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3-(Naphthalen-2-yl(propoxy)methyl)azetidine hydrochloride attenuates MPP<sup>+</sup>-induced cytotoxicity by regulating oxidative stress and mitochondrial dysfunction in SH-SY5Y cells

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**ABSTRACT**

Parkinson's disease (PD) is a common chronic neurodegenerative disease mainly caused by the death of dopaminergic neurons. However, no complete pharmacotherapeutic approaches are currently available for PD therapies. 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>)-induced SH-SY5Y neurotoxicity has been broadly utilized to create cellular models and study the mechanisms and critical aspects of PD. In the present study, we examined the role of a novel azetidine derivative, 3-(naphthalen-2-yl(propoxy)methyl)azetidine hydrochloride (KHG26792), against MPP<sup>+</sup>-induced neurotoxicity in SH-SY5Y cells. Treatment of KHG26792 significantly attenuated MPP<sup>+</sup>-induced changes in the protein levels of Bcl-2 and Bax together with efficient suppression of MPP<sup>+</sup>-induced activation of caspase-3 activity. KHG26792 also attenuated mitochondrial potential and levels of ROS, Ca<sup>2+</sup>, and ATP in MPP<sup>+</sup>-treated SH-SY5Y cells. Additionally, KHG26792 inhibited the induced production of nitric oxide and malondialdehyde. Moreover, the protective effect of KHG26792 is mediated through regulation of glutathione peroxidase and GDNF levels. Our results suggest a possibility that KHG26792 treatment significantly protects against MPP<sup>+</sup>-induced neurotoxicity in SH-SY5Y cells and KHG26792 may be a valuable therapeutic agent for the treatment of PD induced by an environmental toxin.

*Keywords:* MPP<sup>+</sup>, Mitochondrial dysfunctions, Oxidative stress, Azetidine derivative

## INTRODUCTION

Parkinson's disease (PD) is a disorder with progressive and selective neurodegeneration of dopaminergic neurons located mainly in the substantia nigra pars compacta region [1]. The brain uses an excess amount of oxygen compared to other organs, and neurons are one of the most metabolically active cell types [2]. Many studies have reported that increased oxidative stress and mitochondrial membrane impairment play a role as a crucial contributor to the occurrence of PD pathogenesis [3]. Previous studies have reported that 1-methyl-4-phenylpyridinium ion (MPP<sup>+</sup>), a commonly used neurotoxin in experimental models of PD, inhibits mitochondrial respiratory function through the overproduction of reactive oxygen species (ROS) and defective mitochondrial energy metabolism injury [4-6]. Therefore, agents with the potential to attenuate radical oxidative stress and mitochondrial dysfunction can be effective in the protection of MPP<sup>+</sup>-induced neuronal death and subsequently the improvement of neurological outcome in PD [7]. However, no complete pharmacotherapeutic approaches are available for PD therapies.

We have examined various compounds with distinct structures in several models of neurodegenerative disease to develop effective neuroprotective drugs. Azetidine derivatives have been suggested to be useful therapeutic agents for the development of CNS-focused lead-like libraries [8]. For instance, biological activities of azetidine derivatives against serotonin, norepinephrine, and dopamine transporters have been reported [9]. In addition, a previous study also showed the memory-restorative effect of ezetimibe, a well-known azetidine derivative, in memory dysfunctions associated with Alzheimer's disease dementia [10]. Previously, we synthesized 3-(naphthalen-2-yl(propoxy)methyl)azetidine hydrochloride (KHG26792) and reported that KHG26792 significantly decreased hypoxia-induced toxicity by attenuating oxidative stress [11,12]. However, the precise mechanisms for the action of KHG26792 have not been elucidated, particularly for MPP<sup>+</sup>-induced neurotoxicity.

In the present study, we examined the neuroprotective properties of KHG26792 in terms of its ability to attenuate apoptosis and oxidative responses in MPP<sup>+</sup>-induced SH-SY5Y cells. Our results show that KHG26792 treatment significantly protects against MPP<sup>+</sup>-induced toxicity in SH-SY5Y cells by the regulation of several pathways.

## RESULTS AND DISCUSSION

### **KHG26792 attenuates MPP<sup>+</sup>-induced cell death in SH-SY5Y cells**

MPP<sup>+</sup> is one of the most frequently used neurotoxins in creating cellular models and studying critical aspects of PD in SH-SY5Y cells. Some azetidine derivatives show neuroprotective effects in the treatment of PD and other neurodegenerative diseases [9-14]. In the present study, we focused to examine the role of a novel azetidine derivative, KHG26792, against MPP<sup>+</sup>-induced neurotoxicity in SH-SY5Y cells. First, we examined the effects of KHG26792 on the cytotoxicity induced by MPP<sup>+</sup> on SH-SY5Y cells to find an appropriate concentration for the subsequent study. Cultured cells were pretreated with various concentrations of KHG26792 for 1 h, followed by MPP<sup>+</sup> (2 mM) for 24 h. MPP<sup>+</sup> caused cell death as determined both by MTT (Fig. 1A) and LDH assay (Fig. 1B). However, we found that KHG26792 dose-dependently protected SH-SY5Y cells against MPP<sup>+</sup>-induced cell death (Fig. 1A-B). Therefore, these results suggest that KHG26792 effectively attenuates MPP<sup>+</sup>-induced cell death in SH-SY5Y cells. For all subsequent studies, the concentration of KHG26377 was fixed at 50  $\mu$ M.

Next, we determined the anti-apoptotic features of KHG26792 in the MPP<sup>+</sup>-treated SH-SY5Y cells because mitochondrial apoptosis is one of the main pathways involved in dopaminergic neuronal cell death [15,16]. The Bcl-2/Bax ratio is a useful apoptotic marker affected by the MPP<sup>+</sup>-regulated apoptotic pathway, and caspase-3 also mediates cell degradation and protein functions through its cleavage function [17]. Consistent with previous study [16], western blot analysis showed that MPP<sup>+</sup> dramatically induced the protein levels of Bax together with a decrease in Bcl-2 level (Fig. 2A-C). The Bcl-2/Bax ratio was significantly lower in the MPP<sup>+</sup>-treated cells. The protein levels of  $\beta$ -actin remained relatively constant in all groups. Additionally, caspase-3 activity was increased in the MPP<sup>+</sup>-treated cells (Fig. 2D). However, treatment of KHG26792 significantly attenuated the MPP<sup>+</sup>-induced changes in the protein levels of Bcl-2 and Bax (Fig. 2A-C). KHG26792 also efficiently blocked MPP<sup>+</sup>-induced activation of caspase-3 activity (Fig. 2D). Together, our results showed that a decrease in the Bcl-2/Bax ratio as well as activation of caspase-3 induced by MPP<sup>+</sup> can be effectively protected by the anti-apoptotic property of KHG26792.

### **KHG26792 attenuates mitochondrial potential and levels of ROS, Ca<sup>2+</sup>, and ATP in MPP<sup>+</sup>-treated SH-SY5Y cells**

MPP<sup>+</sup>-induced translocation of Bax to the mitochondrial membrane causes a loss of

mitochondrial potential and an activation of caspase-3 [18]. Previous study also suggested a possibility for neuroprotection by a mitochondria-targeted drug in a PD model [19]. In this study, we further investigated the effects of KHG26792 on mitochondrial dysfunction in MPP<sup>+</sup>-treated SH-SY5Y cells by measuring impaired mitochondrial membrane potential. In MPP<sup>+</sup>-treated SH-SY5Y cells, the mitochondrial membrane potential was dramatically reduced compared to that of the control group (Fig. 3A). However, treatment with KHG26792 exerted a protective effect by attenuating the mitochondrial membrane potential relative to those observed in the control group (Fig. 3A). These results indicate that KHG26792 may prevent MPP<sup>+</sup>-induced toxicity by restoring impaired mitochondrial dysfunction.

Oxidative stress plays an important role in various neurodegenerative diseases exhibiting complex features of apoptosis mediated by the apoptosis-related proteins. An injury of the integrity of cell organelles can increase the production of ROS. ROS are produced during most cellular processes involving the metabolism of oxygen, and excess ROS generation induces oxidative stress, which is an important factor in physiological signaling pathways [20]. ROS-mediated oxidative stress also induces large increases in intracellular Ca<sup>2+</sup> and subsequently causes mitochondrial dysfunction, resulting in permanent membrane injury and neuronal cell death [20,21]. Furthermore, the excessive generation of ROS is also involved in the MPP<sup>+</sup>-induced dopaminergic cytotoxicity [20,21]. We, therefore, investigated whether the protective properties of KHG26792 in MPP<sup>+</sup>-treated SH-SY5Y cells were related with the suppression of ROS generation. As shown in Fig. 2B, MPP<sup>+</sup>-induced oxidative stress enhanced ROS production and intracellular Ca<sup>2+</sup> levels by 3.4-fold (Fig. 3B) and 2.7-fold (Fig. 3C), respectively, relative to levels of the untreated control group. Once again, KHG26792 treatment resulted in a significant reduction in both the MPP<sup>+</sup>-induced ROS production and the Ca<sup>2+</sup> level (Fig. 3B-C).

Previous studies have reported that oxidative stress destroys the electron transport chain accompanied by reduction in ATP generation [22]. It also has been reported that MPP<sup>+</sup> caused ATP deficiency in neuronal SHSY5Y cells [6,23]. Therefore, we determined intracellular ATP levels to examine the effect of KHG26792 on mitochondrial function. As shown in Fig. 3D, MPP<sup>+</sup> treatment caused a reduction in ATP concentration. In contrast, KHG26792 treatment significantly recovered the MPP<sup>+</sup>-induced reduction of ATP content (Fig. 3D).

Taken together, our results suggest that suppression of mitochondrial injury is involved in a putative scavenging activity of KHG26792 against MPP<sup>+</sup> toxicity.

### **KHG26792 attenuates the levels of NO, MDA, GPx activity, and GDNF in MPP<sup>+</sup>-treated SH-SY5Y cells**

Nitric oxide (NO) plays an important role in cellular communication. An excess amount of NO is implicated in MPP<sup>+</sup>-induced oxidative damage and apoptosis and subsequently in the pathogenesis of neurodegenerative diseases [24,25]. Therefore, we assessed the effect of KHG26792 on NO production by measuring the level of nitrite in SH-SY5Y cells. As shown in Fig. 4A, MPP<sup>+</sup> caused a significant release of NO by nearly 2-fold, whereas KHG26792 treatment ameliorates this increase in SH-SY5Y cells. These results indicate that KHG26792 possesses neuroprotective effect against MPP<sup>+</sup>-induced generation of NO in SH-SY5Y cells.

The process of lipid peroxidation begins when a free radical removes a hydrogen atom from an unsaturated fatty acid, resulting in the production of malondialdehyde (MDA) [14]. We further examined the neuroprotective properties of KHG26792 on the lipid peroxidation by measuring the formation of MDA, an indicator of lipid peroxidation and oxidative damage [18]. MPP<sup>+</sup> treatment dramatically increased MDA level to nearly 200% compared with the control (Fig. 4B). Our results are similar to what has been reported after MPP<sup>+</sup>-induced neurotoxicity [26]. In contrast, KHG26792 significantly reduced the MPP<sup>+</sup>-induced MDA level (Fig. 4B), indicating that KHG26792 treatment efficiently attenuated MPP<sup>+</sup>-induced oxidative stress.

Glutathione conjugating enzymes such as glutathione peroxidase (GPx) have essential roles in maintaining the redox homeostasis within cells against ROS production and lipid peroxidation [2]. GPx has an important role in decreasing toxicity of exogenous hydrogen peroxide by reducing hydrogen peroxide to water. Therefore, overexpression of GPx, suppresses lipid peroxidation in neurotoxic conditions and subsequently protects neurons against neurotoxic conditions, including oxidative stress [2]. Moreover, previous studies showed decreased values of GPx in an MPP<sup>+</sup>-treated experimental model of PD [2,26]. To determine the effect of KHG26792 on GPx activity, SH-SY5Y cells were treated with MPP<sup>+</sup> in the presence or absence of KHG26792. We observed a decrease in GPx level in MPP<sup>+</sup>-treated cells (Fig. 4C) that correlated with a parallel enhancement in NO and MDA levels (Fig. 4A-B), whereas pretreatment with KHG26792 markedly recovered GPx activity close to the control cells (Fig. 4C). Since the predominant source of ROS is mitochondrial oxidative phosphorylation, it is possible that the suppression of ROS generation by KHG26792 (Fig. 3B) may be due, at least in part, to the prevention of mitochondrial injury via the recovery of GPx activity.



Glial cell line-derived neurotrophic factor (GDNF) is a member of the TGF- $\beta$  superfamily [27]. Previous studies reported the role of GDNF as an important neurotrophic factor in regulating dopaminergic cell survival [28]. Therefore, GDNF has been regarded as one of the candidates for a novel type of disease-modifying treatment [29]. In this study, we measured GDNF level to assess the potential function of KHG26792 in GDNF release. As shown in Fig. 4D, GDNF level was reduced by approximately 30% in the MPP<sup>+</sup>-treated group. Our results are consistent with a previous study reported by Cheng et al [30], where incubation with MPP<sup>+</sup> decreased the GDNF level in SH-SY5Y cells. However, the MPP<sup>+</sup>-suppressed GDNF production in SH-SY5Y cells was significantly attenuated by KHG26792 treatment (Fig. 4D).

In summary, the results of the current study show that KHG26792 treatment significantly protects against MPP<sup>+</sup>-induced toxicity in SH-SY5Y cells by the regulation of multiple pathways: 1) inhibiting apoptosis via Bcl-2, Bax, and caspase 3-dependent pathways, 2) modulating mitochondrial dysfunction, and 3) attenuating ROS/NO generation. Moreover, the protective effect of KHG26792 may be mediated through regulation of GPx and GDNF levels. Our results further suggest a possibility that KHG26792 may be a valuable therapeutic agent for the treatment of PD induced by an environmental toxin, although the neuroprotective effects of KHG26792 *in vivo*, as well as its long-term consequences on neurodegenerative diseases, remain to be studied.

## MATERIALS AND METHODS

### Materials

MPP<sup>+</sup>, fetal bovine serum, penicillin, streptomycin, glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acetyl-DEVD-p-nitroanilide, protease inhibitor cocktail, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY). Antibodies against Bcl-2, Bax, and  $\beta$ -actin were purchased from Cell Signaling Technology (Beverly, MA). 3-(Naphthalen-2-yl(propoxy)methyl)azetidine hydrochloride (KHG26792) was synthesized and purified as previously described [11]. All other commercial reagents were of the highest available purity.



### Cell culture and drug treatments

Human neuroblastoma SH-SY5Y cell culture was performed as described elsewhere, with a slight modification [6]. Briefly, SH-SY5Y cells were cultured in DMEM with 10% fetal bovine serum, 100 units/mL penicillin/streptomycin/glutamine at 37 °C in a humidified environment with 5% CO<sub>2</sub>. Cells were passaged using a trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.05% trypsin and 0.02% EDTA). To perform experiments, cells were washed with DMEM and cultured in serum-free medium for 48 h. For drug treatment, cultured cells were pretreated with various concentrations of KHG26792 for 1 h, followed by MPP<sup>+</sup> (2 mM) for 24 h. Cell viability was then determined by the MTT assay using a microplate reader as previously described [11]. LDH activity was measured using an assay kit (Roche Applied Science, Pleasanton, CA) per manufacturer's instructions. KHG26792 was dissolved as a stock solution (10 mM) in DMSO and diluted to the desired concentrations with treatment medium. Equivalent amounts of DMSO were used for controls and MPP<sup>+</sup>-treated cells throughout the entire experiment.

### Measurement of mitochondrial potential and levels of Ca<sup>2+</sup>, ATP, and ROS

The mitochondrial potential was measured using a lipophilic JC-1 dye (Invitrogen) as described before [13]. The change in the mitochondrial potential was quantified by comparing the ratio of the intensity of green fluorescent monomers (excitation at 485 nm and emission at 535 nm) in non-healthy cells to that of red fluorescent aggregates (excitation at 590 nm and emission at 600 nm) in healthy mitochondria.

The generation of Ca<sup>2+</sup> was determined with the fluorescent probe Fluo-4/AM by fluorescence-activated cell sorting (FACS) analysis on a FACS Calibur (Becton Dickinson, Heidelberg, Germany) according to the protocols described in our previous study [13]. The values were calculated as the relative intensity of fluorescence compared with the control (untreated group) cells.

Measurement of ATP was performed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, MA) according to the manufacturer's instructions.

As an indicator of ROS generation, we measured production of 2',7'-dichlorofluorescein (DCF), which is the oxidized fluorescent product of 2',7'-dichlorofluorescein diacetate (DCF-DA) [13]. All experiments were performed in the dark. After incubation with 1 μM redox-sensitive DCF-DA dye for 1 h, the cell supernatants containing unabsorbed dye were aspirated and washed with phosphate buffered saline. The fluorescence intensity of DCF was obtained

to evaluate the relative levels of ROS in SH-SY5Y cells using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) at excitation of 485nm and an emission of 530nm, normalized by the total number of cells.

### **Measurement of the levels of NO, MDA, and GDNF**

The concentration of NO was quantified by measuring nitrite, a major product of NO, with Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H<sub>3</sub>PO<sub>4</sub>] as previously described [14]. Optical density was measured at 570 nm on a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The NO concentration was calculated based on a reference standard curve for sodium nitrite

Measurement of malondialdehyde (MDA) level as an index of peroxidation of lipids was performed as described elsewhere [14] in a reaction mixture containing 100 mL of 8.1% sodium dodecyl sulfate (SDS), 750 mL of 20% acetic acid (pH 3.5), 750 mL of 0.8% thiobarbituric acid, and 300 mL of distilled water. After boiling at 95 °C for 1 h, samples were centrifuged and the absorbance of the supernatant was measured at 532 nm by spectrophotometry.

The GDNF assay was performed using an ELISA kit (Rat GDNF PicoKine™ ELISA Kit, Boster, CA) according to manufacturer's instructions. The absorbance at 450 nm was determined in a microplate reader (Molecular Devices Corp., Sunnyvale, CA).

### **Measurement of caspase-3 and GPx activities**

To measure the activity of caspase-3, cell lysates were incubated with the substrate working solution (acetyl-DEVD-p-nitroanilide in 30 mM HEPES, 10 mM DTT, 0.15% Triton X-100, 100 mM NaCl, 0.3 mM EDTA, and 1 mM protease inhibitor cocktail) at 37 °C for 1 h. The changes in optical density at 405 nm were measured using a spectrophotometer.

GPx activity was assayed by monitoring the enzymatic reaction initiated by the addition of H<sub>2</sub>O<sub>2</sub> to a reaction mixture containing glutathione reductase together with reduced glutathione and NADPH, and GR [13]. The absorbance change was monitored with a spectrophotometer at 412 nm.

### **Western blot analysis**

Cell lysates were applied to SDS-polyacrylamide gel electrophoresis and then analyzed by Western blotting to compare the protein expression of Bcl-2 and Bax. Protein expression of  $\beta$ -

actin was also measured as a loading control. Protein bands were detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Buckinghamshire, UK) and analyzed using a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA).

### Statistical analysis

Statistical data are expressed as means  $\pm$  standard deviation (SD) in the text and figure legends. Data were obtained from three independent experiments, and significant differences in mean values between the MPP<sup>+</sup> and MPP<sup>+</sup> with KHG26792 groups were evaluated by one-way analysis of variance (ANOVA) followed by Student's t-tests. Values of  $P < 0.05$  were considered to indicate statistically significant differences.

### Conflict of interest

The authors have no conflicts of interest.

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## Figure legends

**Fig. 1.** Effects of KHG26792 on MPP<sup>+</sup>-induced cell death in SH-SY5Y cells. SH-SY5Y cells were pretreated with KHG26792 (0, 1, 5, 20, or 50  $\mu$ M) for 1 h, followed by MPP<sup>+</sup> (2 mM) for 24 h. (A) Chemical structure of KHG26792. (B) MTT assay. (C) LDH assay.

**Fig. 2.** Effects of KHG26792 on MPP<sup>+</sup>-induced imbalance in the expression of Bax and Bcl-2, and caspase-3 activity. (A) Total cell extracts were subjected to Western blotting analysis with antibodies against Bcl-2 and Bax.  $\beta$ -actin was used as a loading control. (B-C) Densitometric analysis is shown for the relative expression level of each protein. (D) Caspase-3 activity. Data from three independent experiments are presented as the means  $\pm$  SD. (\*  $p < 0.05$  relative to MPP<sup>+</sup>-treated cells).

**Fig. 3.** Effects of KHG26792 on mitochondrial potential, and levels of ROS, Ca<sup>2+</sup>, and ATP in MPP<sup>+</sup>-treated SH-SY5Y cells. SH-SY5Y cells were treated as described in Fig. 1. Mitochondrial membrane potential (A), ROS (B), Ca<sup>2+</sup> (C), and ATP (D) levels were measured as described in Materials and Methods section. Data from three independent experiments are presented as the means  $\pm$  SD. (\*  $p < 0.05$  relative to MPP<sup>+</sup>-treated cells).

**Fig. 4.** Effects of KHG26792 on the levels of NO, MDA, GPx, and GDNF in MPP<sup>+</sup>-treated SH-SY5Y cells. SH-SY5Y cells were treated as described in Fig. 1. NO (A), MDA (B), GPx activity (C), and GDNF (D) levels were measured as described in Materials and Methods section. Data from three independent experiments are presented as the means  $\pm$  SD. (\*  $p < 0.05$  relative to MPP<sup>+</sup>-treated cells).



Fig. 1

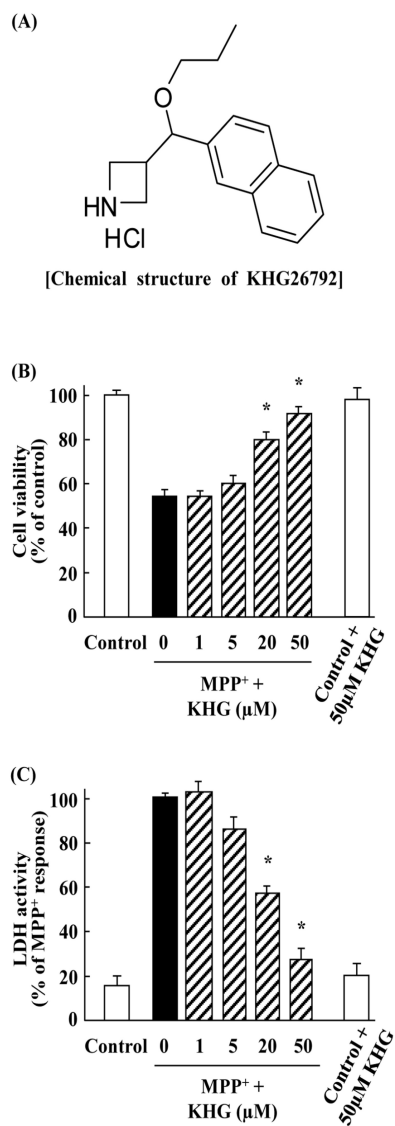


Fig. 1.

Fig. 2

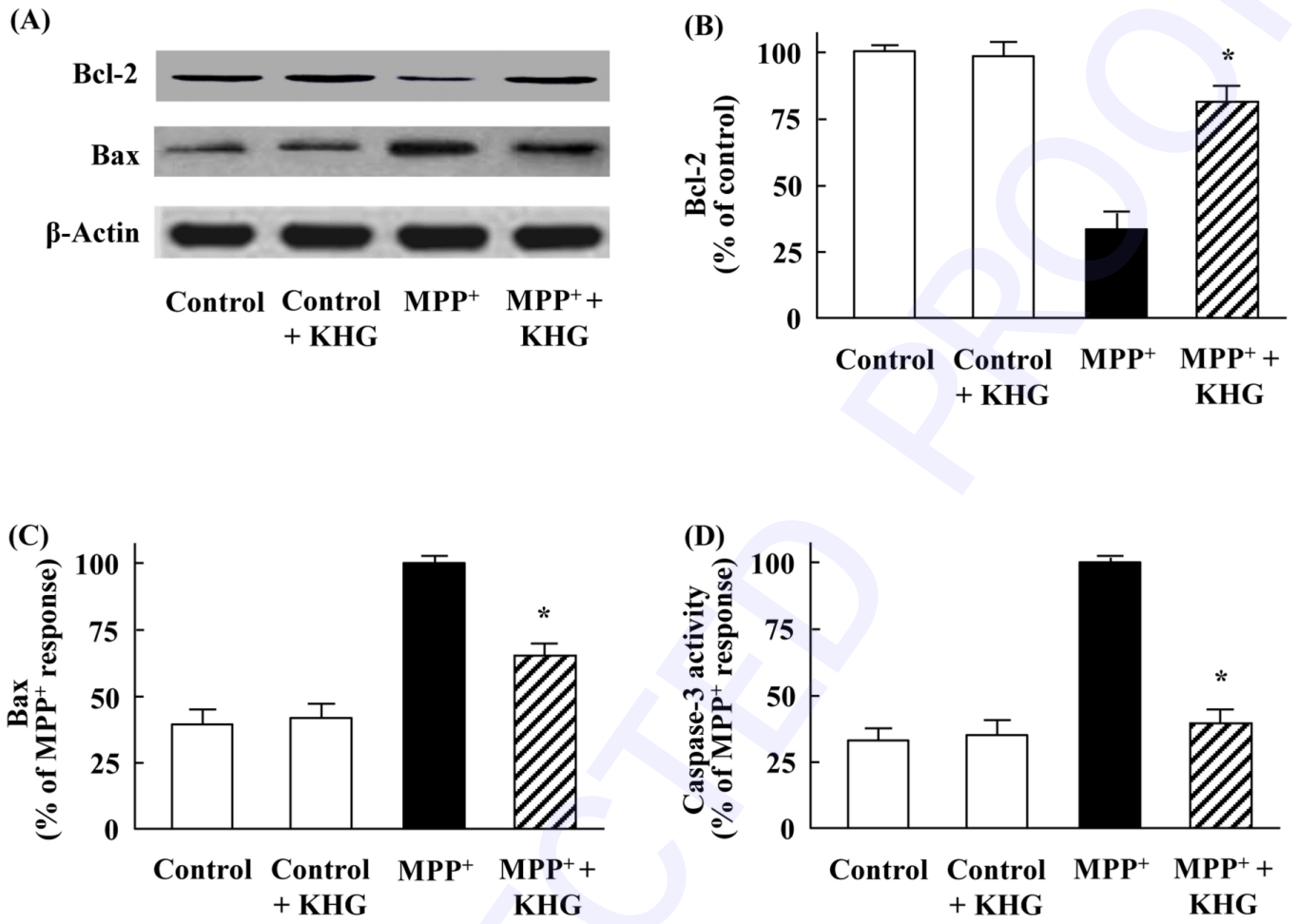


Fig. 2.

Fig. 3

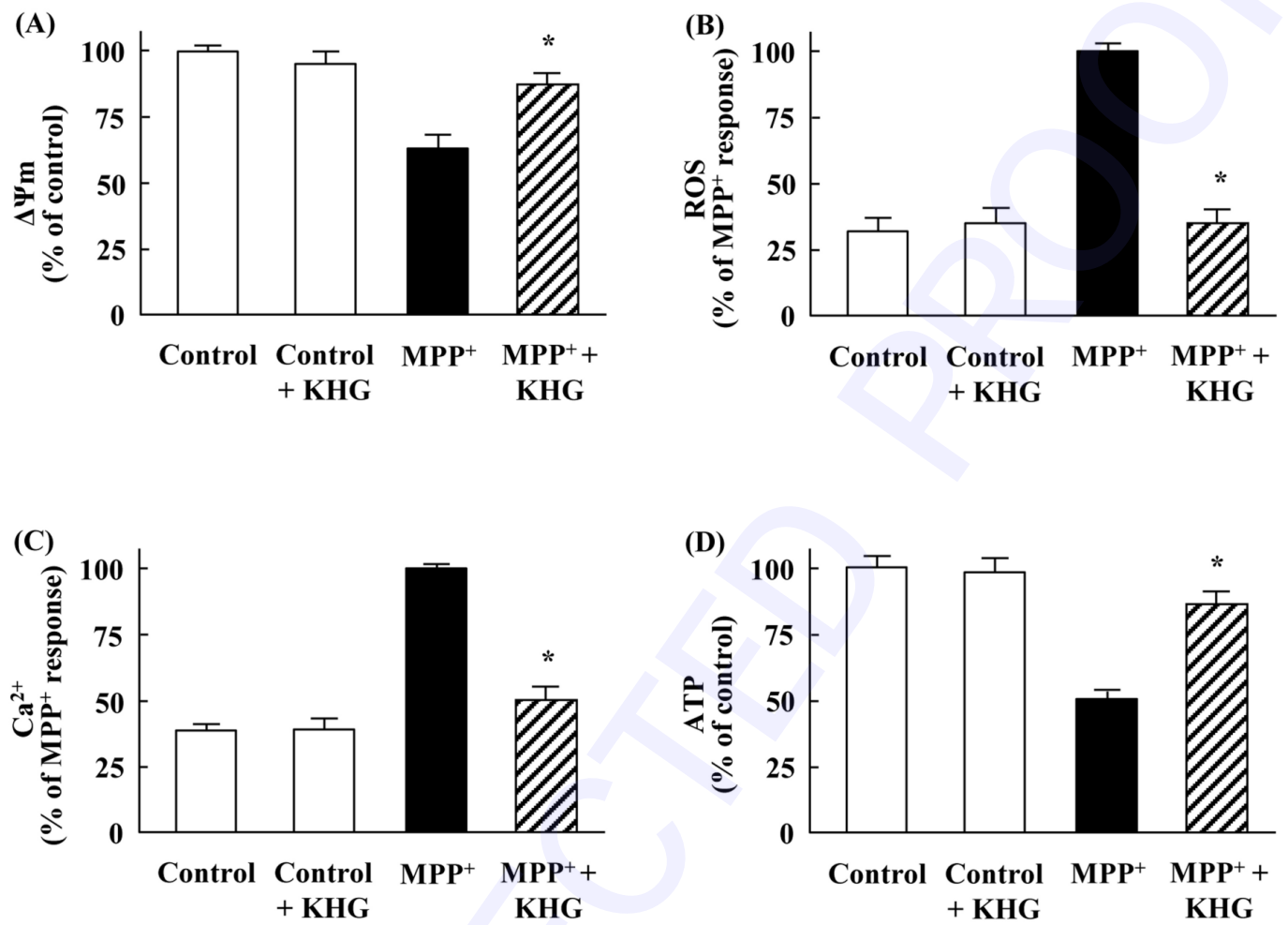


Fig. 3.

Fig. 4

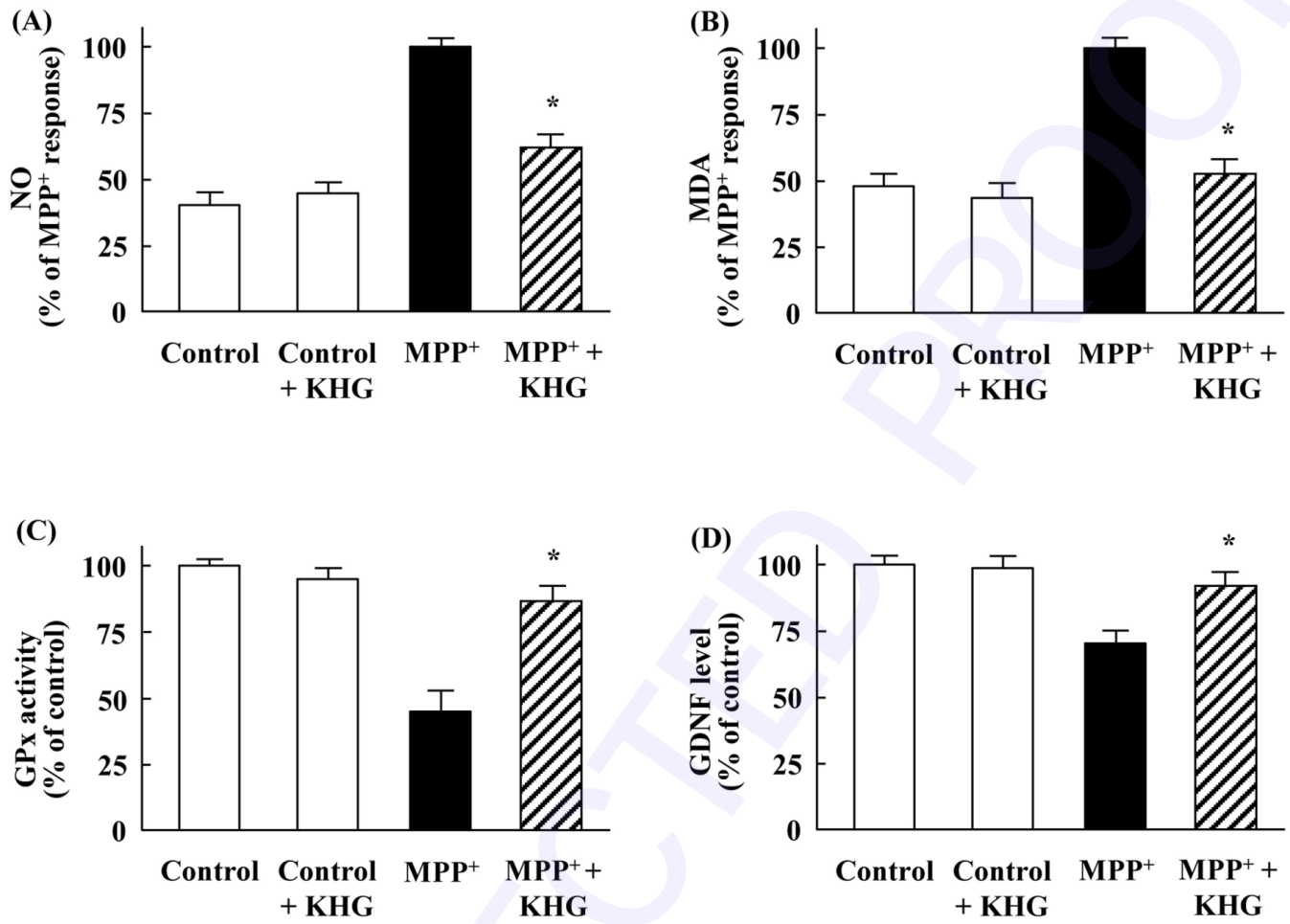


Fig. 4.