COA-Cl evokes protective responses against H 2 O 2 -and 6-OHDA-induced toxic injury in PC12 cells

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Abstract

COA-Cl, a novel adenosine-like nucleic acid analog, has recently been shown to exert neuroprotective effects and to increase dopamine levels both *in vivo* and *in vitro*. Therefore, we hypothesized that COA-Cl could protect dopaminergic neurons against toxic insults. Thus, the present study aimed to investigate the protective effects of COA-Cl against hydrogen peroxide (H_2O_2)- and 6-hydroxydopamine (6-OHDA)-induced toxicity in PC12 cells, and to elucidate the possible mechanisms. PC12 cells were incubated with COA-Cl (100 μM) with or without H_2O_2 or 6-OHDA (200 μM) for 24 h. Exposure to H_2O_2 resulted in a decrease in cell viability in a dose-dependent manner. H_2O_2 enhanced lactate dehydrogenase (LDH) release, reactive oxygen species (ROS) production and caspase-3 activity, and decreased superoxide dismutase (SOD) activity and the Bcl-2/Bax protein ratio. H_2O_2 also triggered apoptosis as measured by TUNEL analysis. However, treatment with COA-Cl attenuated the decrease in cell viability, SOD activity and the Bcl-2/Bax ratio caused by H_2O_2. In addition, COA-Cl attenuated the increase in LDH release, ROS production, caspase-3 activity and apoptosis. 6-OHDA also dose-dependently decreased cell viability and the Bcl-2/Bax ratio, and increased LDH release. However, COA-Cl greatly protected PC12 cells against the toxicity caused by 6-OHDA, as evidenced by an increase in cell viability and the Bcl-2/Bax ratio, and a decrease in LDH release. Our results are the first to demonstrate that COA-Cl potentially protects PC12 cells against toxicity induced by H_2O_2 and 6-OHDA, implying that COA-Cl could be a promising neuroprotective agent for the treatment of Parkinson's disease.

Introduction

COA-Cl (6-amino-2-chloro-9-[trans-trans-2,bis (hydroxymethyl)cyclobutyl] purine) is a novel adenosine-like nucleic acid analog, which has recently been shown to have a number of beneficial effects in cell cultures and animal models. For example, COA-Cl promotes peri-infract angiogenesis and synaptogenesis both *in vivo and in vitro* (Tsukamoto et al., 2010; Okabe et al., 2013; Sakamoto et al., 2021). COA-Cl also exerts neuroprotective effects and enhances spatial memory (Kishimoto et al., 2018). Recent studies have demonstrated a remarkable beneficial effect of COA-Cl on acute ischemic diseases in mice, including myocardial infarction and stroke (Tsukamoto et al., 2010; Okabe et al., 2013; Nishikido et al., 2019). COA-Cl significantly reduces infarct volume and brain edema, and improves neurological deficits, in animal models of brain ischemia and hemorrhage (Okabe et al., 2013; Feng et al., 2016). Moreover, COA-Cl has been shown to reduce perihematomal TUNEL- and 8-hydroxydeoxyguanosine (8-OHdG)-positive cells in a rat model of intracerebral hemorrhage, indicating that COA-Cl may exert an anti-oxidative effect on hemorrhagic stroke (Feng et al., 2016). However, the neuroprotective effects of COA-Cl against toxic insults and its potential mechanisms in neuron-like PC12 cells are far from being understood.

Hydrogen peroxide (H_2O_2) has been extensively used as an inducer of oxidative stress in *in vitro* models (Satoh et al., 1996; Cheng et al., 2010). H_2O_2 can elicit apoptosis *in vitro* by altering cellular antioxidant defenses, inducing ROS generation and damaging DNA, which, in turn, lead to mitochondrial dysfunction, cell damage, and death. 6-hydroxydopamine (6-OHDA) has long been used as a model neurotoxin to

investigate Parkinson's disease (PD) pathology (Blum et al., 2001; Simola et al., 2007). 6-OHDA selectively damages dopaminergic neurons, which produces PD-like symptoms (Burns et al., 1983; Latchoumycandane et al., 2011; Ma et al., 2021). Its neurotoxic effects have been related to the production of reactive oxygen species (ROS) that cause oxidative stress, mitochondrial damage and, ultimately, apoptotic cell death in neurons (Tsao et al., 1996; Bernstein et al., 2011; Pantic et al., 2021). Oxidative stress results from an imbalance between ROS and antioxidant molecules (Poljsak et al., 2013), and excess ROS production is associated with oxidative stress and mitochondrial anomalies (Nesi et al., 2017; Sundqvist et al., 2017; Franco-Iborra et al., 2018). Apoptosis is largely mediated by the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax (Tsujimoto, 1998; D'orsi et al., 2017). Caspase-3, a key component of the apoptotic process, is activated to generate cleaved-caspase-3, leading to DNA degradation or cell death (Choudhary et al., 2015; Porter and Janicke, 1999). It has been demonstrated that cell apoptosis caused by oxidative stress may be related to various neurodegenerative diseases, including Alzheimer's disease and PD (Albers and Beal, 2000; Dias et al., 2013). Therefore, blocking cleaved-caspase-3 and Bax, or Bcl-2 upregulation, can promote neuronal apoptosis.

Oxidative stress has long been associated with the loss of dopaminergic neurons in PD (Dauer and Przedborski, 2003; Michel et al., 2016; Trist et al., 2019). COA-Cl, on the other hand, promotes dopamine (DA) release through the phosphorylation of tyrosine hydroxylase in PC12 cells (Jamal et al., 2019). This finding suggests a potential therapeutic role for COA-Cl in the treatment of PD associated with low dopamine, and warrants investigation of its neuroprotective effects in PC12 cells as a model of PD. To this end, the current study aimed to assess whether COA-Cl exerts salutary effects on the neurotoxicity caused by H_2O_2 or 6-OHDA in PC12 cells, and to identify its underlying mechanisms, with a focus on oxidative stress and apoptosis. PC12 cells have been used extensively as a dopaminergic nerve cell model for PD. Thus, we chose PC12 cells as a neuronal model in the present study to investigate how COA-Cl exerts its neuroprotective effects. Thus, the current study, for the first time, applied the dopaminergic model of PC12 cells to test the effects of COA-Cl on toxin-evoked cellular dysfunction and apoptosis.

Material And Methods

Reagents

COA-Cl was synthesized as described previously (Sakakibara et al., 2015). Dulbecco's modified Eagle's medium (DMEM), H_2O_2 and 6-OHDA were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Bcl-2 and Bax polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). CCK-8 (Cell Counting Kit-8), superoxide dismutase (SOD) assay kit-WST, and cytotoxicity lactate dehydrogenase (LDH) assay kit-WST were purchased from Dojindo (Kumamoto, Japan). The DeadEnd™ Fluorometric TUNEL System kit was purchased from Promega (Madison, WI, USA) and the OxiSelect Intracellular ROS assay kit was purchased from Cell Biolabs (San Diego, CA). The Caspase-3 fluorometric assay kit was purchased from MBL (Nagoya, Japan).

PC12 cell culture and drug treatments

PC12 cells were obtained from the Riken BRC cell bank (Ibaragi, Japan). The cells were grown in DMEM supplemented with 10% fetal bovine serum, 5% horse serum, penicillin (100 U/ml), and streptomycin (50 U/ml) at 37°C in a humidified incubator containing 5% CO_2. The cells were incubated with COA-Cl (100 μM) with or without H_2O_2 (200 μM) or 6-OHDA (200 μM) for 24 h. Untreated cells and cells treated with H_2O_2 or 6-OHDA alone were used as normal and H_2O_2 or 6-OHDA controls, respectively. To confirm the non-cytotoxic doses of COA-Cl, PC12 cells were treated with four different concentrations of COA-Cl (10–200 μM). To investigate the neurotoxicity of H_2O_2 or 6-OHDA, the PC12 cells were treated with four different concentrations of H_2O_2 (50–500 μM) or 6-OHDA (50–500 μM) for 24 h.

Cell viability assay

Cell viability was measured by using CCK-8. Briefly, PC12 cells were seeded in a 96-well plate at a density of 2 x 10^4 cells/well. After an overnight incubation, the cells were incubated with COA-Cl with or without H_2O_2 or 6-OHDA for 24 h. Following this, 1/10 volumes of CCK-8 solution/well were added and the samples were incubated for another 2 h. Absorbance was measured at 450 nm using a microplate reader (SH-9000Lab, Corona), which reflects the cell growth condition. Control cells were treated in the same way without H_2O_2 or 6-OHDA, and absorbance values were expressed as a percentage of the control.

LDH release assay

A LDH assay was used to measure cell membrane damage as a function of the amount of cytoplasmic LDH released into the medium. LDH content in the culture supernatant was quantified using a LDH assay kit according to the manufacturer's instructions. Briefly, PC12 cells were spun down and supernatants were transferred into new wells. The absorbance was read at 440 nm and all values of % LDH released were normalized to the control. All LDH values were expressed as a percentage compared with the control.

Immunoblot for Bcl-2 and Bax

PC12 cells were lysed in RIPA lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium dodecyl sulfate, and protease inhibitor cocktail. Protein concentration was determined using the Bio-Rad Protein Assay according to the manufacturer's instructions. Equal amounts of protein samples were electrophoresed and transferred to a polyvinylidene difluoride membrane. The blots were blocked at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20. Membranes were subsequently incubated at 4°C overnight with rabbit anti-Bax and anti-Bcl-2 antibodies (1:1000, Cell Signaling Technology). The immunoblots were then incubated with a

horseradish peroxidase-conjugated anti-rabbit IgG (1:3000; GE Healthcare Japan, Tokyo, Japan), and the signals were visualized using ECL-plus detection reagents (GE Healthcare Japan). The chemiluminescent signals from the protein blots were detected using a ImageQuant LAS 4000 (GE Healthcare Japan).

SOD enzyme activity

Intracellular SOD activity was measured using a SOD assay kit-WST at a wavelength of 450 nm according to the manufacturer's protocol. In brief, the cells were washed with PBS and lysed by sonication. After centrifugation, the supernatant was used to measure SOD activity. Values for each treatment group are expressed as a percentage of the control value.

ROS activity

ROS production was determined using a 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence probe. Briefly, the cells were incubated with DCFH-DA at 37 ^{o}C for 30 min, and then treated with COA-Cl with or without H_2O_2 after change of medium. At the end of treatment, cells were washed with PBS and then lysed with RIPA buffer. The fluorescence intensity of DCFH-DA in lysate was measured using a microplate reader. An excitation wavelength of 480 nm and an emission wavelength of 530 nm were selected to determine the fluorescence intensity. All ROS values were expressed as a percentage compared with the control.

TUNEL assay

TUNEL analysis was performed to measure the DNA fragmentation in PC12 cells as described previously (Takata et al., 2005). Briefly, the cells were plated at a density of 2 x 10^4 cells/well onto 8-chamber polylysine mimic-coated glass slides and treated with COA-Cl with or without H_2O_2 for 24 h. Subsequently, the treated-cells were fixed in 4% methanol-free formaldehyde solution in PBS (pH 7.4) at 4°C for 25 min. The fixed cells were permeabilized in lysis buffer (0.2% Triton X-100) for 5 min at room temperature and then degraded DNA strands were labeled with the TUNEL reaction mixture for 1 h. After stopping the reaction with 2× SSC, the cells were stained with propidium iodide and analyzed using fluorescence microscopy. Apoptotic cells were detected as localized bright green cells (positive cells) on a red background. At least four fields were observed and more than 500 cells were counted to determine a statistically significant percentage of apoptotic cells.

Caspase-3 activity

A caspase-3 assay kit was used for the measurement of caspase-3 enzymatic activity. Briefly, cultured PC12 cells were lysed for 10 min on ice, and the lysates were centrifuged (12,000 rpm) for 5 min at 4°C.

The supernatant and the reaction buffer (containing caspase-3 substrate) were mixed and incubated for 1 h at 37°C. Fluorescence intensity (380 nm excitation and 460 nm emission) was measured using a spectrophotometer (SH900Lab). All caspase-3 values were expressed as a percentage compared with the control.

Statistical analysis

All data were presented as mean ± SE for at least four repeated experiments. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by the post hoc Scheffe test or Student's *t*-test, where appropriate, using Statcel3 software (OMS, Saitama, Japan). A *P*-value < 0.05 was considered statistically significant.

Results

COA-Cl attenuates H_2O_2- and 6-OHDA-induced viability

We investigated the viability of PC12 cells after incubation with various concentrations of H_2O_2 COA-Cl (10–200 μM), (50–500 μM) and 6-OHDA (50–500 μM) as shown in Figs. 1A–C. The treatment of PC12 cells with COA-Cl had no effect on cell viability at concentrations up to 200 μM (Fig. 1A), indicating that COA-Cl was not toxic to PC12 cells under these treatment conditions. However, a significant decline in PC12 cell viability was seen following 24 h of incubation with an increasing concentrations of H_2O_2 and 6-OHDA up to 500 μM (Figs. 1B,C). The decrease in cell viability was found to occur in a dose-dependent manner. Interestingly, the decrease in cell viability mediated by H_2O_2 and 6-OHDA was drastically increased by 67.9% and 62. %, respectively, in the presence of COA-Cl (100 μM; Figs. 1D,E). Although 500 μM H_2O_2 and 6-OHDA induced the largest decrease of cell viability, we chose to use H_2O_2 and 6-OHDA at a dose of 200 μM for further experiments.

COA-Cl attenuates H_2O_2- and 6-OHDA-induced cell injury

To further explore the protective effects of COA-Cl, we examined the release of LDH, an indicator of cell injury in PC12 cells. Exposure of the cells to H_2O_2 or 6-OHDA at 200 μM increased LDH leakage compared to the control (Figs. 2A,B), but this value was markedly reduced by 20.1% and 31.6% in the H_2O_2 and 6-OHDA groups, respectively, after COA-Cl treatment at 100 μM (Figs. 2A,B). These results show that COA-Cl has potent protective effects against H_2O_2- and 6-OHDA-induced cell injury.

COA-Cl inhibits H_2O_2-induced intracellular ROS production

As shown in Fig. 3, the cells exposed to H_2O_2 (200 μM) exhibited an increase in intracellular ROS levels compared to the control. However, COA-Cl (100 μM) treatment significantly attenuated H_2O_2-induced ROS

accumulation by 43.0% compared to incubation with H_2O_2 alone. This result indicates that the treatment of cells with COA-Cl inhibits ROS production induced by H_2O_2.

COA-Cl attenuates H_2O_2- and 6-OHDA-induced apoptotic proteins

We used western blots to further investigate the effects of COA-Cl on apoptosis induced by H_2O_2 and 6-OHDA. H_2O_2 and 6-OHDA (200 μM) significantly decreased the Bcl-2/Bax ratio compared to the control group (Figs. 4B,D). However, COA-Cl (100 μM) treatment significantly increased the Bcl-2/Bax ratio by 21.7% and 102.1% in the H_2O_2 and 6-OHDA groups, respectively, compared incubation with H_2O_2 or 6-OHDA alone. COA-Cl itself had no effect on the Bcl-2/Bax ratio (data not shown).

COA-Cl attenuates H_2O_2-induced apoptosis

To further investigate the protective effect of COA-Cl on H_2O_2-induced apoptosis in PC12 cells, a TUNEL assay was performed. A few positive-staining cells were noted in the control cultures as shown in Fig. 5A. H_2O_2 (200 μM) significantly increased the percentage of apoptotic PC12 cells compared to the control (Figs. 5A,B). However, treatment with 100 μM COA-Cl significantly suppressed the increase in TUNEL-positive apoptotic cells by 52.5% compared to cells treated with H_2O_2 alone. This result suggests that COA-Cl treatment markedly inhibits H_2O_2-induced apoptosis. In order to further verify the anti-apoptosis effect of COA-Cl, we investigated caspase-3 activity in PC12 cells. It was found that H_2O_2 induces a significant increase in caspase-3 activity compared to the control (Fig. 5C). However, COA-Cl (100 μM) reversed the H_2O_2-induced increase in the activity of caspase-3 by 30.6% compared to cells treated with H_2O_2 alone. This result suggests that COA-Cl can decrease cleaved caspase-3 activity to inhibit H_2O_2-induced apoptosis.

COA-Cl attenuates H_2O_2-induced SOD activity

To further explore the mechanisms involved in the protective action of COA-Cl we monitored the activity of the antioxidant enzyme SOD in PC12 cells. As shown in Fig. 6, H_2O_2 (200 μM) significantly decreased the activity of SOD compared to the control. However, COA-Cl (100 μM) significantly increased SOD activity by 16.6% compared to incubation with H_2O_2 alone, indicating that COA-Cl acts an inducer of antioxidant enzymes *in vitro*.

Discussion

In this study, we utilized PC12 cells to explore the protective properties of COA-Cl against oxidative damage induced by H_2O_2 and 6-OHDA. Both H_2O_2 and 6-OHDA are cytotoxic agents. H_2O_2 is used to cause oxidative damage in cellular models and 6-OHDA is used as an *in vitro* model of DA neuron degeneration. COA-Cl is known to possess antioxidant and neuroprotective effects in both *in vitro* and *in vivo* ischemia models (Feng et al., 2016). In addition, COA-Cl has been shown to induce striatal dopamine release both *in vivo* and *in vitro* (Jamal et al., 2019). Considering these effects, we wanted to test whether COA-Cl could exert neuroprotective effects on dopaminergic neurons in cellular model of PD. Therefore, we chose the PC12 cell line to study the effects of COA-Cl on H_2O_2-induced neurotoxicity. Our results demonstrate that H_2O_2 triggered cell injury, accompanied by an increase in intracellular ROS generation and apoptosis via increasing the Bcl-2/Bax ratio and caspase-3 activity, consistent with previous studies (Matsura et al., 1999; Dumont et al., 1999; Redza-Dutordoir and Averill-Bates, 2017). However, COA-Cl attenuated these effects, indicating that COA-Cl can inhibit H_2O_2-induced neurotoxicity, most likely by preventing oxidative stress and apoptosis. In accordance with the above findings, 6-OHDA dose-dependently decreased cell viability and the ratio of Bcl-2/Bax protein, and increased LDH release. However, these changes were also attenuated by treatment with COA-Cl. These data provide the first evidence that COA-Cl protects PC12 cells against H_2O_2- and 6-OHDA-induced oxidative injury and cell demise. Thus, our findings raise the possibility of the therapeutic application of COA-Cl for treating PD.

Several lines of evidence have confirmed that H_2O_2 and 6-OHDA produce oxidative stress, which is closely associated with neuronal damage in various neurodegenerative diseases, including PD (Milton, 2004; Simola et al., 2007; Yang et al., 2016; Lee et al., 2021). COA-Cl exhibits neuroprotective and anti-oxidative effects, which may prove beneficial in intracerebral hemorrhage and ischemic stroke (Okabe et al., 2013; Feng et al., 2016). PC12 cells are widely used as neuronal cell models *in vitro* and exhibit some features of mature dopaminergic neurons (Greene and Tischler, 1976; Duan et al., 2015; Heusinkveld et al., 2017). To explore whether COA-Cl might protect dopaminergic neurons against oxidative stress-induced damage, we investigated the effects of COA-Cl in H_2O_2- and 6-OHDA-treated PC12 cells. It was found that treatment with COA-Cl produced significant neuroprotection against injury in PC12 cells.

PC12 cells were treated with H_2O_2 and 6-OHDA at concentrations ranging from 50–500 μM for 24 h. Our data revealed that H_2O_2 and 6-OHDA produced a loss of cell viability in a concentration-dependent manner, with significant cytotoxicity being observed at a concentration of 200 μM (Figs. 1B,C). The 200 μM of H_2O_2 and 6-OHDA resulted in decrease in cell viability of 42.3 to 43.1% compared to untreated cells (viability of 100%), which are in agreement with other studies (Cho et al, 2008; Oraki Kohshour et al., 2013; Zou et al., 2016; Ramazani et al., 2019). Therefore, we used 200 μM H_2O_2 and 6-OHDA (56.9–57.7% inhibition) to examine the protective effects of COA-Cl in PC12 cells. Our data showed that COA-Cl (100 μM) pretreatment significantly attenuated the decreased cell viability induced by H_2O_2 and 6-OHDA (Figs. 1D,E). We also assayed the LDH released to further investigate the protective effects of COA-Cl against cell injury induced by H_2O_2 and 6-OHDA in PC12 cells. The results revealed that exposure to H_2O_2 and 6-OHDA increased LDH release. Of note, treatment with COA-Cl attenuated the LDH release induced

by H_2O_2 and 6-OHDA (Figs. 2A,B). We also found that COA-Cl (10–200 μM) was non-toxic to PC12 cells (Fig. 1A), indicating that COA-Cl itself did not cause any cell injury, consistent with our previous study (Jamal et al., 2019). H_2O_2- and 6-OHDA-induced alteration of cell viability and LDH release was almost similar, which is in line with earlier evidence demonstrate that H_2O_2 and 6-OHDA produced similar morphological changes in various sites of the brains of cats and rats (Poirier, 1975). Together, these results show that COA-Cl protects PC12 cells against injury caused by H_2O_2 and 6-OHDA and led us to hypothesize that COA-Cl could protect against injury by inhibiting ROS generation.

ROS production causes oxidative stress and mitochondrial dysfunction, leading to cell apoptosis and contributing to many neurodegenerative diseases, including PD (Cui et al., 2004). H_2O_2 generates hydroxyl radicals, which are highly toxic and can cause serious damage to cells, leading to apoptosis or necrosis (McKeague et al., 2003; Minjie et al., 2005). Hence, the removal of excess ROS and the inhibition of its production may have a protective effect on cell death caused by oxidative stress. Thus, in this study, H_2O_2 was used to stimulate PC12 cells to explore whether COA-Cl could prevent H_2O_2-stimulated ROS generation and the resulting oxidative stress. Our data confirmed that H_2O_2 markedly increases intracellular ROS production, which initiates DNA and mitochondrial damage, resulting cell apoptosis (Rhee, 1999; Ding et al., 2016). However, treatment with COA-Cl suppressed the production of intracellular ROS in H_2O_2-treated cells (Fig. 3), suggesting that COA-Cl acts as an anti-apoptotic agent by suppressing ROS under H_2O_2-induced neurotoxic conditions. Collectively, our data strongly suggest that COA-Cl promotes PC12 cells survival by decreasing the amount of ROS, which, in turn, preserves mitochondrial activity.

Antioxidant enzymes, including SOD, play a major role in ROS scavenging and preventing the damage caused by oxidation. Overexpression of SOD protects against oxidative stress-induced cell death and injury (Kiningham et al., 1999). As previously shown, H_2O_2 likely provokes astrocyte death, in part, through a reduction in the activity of the antioxidant enzymes SOD and catalase (Sokolova et al., 2001; Lopez et al., 2007). Thus, we hypothesized that COA-Cl-induced neuroprotection is mediated through a modulation of antioxidant enzymes. Indeed, it was found that H_2O_2 decreased the activity of SOD (Fig. 6), and that this effect was reversed by COA-Cl, suggesting that the neuroprotective effect of COA-Cl is also related to its antioxidant effects. A previous study showed that COA-Cl could effectively reduce the oxidative stress marker 8-OHdG in a rat intracerebral hemorrhage model (Lu et al., 2016). Consistent with this previous finding, the present data strongly suggest that COA-Cl can protect against PD-related neuronal injury by enhancing the antioxidant status via a lowering of the level of ROS and inhibiting the production of oxidative enzymes. It has been shown that expression of SOD in cultured glial cells depends of protein kinase C activation (Huang et al., 2001). Hence, we suggest that COA-Cl is able to stimulate SOD activity in PC12 cells possibly through activation of protein kinase C; however, further investigation is needed to determine how COA-Cl activates SOD.

We also examined the effects of COA-Cl on PC12 cell apoptosis induced by H_2O_2 and 6-OHDA. Previous work has found that H_2O_2 and 6-OHDA induce apoptosis in PC12 cells (Jiang et al., 2003; Hanrott et al.,

2006; Hadipour et al., 2020). Apoptosis occurs by a series of molecular events, including an up-regulation of Bcl-2, a down-regulation of Bax, decreased cytoplasmic release of cytochrome C, and reduced cleavage of caspases 9 and 3. For example, Bcl-2 inhibits apoptosis (Tsujimoto, 1998), whereas Bax promotes apoptosis (Lindenboim et al., 2000. Therefore, the ratio of Bcl-2 to Bax plays a pivotal role in cell survival and death. We hypothesized that COA-Cl could effectively attenuate the apoptosis caused by H_2O_2 and 6-OHDA via an increase in the Bcl-2/Bax ratio and an inactivation of caspase-3. Thus, we investigated if COA-Cl could attenuate apoptosis in H_2O_2-and 6-OHDA-treated PC12 cells. As shown in Fig. 4, both H_2O_2 and 6-OHDA decreased the Bcl-2/Bax expression ratio in PC12 cells, indicating that H_2O_2 and 6-OHDA are able to stimulate mitochondria-dependent apoptosis. However, COA-Cl markedly increased the Bcl-2/Bax ratio (Fig. 4B,D), suggesting that the anti-apoptotic effect of COA-Cl may be associated with its modulation of the expression of the Bcl-2 family. In addition, we evaluated apoptotic cells using TUNEL assays. It was found that COA-Cl significantly decreased the percentage of TUNEL-positive cells (Fig. 5A,B), suggesting that COA-Cl consistently reduces apoptosis in PC12 cells. Our results suggest that the H_2O_2- and 6-OHDA-induced decrease in the Bcl-2/Bax ratio alters mitochondrial membrane permeability, which, in turn, activates the caspase-3 cascade, leading to apoptosis. Several studies have reported that H_2O_2 induces apoptosis through activation of caspase-3 *in vitro* (Juknat et al., 2005; Shin et al., 2009; Matsura et al., 1999; Wu et al., 2011). Thus, we investigated the effects of COA-Cl on H_2O_2-induced caspase-3 activation in PC12 cells. Consistent with previous findings, the present results showed that H_2O_2 induces caspase-3 cleavage in PC12 cells (Fig. 5C). However, COA-Cl attenuated caspase-3 activity, indicating that the protective effect of COA-Cl can be accounted for by an inhibition of caspase-3 activity, likely resulting from oxidative stress suppression. These findings indicate that COA-Cl suppression of the Bcl-2 family in PC12 cells is mediated, at least in part, through the caspase 3-dependent apoptosis pathway. COA-Cl-induced attenuation of the apoptotic and necrotic processes induced by H_2O_2 is mostly in line with the findings from the model of 6-OHDA-induced cell damage. In combination, these results provide the first evidence that COA-Cl can protect against H_2O_2- and 6-OHDA-induced cell death, possibly through the induction of antioxidant enzymes and a regulation of oxidative stress, mitochondrial dysfunction, and apoptotic events.

In conclusion, the novel finding of the current study is that COA-Cl exerts neuroprotective effects against H_2O_2- and 6-OHDA-induced neuronal injury in PC12 cells. The protective effects of COA-Cl may be mediated by preventing oxidative stress and neuronal apoptosis. On the basis of these findings, COA-Cl could be a plausible therapeutic agent for the treatment of PD. However, further research is needed to verify and explore potential mechanisms underlying these findings.

Declarations

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Conflict of interest:

The authors declare that there is no conflict of interest

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Figures

Figure 1

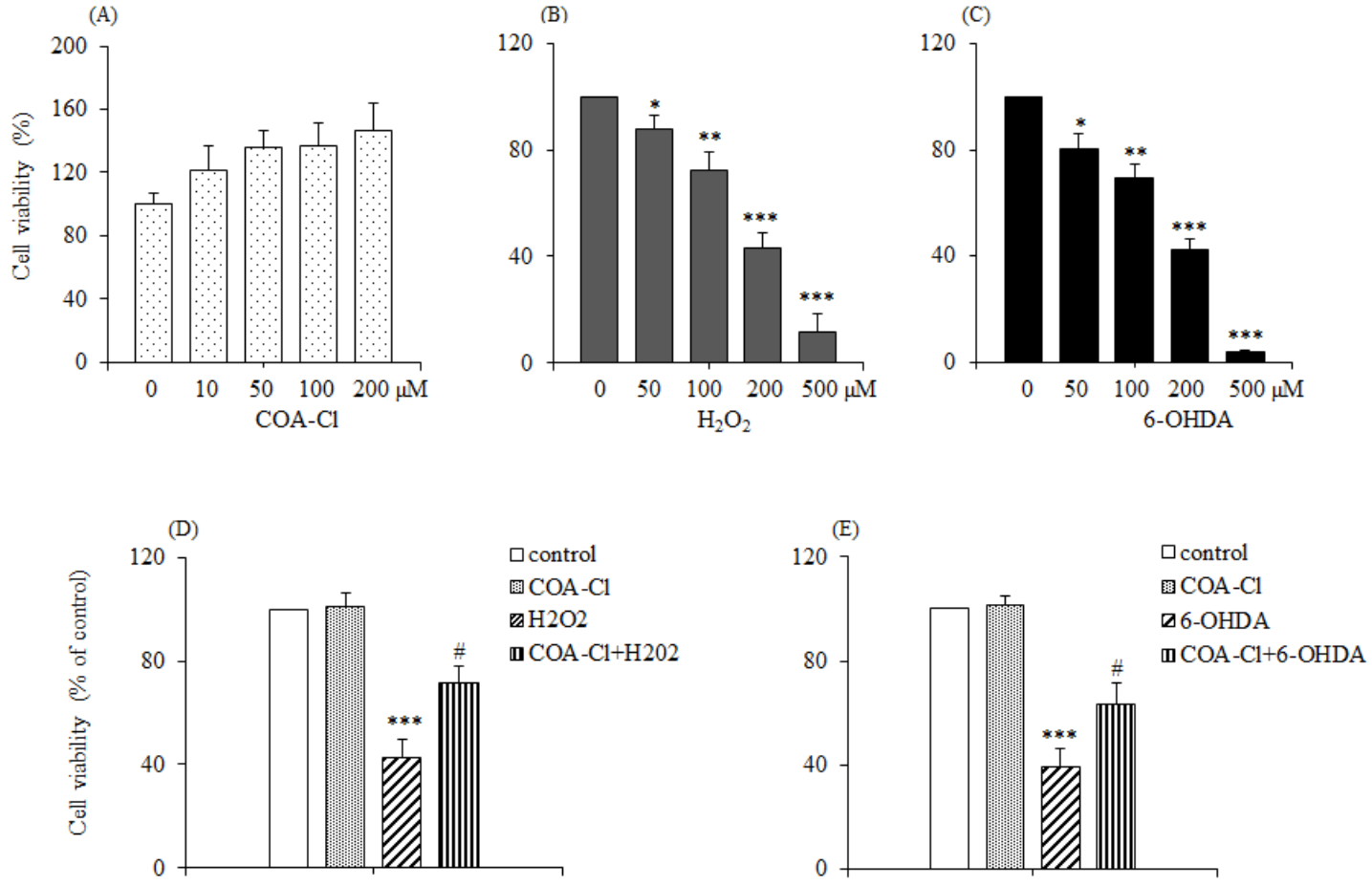

Figure 1

Effect of COA-Cl on cell viability in PC12 cells. Cells were incubated with various concentrations of COA-Cl (0–200 μM) (A), H_2O_2 (0–500 μM) (B) and 6-OHDA (0–500 μM) (C) or COA-Cl (100 μM) alone in combination either with 200 μM H_2O_2 (D) or 6-OHDA (E). All data are shown as mean ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control, # $p < 0.05$ versus H_2O_2 or 6-OHDA alone group. H_2O_2, hydrogen peroxide; 6-OHDA, 6-hydroxydopamine.

Figure 2

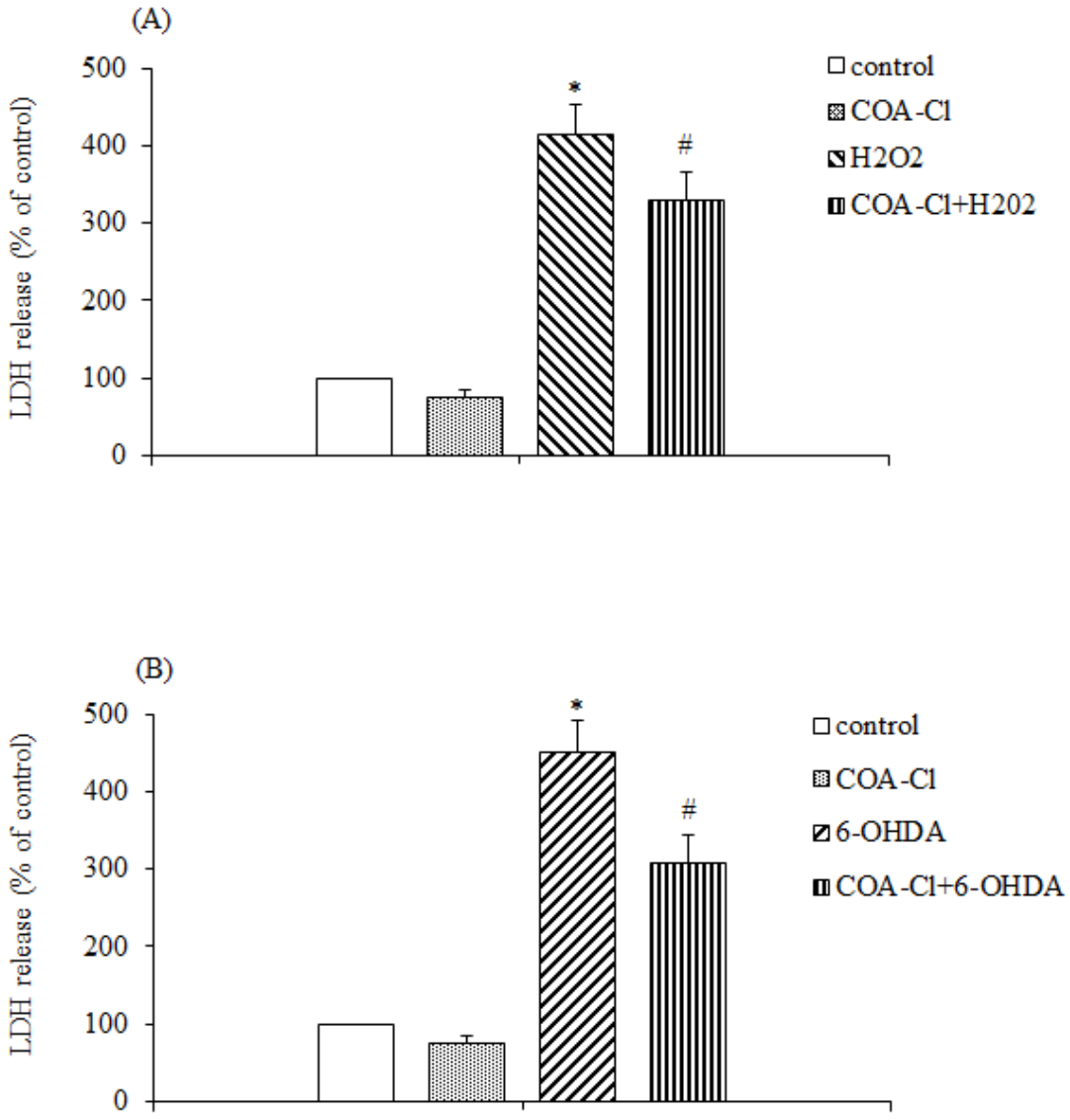

Figure 2

Effect of COA-Cl (100 μM) on LDH release in PC12 cells treated with 200 μM H_2O_2 (A) or 6-OHDA (B). All data are shown as mean ± SE. * p <0.001 versus control; # p <0.05 versus H_2O_2 or 6-OHDA alone group. LDH, lactate dehydrogenase; H_2O_2, hydrogen peroxide; 6-OHDA, 6-hydroxydopamine.

Figure 3

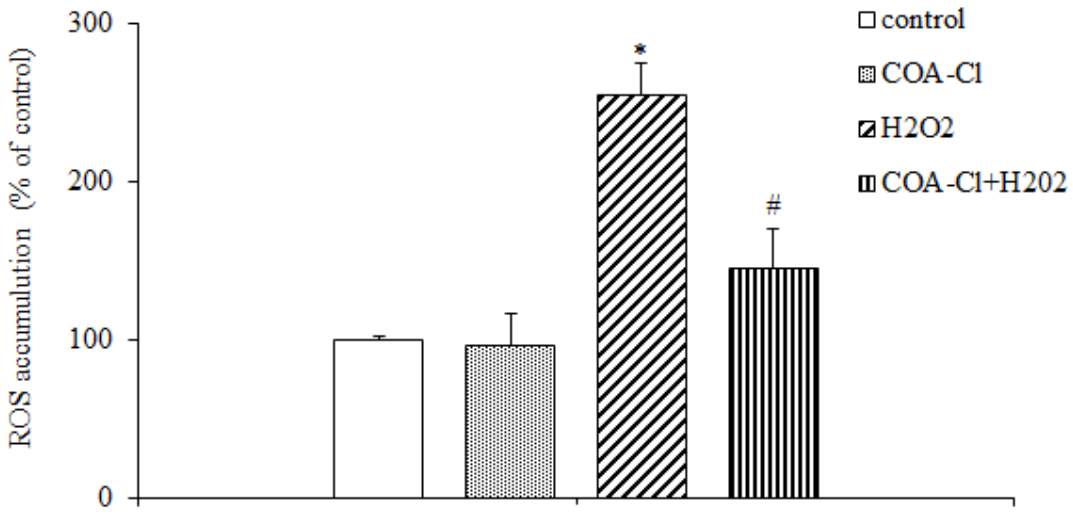

Figure 3

Effect of COA-Cl (100 μM) on ROS accumulation in PC12 cells treated with 200 μM H_2O_2. All data are shown as mean ± SE. * p <0.001 versus control; # p <0.01 versus H_2O_2 alone group. ROS, reactive oxygen species; H_2O_2, hydrogen peroxide.

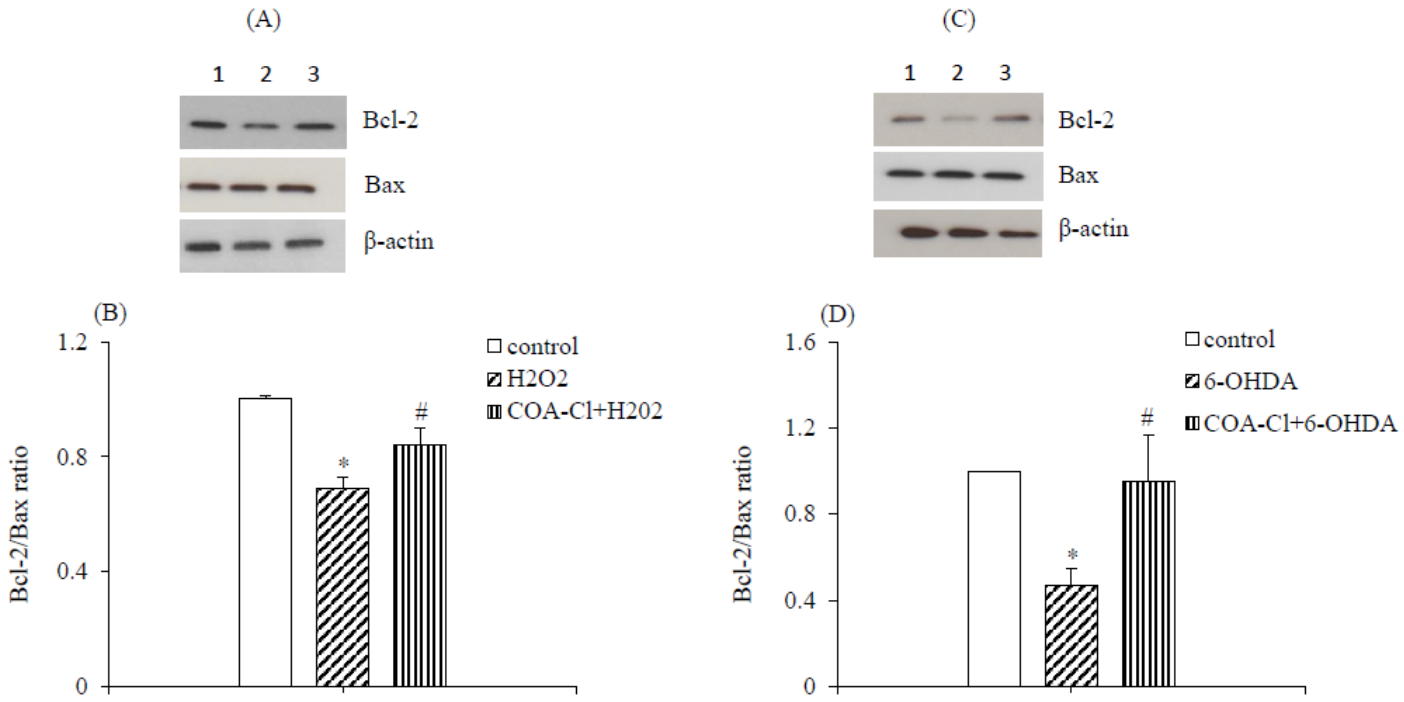

Figure 4

Effect of COA-Cl (100 μM) on expression of apoptotic proteins in PC12 cells treated with 200 μM H_2O_2 or 6-OHDA. Western blot analysis of the expression of Bcl-2/Bax (A, C) and quantitative analysis of Bcl-2/Bax ratio (B, D). All data are shown as mean ± SE. Control (1), H_2O_2 or 6-OHDA (2), COA-Cl + H_2O_2/6-OHDA (3). * $p < 0.001$ versus control; # $p < 0.05$ versus H_2O_2 or 6-OHDA alone group. H_2O_2, hydrogen peroxide; 6-OHDA, 6-hydroxydopamine.

Figure 5

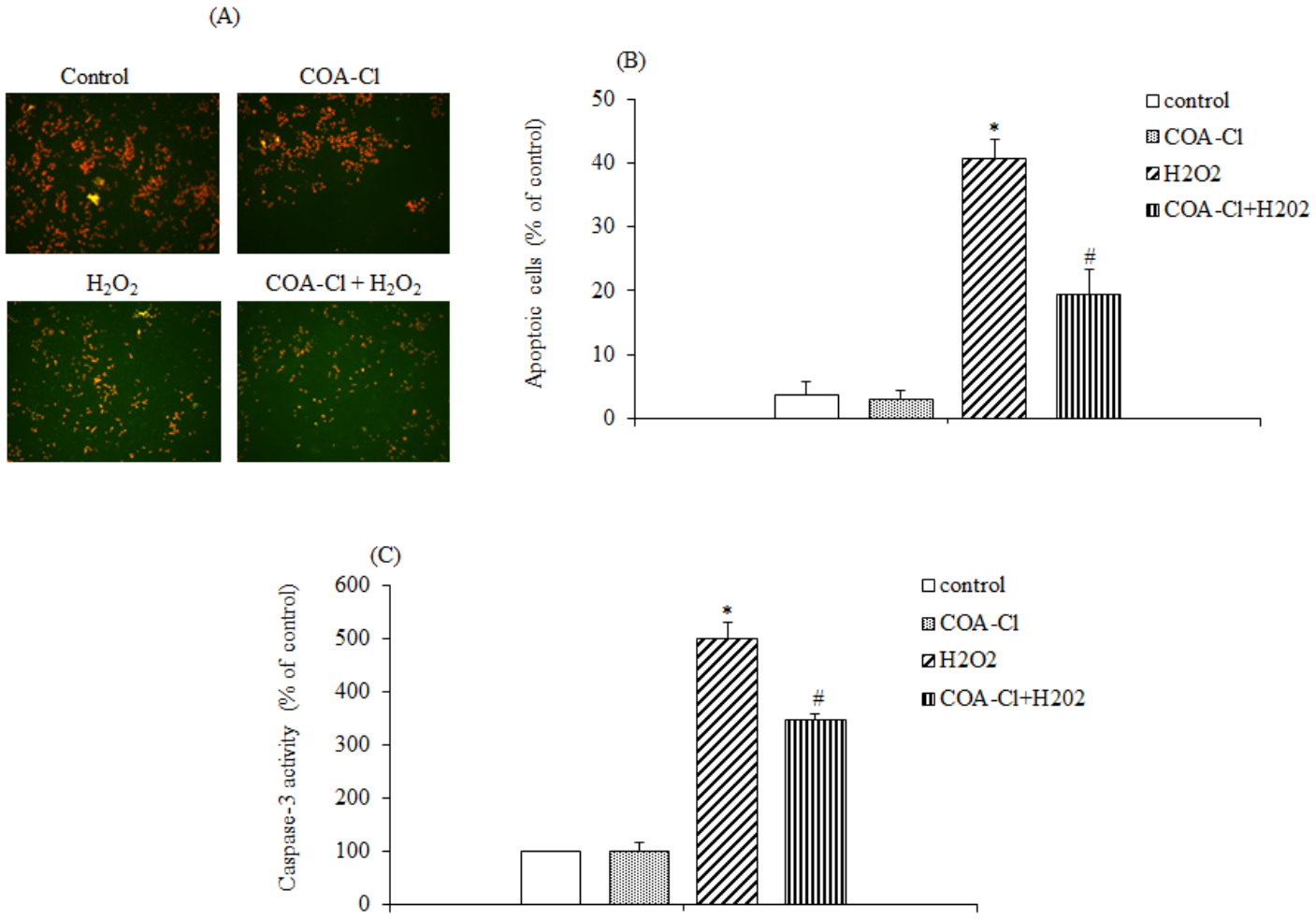

Figure 5

Effect of COA-Cl (100 μM) on 200 μM H_2O_2-induced apoptosis and caspase-3 activity in PC12 cells. Represented images of TUNEL assay (400 X) (A), quantification of TUNEL-positive cells stained red (B), and casepase-3 activity (D). All data are shown as mean ± SE. * p <0.001 versus control; # p <0.01 versus H_2O_2 alone group. H_2O_2, hydrogen peroxide.

Figure 6

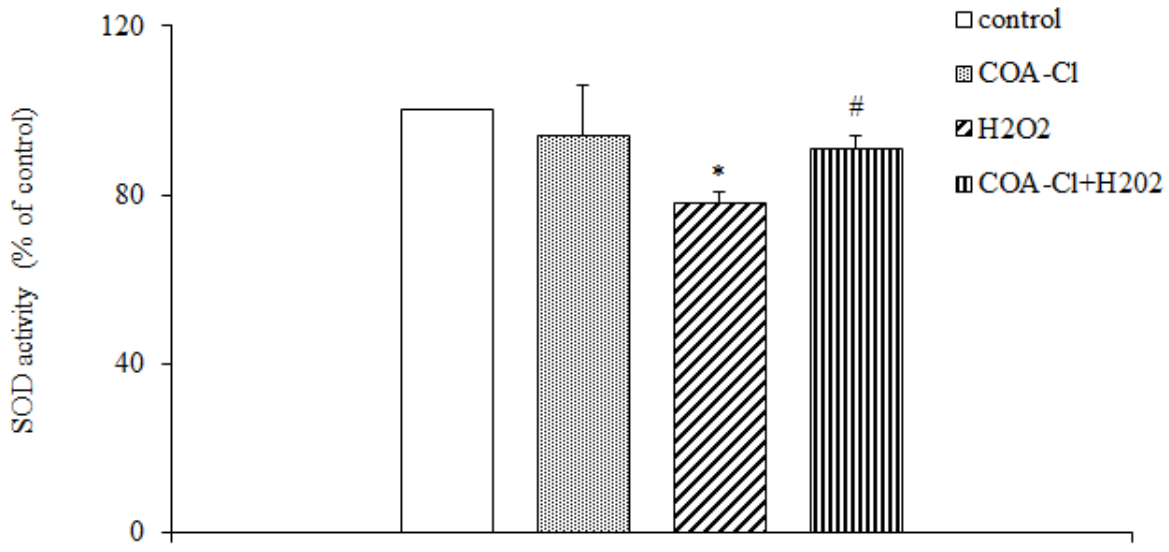

Figure 6

Effect of COA-Cl (100 μM) on SOD activity in PC12 cells treated with 200 μM H_2O_2. All data are shown as mean ± SE. * p <0.01 versus control; # p <0.05 versus H_2O_2 alone group. H_2O_2, hydrogen peroxide; SOD, superoxide dismutase.