mice. The phosphorylation of redox-sensitive transcription factor Nrf2 (B) and the expression of antioxidant enzymes (C) were measured to compare the cellular defense capacity. Relative intensity of protein expression normalized by housekeeping control was represented in the right panel. Data are shown as mean ± SEM. Significantly different between groups: $^{**}p<0.01$ saline-treated control mice vs METH-treated mice, $^{\#\#}p<0.01$ METH-treated mice vs APN-treated mice (n=5).

trol group, which was significantly decreased by 10 mg/kg of APN pretreatment. Particularly, this study is the first to evaluate APN as a novel treatment for METH-induced dopaminergic neurotoxicity. In addition, repeated METH administration induced long-term dopamine terminal damage as evidenced by the decrease in dopaminergic markers such as TH and D2R whereas APN restored the losses in TH and D2R. These results of the present study correspond well with those found in the previous studies. Several postmortem studies have shown that the levels of DA, TH, and DAT are significantly reduced in the striatum of chronic METH users (Volz *et al*., 2007a; Nguyen *et al*., 2019). Neuroimaging techniques using PET have shown decreases in DAT level and D2R in the striatum of METH abusers (Volkow *et al*., 2001a; Volz *et al*., 2007b). We found that APN pretreatment (10 mg/kg) effectively inhibited the stereotyped behavior and the changes in the levels of TH and D2R by repeated METH exposure. Our results suggest that APN exhibited protective and restorative effect against METH-induced dopaminergic neurotoxicity. Furthermore, there are two major theories of addiction, which are the incentive sensitization theory and the opponent process theory. According to the opponent process theory, drug-seeking and craving are based on negative reinforcement, which means that drugs are used continuously to alleviate negative affective states that accompany drug withdrawal. The neurobiological changes involve a decrement of neurotransmission in the mesolimbic dopamine system and activation of the brain stress system. In the present study, our results suggest that α-pinene might reduce METH-induced CPP by normalizing a marked deficiency of the mesolimbic dopamine neurotransmission, leading to suppressing negative affective states and motivated drug-seeking behaviors.

Although the pathogenesis on the METH-induced dopaminergic neurotoxicity remains to be further elucidated, this neurotoxicity may be, at least in part, related to apoptosis, oxidative stress, and inflammatory changes. Single high dose of METH selectively induced pro-apoptosis in the striatum (Tulloch *et al*., 2011). This range of METH dose also induced the loss of striatal DA terminal markers such as DAT, TH, and tissue dopamine contents. The apoptotic signaling pathway after METH exposure was investigated. Neurotoxic dose of METH caused differential regulation of several Bcl-2 family genes with two distinct clustering consisting of upregulation of pro-apoptotic and downregulation of anti-apoptotic gene expression (Jayanthi *et al*., 2001). The ratio of Bcl-2 to Bax determines the susceptibility of a cell to apoptosis. A decrease of Bcl-2/Bax ratio indicates an increased susceptibility of a cell to apoptosis (Raisova *et al*., 2001). METH treatment increased Bax and lowered Bcl-2 expression, while APN administration prevented these changes. Moreover, Bcl-2 has been reported to protect against generators of ROS to increase antioxidant defenses and to decrease levels of ROS and oxidative damage (Chen and Pervaiz, 2009). METH-induced mitochondrial oxidative stress and dysfunction promotes have been reported to cause dopaminergic degeneration (Yang *et al*., 2018). Excess ROS further oxidizes biomolecules including lipids, proteins, and nucleic acids and induces the malfunction of cellular components and damage (Juan *et al*., 2021). Impairments in endogenous antioxidant defense systems play a causal role in METH-induced oxidative damage. In clinical studies significant reduction of SOD activity or compensative elevation of SOD activity against superoxide radicals has been observed (Younus, 2018). In this study, repeated administration of METH caused oxidative stress in C57BL/6 mice. METH increased lipid peroxidation and decreased expression of antioxidant enzymes such as MnSOD and GCS, which were significant restored by APN. This finding is in accordance with results of previous study which showed that MnSOD overexpression attenuated dopaminergic toxicity induced by METH and protected cells from apoptosis (Maragos *et al*., 2000). In addition, other study has demonstrated that melatonin protects METH-induced neuroinflammation via upregulation of GCS in C6 glioma cell line (Jumnongprakhon *et al*., 2015). It is also known that microglial activation or neuroinflammation is involved in METH-elicited neurotoxicity as much as oxidative stress is. The present study showed that APN significantly prevented METH-induced Iba-1 expression, indicating that protective effect of APN is mediated by inhibiting METH-induced microglial activation or neuroinflammation. This interpretation is in accordance with previous findings from other studies which showed that Ginsenoside Re attenuated METH-induced dopaminergic degeneration by pro-inflammatory microglial activation (Shin *et al*., 2014).

In conclusion, evidence from the present study appears to indicate that APN may inhibit the neuronal damages induced by repeated METH exposure as evidenced by the significant attenuation of METH-induced stereotyped behavior as well as the inhibition of reductions in TH and D2R expression levels with APN pretreatment. In addition, APN appears to afford restorative effects on the METH-induced dopaminergic toxicity, as evidenced by the significant recovery of apoptotic cell death, inflammation, and oxidative damages by augmentation of antioxidant capacity via activation of redox-sensitive transcription factor Nrf2. Taken together, results from our present study document that APN may be considered a promising therapeutic agent in patients with METH use disorder.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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