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Protective effect of boldine on dopamine-induced membrane permeability transition in brain mitochondria and viability loss in PC12 cells

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Abstract

Boldine ([*S*]-2,9-dihydroxy-1,10-dimethoxyaporphine) has been shown to exert antioxidant and anti-inflammatory effects. The present study elucidated the protective effect of boldine on catecholamine-induced membrane permeability transition in brain mitochondria and viability loss in PC12 cells. Dopamine (200 μM) and 6-hydroxydopamine (6-OHDA, 100 μM) attenuated Ca^{2+} and succinate-induced mitochondrial swelling and membrane potential formation. Boldine (10–100 μM) and 10 μg/mL of superoxide dismutase (SOD) or catalase reduced the effect of catecholamine oxidation on brain mitochondria. Boldine, SOD, and catalase decreased catecholamine-induced mitochondrial cytochrome *c* release. Antioxidant enzymes attenuated the depressant effect of catecholamines on mitochondrial electron flow, whereas boldine did not reduce it. Boldine inhibited the catecholamine-induced decrease in thioredoxin reductase activity and the increase in thiol oxidation in mitochondria. It also showed a scavenging action on hydrogen peroxide and hydroxyl radicals and decreased the formation of melanin from dopamine. Boldine and antioxidant enzymes decreased the dopamine-induced cell death, including apoptosis, in PC12 cells. The results suggest that boldine may attenuate the catecholamine oxidation-induced brain mitochondrial dysfunction and decrease the dopamine-induced death of PC12 cells through a scavenging action on reactive oxygen species and inhibition of melanin formation and thiol oxidation.

Keywords: Boldine; Catecholamines; Brain mitochondria; PC12 cells; Protection

1. Introduction

Mitochondrial dysfunction has been shown to participate in the induction of apoptosis as well as in excitotoxic neuronal death [1,2]. Hydrogen peroxide, one of the oxidation products of dopamine, diffuses partly into the mitochondrial matrix and oxidizes glutathione to glutathione disulfide, which induces the alteration of cellular functions, including suppression of thiol-dependent electron transport [3]. Defects in cellular respiration lead to diminished ATP production, increased sensitivity to oxidative attack, and eventually apoptotic or necrotic neuronal cell death [4]. The major mitochondrial defect in Parkinson's disease appears to be associated with complex I of the electron transport chain. 6-OHDA and 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), inhibit the mitochondrial electron transport chain and induce a syndrome closely resembling Parkinson's disease [5,6].

The membrane permeability transition of mitochondria is known as a central event in the course of a variety of toxic and oxidative forms of cell injury as well as apoptosis. Opening of the mitochondrial permeability transition pore has been shown to induce depolarization of the transmembrane potential, release of small solutes, release of Ca^{2+} and cytochrome *c*, osmotic swelling, and loss of oxidative phosphorylation [7–9]. The permeability transition pore is suggested as another target of the oxidation products of dopamine and MPP^+ in mitochondria [10,11].

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Abbreviations: MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SOD, superoxide dismutase; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); MTT, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide.

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Dopamine oxidation produces free radicals and quinone, which cause a significant increase in the swelling of brain and liver mitochondria [8,10]. The neuronal cell death induced by mitochondrial complex I inhibitors and MPP^+ has been suggested to be mediated by the opening of the permeability pore and the collapse of the mitochondrial membrane potential [2,12,13]. The 6-OHDA- and MPP^+-induced mitochondrial membrane permeability transition was attenuated by the addition of antioxidant enzymes and thiol compounds.

Boldine, an alkaloid found in the leaves and bark of boldo, has been shown to exert anti-inflammatory and antipyretic effects and to have antioxidant ability. The compound reduces paw edema and prostaglandin synthesis in the carrageenan-injected guinea pig [14] and depresses experimentally induced colitis in the rat [15]. Boldine has also been found to inhibit the peroxidative and lytic damage caused by *tert*-butyl hydroperoxide in isolated rat hepatocytes [16] and to attenuate ferric-ATP-, iron-cysteine-, and carbon tetrachloride-induced lipid peroxidation in liver microsomes [17,18]. The compound shows a scavenging effect on hydroxyl radicals, while its effect on superoxide anion and hydrogen peroxide is uncertain [17,19]. In addition, boldine has been postulated to exert anticonvulsant and anti-nociceptive effects [20], and although the central effect is not well understood, boldine has a binding affinity to α_1-adrenergic and dopaminergic receptors [21,22].

Antioxidants and monoamine oxidase inhibitors may provide a protective effect on the degeneration of striatal dopaminergic neurons. We have shown that harmalol, which has antioxidant ability and inhibitory action on monoamine oxidase, decreases MPTP-induced neurotoxicity in the mouse [11]. Boldine also has been suggested to exert antioxidant and anti-inflammatory effects. Therefore, the present study examined the protective effect of boldine on dopamine- and 6-OHDA-induced membrane permeability transition in brain mitochondria and PC12 cell death.

The results demonstrated that boldine may attenuate the catecholamine oxidation-induced damage of brain mitochondria and decrease the dopamine-induced death of PC12 cells through a scavenging action on reactive oxygen species and inhibition of melanin formation and thiol oxidation. The boldine alkaloids appear to exhibit a protective effect on neuronal damage associated with catecholamine oxidation.

2. Materials and methods

2.1. Materials

Boldine, dopamine (3-hydroxytyramine), 6-OHDA, SOD (from bovine erythrocytes; 2500–7000 U/mg of protein), catalase (from bovine liver; 10,000–25,000 U/mg of protein), rotenone, antimycin A, myxothiazol, pyruvate, malate, succinic acid, safranin O, melanin, DTNB, NADPH, 2-deoxy-D-ribose, DMSO, mannitol, MTT, heat-inactivated horse serum, and DMEM/F12 medium were purchased from Sigma–Aldrich Inc. An ApoAlert CPP32/caspase-3 assay kit was obtained from CLONTECH Laboratories Inc., fetal bovine serum from Gibco-BRL, and a protein assay kit from Bio-Rad Laboratories. All other reagents were of analytical grade.

2.2. Preparation of rat brain mitochondria

Mitochondria were prepared from the brains (cerebrum) of male Sprague–Dawley rats, weighing between 150 and 200 g, according to the method of Clark and Nicklas [23]. The cerebrum was removed and homogenized in 9 vol. of ice-cold medium (250 mM sucrose, 0.5 mM EDTA, and 10 mM Tris–HCl, pH 7.4). The homogenate was cleared by centrifugation at 2000 *g* for 2 min at 4°, and then the supernatant was centrifuged at 12,500 *g* for 8 min at 4°. The mitochondrial pellet was resuspended in 10 mL of a 3% Ficoll medium (3% Ficoll, 120 mM mannitol, 30 mM sucrose, and 25 μM EDTA, pH 7.4). The suspension was carefully layered onto 20 mL of a 6% Ficoll medium (6% Ficoll, 240 mM mannitol, 60 mM sucrose, and 50 μM EDTA, pH 7.4) and centrifuged at 11,500 *g* for 30 min at 4°. The mitochondrial pellet was resuspended in a KCl–Tris medium (120 mM KCl and 50 mM Tris–HCl, pH 7.4) and centrifuged at 12,500 *g* for 10 min at 4°. The final pellet was suspended in KCl–Tris medium, and protein concentration was determined by the method of Bradford as described in the Bio-Rad protein assay kit.

Animals were cared for in accordance with the NIH guidelines and according to the regulations of our university. Rats were maintained under a 12-hr light and dark cycle in a temperature-regulated (23 ± 1°) animal room with water and food continuously available.

2.3. Measurement of mitochondrial swelling

Mitochondrial swelling was assayed by measuring the decrease in absorbance at 540 nm [24]. The assay mixture contained mitochondria (1 mg of protein/mL), 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 mM succinate, 50 μM $CaCl_2$, 5 μM rotenone, and 10 mM HEPES, pH 7.4. Swelling was induced by the addition of Ca^{2+} in a 30° medium, and absorbance change was measured using a spectrophotometer (Beckman Instruments Inc., DU-70).

2.4. Measurement of membrane potential

The change of membrane potential in mitochondria (1 mg of protein/mL) was assayed by measuring the fluorescence change of safranin O (10 μM) under the same experimental conditions without calcium, as described in the assay to determine swelling [24]. Fluorescence change

was measured at an excitation wavelength of 495 nm and an emission wavelength of 586 nm using a luminescence spectrophotometer (Aminco-Bowman Series 2).

2.5. Measurement of cytochrome c release

Mitochondria were treated with catecholamines for the indicated time, and then the amount of cytochrome *c* in the supernatant, obtained by centrifuging the mixture at 15,000 *g* for 10 min at 25°, was determined. Mitochondria (1 mg of protein/mL) were incubated in a reaction mixture containing 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 3 μM rotenone, 0.8 μM antimycin A, 6 μM myxothiazol, and 5 mM HEPES, pH 7.2, and treated with 100 μM dopamine (or 6-OHDA) for 10 min at 30°. The reaction was performed by the sequential addition of 0.1 mM potassium ferricyanide, 1 mM potassium cyanide, and 0.1 mM sodium dithionite [25–27]. The change in absorbance was measured at 550–540 nm using a dual wavelength spectrophotometer (Aminco Chance). The amount of cytochrome *c* was determined using a molar extinction coefficient of 1.91×10^4 $(M\ cm)^{-1}$.

2.6. Measurement of mitochondrial electron flow

The conversion of the MTT dye to formazan crystals in cells has been shown to be related to the mitochondrial redox state and respiratory chain activity [27,28]. On the basis of these reports, mitochondrial electron flow was measured using MTT. The mitochondrial fraction (0.2 mg of protein/mL) was suspended in a reaction mixture containing 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.2, and treated with 100 μM catecholamines for 10 min at 27°. The reaction was performed by the sequential addition of MTT (0.11 mg/mL) and pyruvate/malate (5 mM each), and absorbance was measured at 592 nm.

2.7. Measurement of mitochondrial thioredoxin reductase activity

Thioredoxin reductase activity was measured as the reduction of DTNB in the presence of NADPH [10,29]. Mitochondria (1 mg of protein/mL) were treated with 100 μM dopamine (or 6-OHDA) in a reaction mixture containing 120 mM KCl, 0.1 mM sodium azide, and 50 mM potassium phosphate, pH 7.0, for 30 min at 37°. The reaction was stopped by the addition of 10 μg/mL of catalase. Mitochondrial suspensions were sonicated two times at 55 W for 15 s using a sonifier cell disruptor (Branson sonifier, model W185D).

Disrupted mitochondrial suspension (0.6 mL) was added to the enzyme assay mixture (3 mL) containing 1 mM EDTA, 0.25 mM NADPH, and 1 mM DTNB. The absorbance change was measured at 412 nm, and the activity was determined using a molar extinction coefficient of 1.36×10^4 $(M\ cm)^{-1}$. One unit was defined as 1 nmol 5-thio-2-nitrobenzoic acid formed/1 min.

2.8. Measurement of mitochondrial thiol content

Brain mitochondria (1 mg of protein/mL) suspended in 100 mM Tris–HCl buffer medium, pH 7.4, were treated with 100 μM dopamine (or 6-OHDA) for 1 hr at 37°. Thiol content in mitochondria was determined using DTNB, as described in the method of Wudarczyk *et al.* [30].

2.9. Measurement of hydrogen peroxide decomposition

Boldine (5–100 μM) or 10 μg/mL of catalase was added to a reaction mixture containing 100 μM H_2O_2, 120 mM KCl, and 50 mM Tris–HCl, pH 7.4. The reaction was performed for 30 min at 37° and terminated by the addition of a stopping solution (25 mg/mL of potassium biphthalate, 2.5 mg/mL of NaOH, 82.5 mg/mL of potassium iodide, and 0.25 mg/mL of ammonium molybdate). The absorbance of the mixture was measured at 350 nm. The hydrogen peroxide remaining was determined using an H_2O_2 solution as the standard [31].

2.10. Measurement of 2-deoxy-D-ribose degradation

The decomposing effect of boldine on hydroxyl radicals was determined by an assay of malondialdehyde chromogen formation due to 2-deoxy-D-ribose degradation [32,33]. The reaction mixtures contained 2 mM 2-deoxy-D-ribose, 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2, 100 μM ascorbate, 150 mM KCl, and 50 mM NaH_2PO_4 buffer, pH 7.4, in a final volume of 1 mL. After a 30-min incubation, the reaction was stopped by adding 1% thiobarbituric acid in 50 mM NaOH and 2.8% trichloroacetic acid. Absorbance was measured at 532 nm.

2.11. Measurement of the melanin formed from dopamine

Melanin, the end product of dopamine oxidation, was measured at 405 nm [34,35]. Mitochondria (1 mg of protein/mL) were incubated in the reaction mixture containing 5 mM dopamine for 2 hr at 37°. The absorbance of the supernatant obtained by centrifugation (15,000 *g* for 10 min at 25°) was measured, and the melanin formed was expressed as micrograms per milliliter using a commercial melanin (oxidation product of tyrosine) as the standard.

2.12. Cell culture

PC12 cells were cultured in Dulbecco's Modified Eagle's Medium/F12 mixture supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Cells were subcultured every 5–7 days [36].

2.13. Cell viability assay

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Cells were plated at a density of 3×10^5/200 μL medium in a 96-well plate and treated with 200 μM dopamine in the presence of boldine for 24 hr. The medium was incubated with 10 μL of a 10 mg/mL MTT solution for 3 hr at 37°. Culture medium was removed, and 100 μL of DMSO was added to each well to dissolve the formazan. Absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Spectra MAX 340). Cell viability was expressed as a percent of the control culture value [36,37].

2.14. Measurement of apoptosis using a caspase-3-activity assay

PC12 cells (2×10^6/1 mL) were treated with 50 μM dopamine in the presence of boldine for 24 hr at 37°. The effect of boldine on apoptosis in dopamine-treated PC12 cells was measured as described in the ApoAlert CPP32/caspase-3 assay kit user manual of CLONTECH. One unit of the enzyme was defined as 1 nmol of the chromophore *p*-nitroanilide produced.

2.15. Data analysis

Statistical analysis on the data obtained was performed by using Student's *t*-test. A *P*-value under 0.05 indicates that data were significantly different from samples that did not contain catecholamines. Data are expressed as means ± SEM. Depiction and expression of the data in Figs. 1-4 and 6 were based on previous reports.

3. Results

3.1. Effect of boldine on membrane permeability transition induced by dopamine oxidation in brain mitochondria

The swelling of brain mitochondria was induced when challenged with 50 μM Ca^{2+}. To elucidate the effect of catecholamine oxidation products on membrane permeability, brain mitochondria were pretreated with the compounds for 10 min. Dopamine (200 μM) attenuated mitochondrial swelling induced by Ca^{2+} (Fig. 1) in agreement with a previous report [10]. The depressant effect of dopamine oxidation on Ca^{2+}-induced mitochondrial swelling was partially inhibited by the addition of 10 μg/mL of SOD, a scavenger of superoxide anion, or of 10 μg/mL of catalase, a scavenger of hydrogen peroxide. Neuroprotection by boldine was assayed by looking at its effect on the oxidative membrane permeability change in brain mitochondria. The concentration of boldine used in this experiment was based on reports in which the compound showed a prominent protective effect on oxidative tissue damage [17–19]. Boldine (10–100 μM) decreased the effect of dopamine on mitochondrial swelling. It alone did not reduce Ca^{2+}-induced mitochondrial swelling.

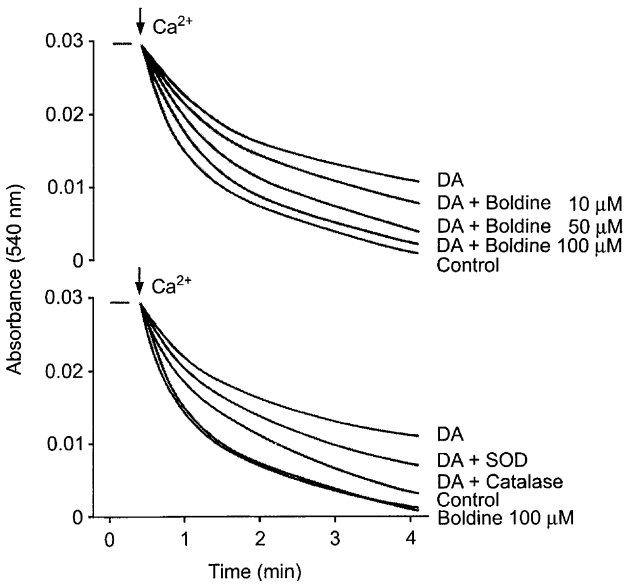

Fig. 1. Effect of boldine on dopamine-induced modification of mitochondrial swelling. Brain mitochondria (1 mg of protein/mL) were treated with 200 μM dopamine (DA) for 10 min in the presence of various concentrations of boldine and 10 μg/mL of SOD or catalase; swelling was induced by the addition of 50 μM Ca^{2+}. The traces are representative of three to five experiments.

Like dopamine, the mitochondria incubated with 6-OHDA (100 μM) for 10 min showed a depression of Ca^{2+}-induced swelling (Fig. 2). The addition of SOD or catalase (10 μg/mL) partially decreased the depressant effect of 6-OHDA oxidation on Ca^{2+}-induced mitochondrial swelling. To exclude the influence of the color of catecholamine quinone on the assay of mitochondrial swelling, we observed the change in absorbance due to the oxidation of 6-OHDA [8]. The absorbance increase due to the oxidation of 6-OHDA at 540 nm reached a plateau level at 10 min post-addition in the mitochondrial swelling assay mixture at 30°, and, after this, no further increase in absorbance occurred, as in the previous report [8]. Boldine decreased the effect of 6-OHDA on Ca^{2+}-induced mitochondrial swelling in a concentration-dependent manner.

Brain mitochondria energized with succinate in the absence of Ca^{2+} demonstrated a rapid fall of membrane potential, and then this potential maintained a steady-state level. Figs. 3 and 4 show that brain mitochondria pretreated with either 200 μM dopamine or 100 μM 6-OHDA for 10 min produced a change in membrane potential consisting of gradual polarization and subsequent depolarization. Both catecholamines caused a similar pattern of membrane potential change. The present study examined the effect of boldine on the change in mitochondrial membrane potential due to catecholamine oxidation. Boldine (10–100 μM)

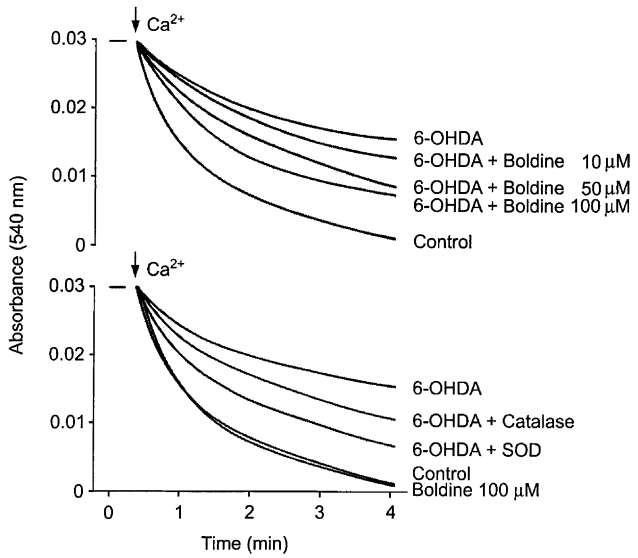

Fig. 2. Effect of boldine on the 6-OHDA-induced modification of mitochondrial swelling. Brain mitochondria were treated with 100 μM 6-OHDA for 10 min in the presence of various concentrations of boldine and 10 μg/mL of SOD or catalase; swelling was induced by the addition of 50 μM Ca^{2+}. The traces are representative of three experiments.

and 10 μg/mL of SOD or catalase attenuated the dopamine- or 6-OHDA-induced alteration of mitochondrial membrane potential. Boldine alone did not disturb the succinate-induced formation of membrane potential.

3.2. Effect of boldine on cytochrome c release from mitochondria

Induction of membrane permeability transition in mitochondria has been suggested to cause the release of cytochrome *c* [9,38]. In the present study, the effect of boldine

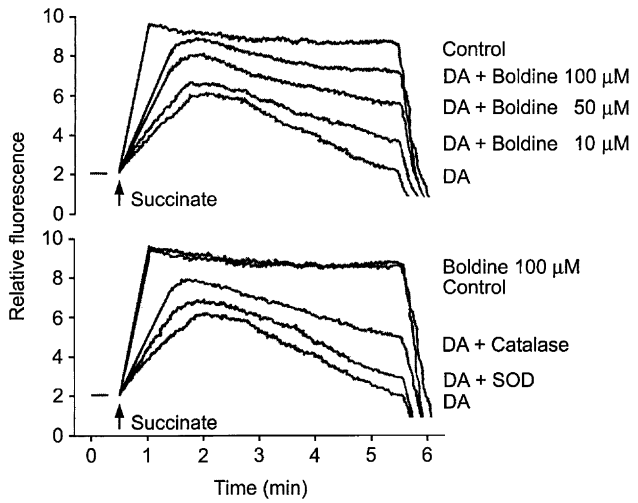

Fig. 3. Effect of boldine on the dopamine-induced alteration of mitochondrial membrane potential. Brain mitochondria (1 mg of protein/mL) were treated with 200 μM dopamine (DA) for 10 min in the presence of various concentrations of boldine and 10 μg/mL of SOD or catalase; then the formation of membrane potential was induced by the addition of 5 mM succinate. The traces are representative of three to five experiments.

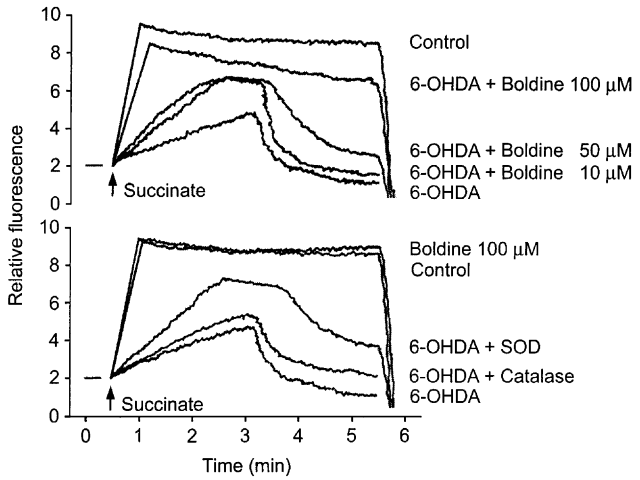

Fig. 4. Effect of boldine on the 6-OHDA-induced alteration of mitochondrial membrane potential. Brain mitochondria were treated with 100 μM 6-OHDA for 10 min in the presence of various concentrations of boldine and 10 μg/mL of SOD or catalase; then the formation of membrane potential was induced by the addition of 5 mM succinate. The traces are representative of three experiments.

on membrane permeability transition was examined with its action on mitochondrial cytochrome *c* release. The cytochrome *c* release from mitochondria was assayed by measuring the increase in absorbance due to the dithionite-dependent reduction of previously oxidized cytochrome *c* [25,26]. Brain mitochondria were treated with 100 μM dopamine or 6-OHDA for 10 min at 37°, and then 0.1 mM potassium ferricyanide and 1 mM potassium cyanide were added to the reaction mixture. The addition of 0.1 mM sodium dithionite to the supernatant obtained by centrifugation caused a fast increase in absorbance that indicated the presence of cytochrome *c* in the reaction mixture. In contrast, a significant change in absorbance was not found in the control mitochondria. Mitochondria treated with 100 μM dopamine or 6-OHDA released 0.27 ± 0.02 and 0.20 ± 0.02 nmol cytochrome *c*/mg of protein ($N = 3$), respectively. Preincubation of mitochondria with 100 μM boldine and 10 μg/mL of antioxidant enzymes for 10 min decreased the release of mitochondrial cytochrome *c* induced by catecholamines (Fig. 5). In the same experiment, boldine alone did not induce the release of cytochrome *c*.

3.3. Effect of boldine on mitochondrial electron flow

The reduction of MTT was used in the assay of mitochondrial electron flow. Incubation with tyramine, dopamine, or benzylamine has been shown to inhibit the ability of mitochondria to reduce MTT during electron flow [3]. Tyramine was found to have a similar effect on both mitochondrial respiration and MTT reduction [27]. Addition of pyruvate and malate induced the reduction of MTT in the reaction mixture containing mitochondria. Fig. 6 shows that preincubation with 100 μM dopamine or 6-OHDA depressed the MTT reduction rate in mitochondria, which

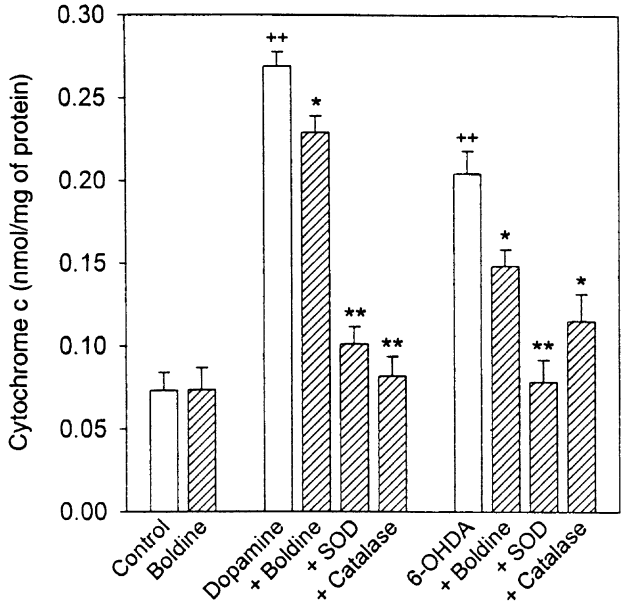

Fig. 5. Effect of boldine on the catecholamine-induced release of mitochondrial cytochrome *c*. Brain mitochondria (1 mg of protein/mL) were treated with 100 μM dopamine or 6-OHDA for 10 min in the presence of 100 μM boldine and 10 μg/mL of SOD or catalase. The amount of cytochrome *c* in the supernatant obtained by centrifugation of the reaction mixture was measured. Data are means ± SEM, $N = 3$. (*) $P < 0.05$ and (**) $P < 0.01$: significantly different from catecholamines alone; (++) $P < 0.01$: significantly different from the control.

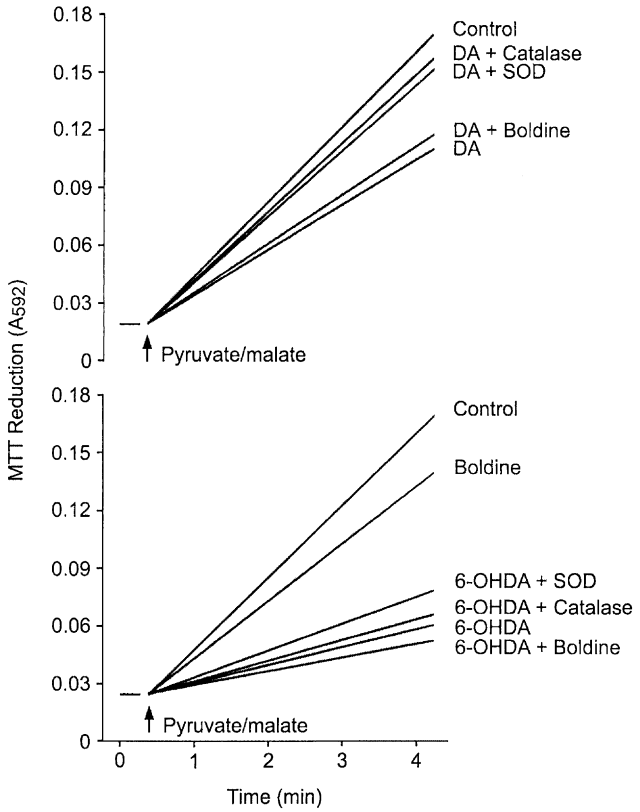

Fig. 6. Effect of boldine on the catecholamine-induced depression of mitochondrial electron flow. Brain mitochondria (0.2 mg of protein/mL) were treated with 100 μM dopamine (DA) or 6-OHDA for 10 min in the presence of 100 μM boldine and 10 μg/mL of SOD or catalase. The traces are representative of three to five experiments.

was reduced by the addition of SOD or catalase (10 μg/mL). Although boldine (100 μM) alone inhibited MTT reduction, the same concentration of compound did not affect the depressant effect of dopamine and 6-OHDA on the MTT reduction.

3.4. Effect of boldine on thioredoxin reductase activity and thiol oxidation in mitochondria

The activity of thioredoxin reductase in brain mitochondria is reduced significantly by additions of 25 μM Zn^{2+} and 50 μM Mn^{2+}, similar to its activity in liver mitochondria [10,29]. Mitochondria were treated with 0.1 mM sodium azide to minimize the decomposing action of glutathione peroxidase and catalase on hydrogen peroxide. For sufficient action of the cell membrane permeable oxidant on thioredoxin reductase (intramitochondrial enzyme), mitochondria were incubated with catecholamines for 30 min. Fig. 7 shows that when mitochondria were incubated with 100 μM dopamine or 6-OHDA for 30 min at 37°, thioredoxin reductase activity was decreased by about 21%. The effect of boldine on the catecholamine-induced alteration of mitochondrial membrane permeability was examined in relation to thioredoxin reductase activity. Boldine (100 μM) exerted a 52 and 53% inhibitory effect on the enzyme against dopamine and 6-OHDA, respectively. Unlike 6-OHDA, the effect of dopamine on thioredoxin reductase was not reduced by 10 μM boldine.

A decrease in reduced glutathione may be associated with formation of the mitochondrial permeability transition [39]. The thiol content in intact mitochondria was 35.03 ± 0.54 nmol/mg of protein. When mitochondria were treated with either dopamine or 6-OHDA (100 μM) for 1 hr, they showed 9.54 and 8.82 nmol of oxidized thiol/mg of protein, respectively. SOD and catalase inhibit dopamine-induced thiol oxidation in mitochondria [11]. As shown in Table 1, boldine inhibited both dopamine- and 6-OHDA-induced thiol oxidation in mitochondria in a concentration-dependent manner. Boldine alone did not induce a change in the enzyme activity and thiol content in brain mitochondria. The indicated time of incubation did not induce a significant change in thioredoxin reductase activity and in reduced thiols in intact mitochondria (data not shown).

3.5. Decomposing effect of boldine on reactive oxygen species and melanin formation

The effect of boldine on oxidative mitochondrial damage was examined with the decomposing effect on hydrogen peroxide and hydroxyl radicals. Hydrogen peroxide is attained from the dismutation of the superoxide anion and is well known as a precursor of highly reactive

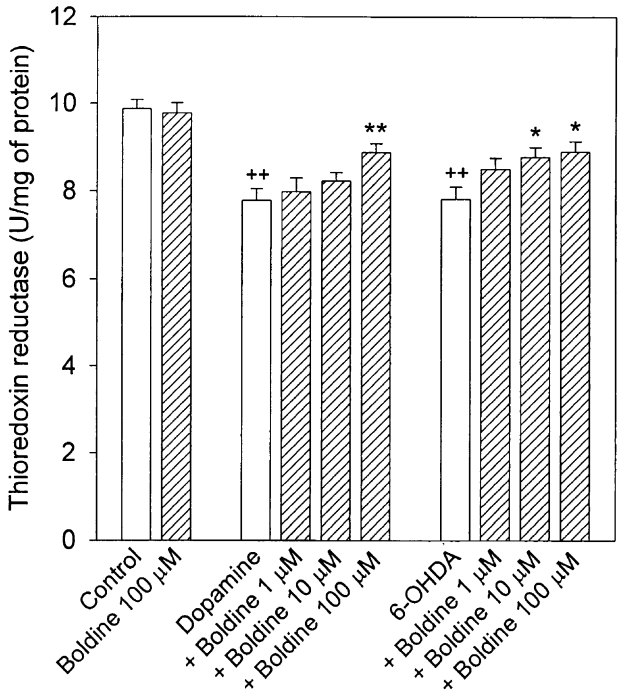

Fig. 7. Effect of boldine on mitochondrial thioredoxin reductase activity. Brain mitochondria (1 mg of protein/mL) were treated with 100 μM dopamine or 6-OHDA in the presence of boldine for 30 min. The enzyme activity in intact mitochondria was 9.87 ± 0.34 U/mg of protein. Data are means ± SEM, $N = 6$. (*) $P < 0.05$ and (**) $P < 0.01$: significantly different from catecholamines alone; (++) $P < 0.01$: significantly different from the control.

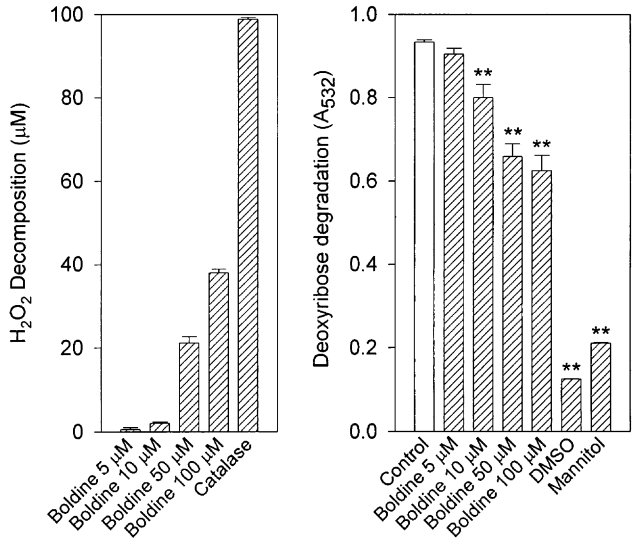

Fig. 8. Effect of boldine on hydrogen peroxide decomposition and 2-deoxy-D-ribose degradation. Boldine and oxidant scavengers (10 μg/mL of catalase, 10 mM DMSO, or 10 mM mannitol) were added to the reaction mixtures containing either 100 μM H_2O_2 or 2 mM 2-deoxy-D-ribose, 50 μM Fe^{3+}, 50 μM EDTA, 500 μM H_2O_2, and 100 μM ascorbate. The reaction was performed for 30 min. Data represent means ± SEM, $N = 3$–5. (**) $P < 0.01$: significantly different from the control.

oxidants [33]. The oxidation of dopamine caused by mitochondrial monoamine oxidase releases hydrogen peroxide. Fig. 8 shows that boldine directly decomposed hydrogen peroxide in a concentration-dependent manner, and the effect of boldine was supported by the scavenging action of catalase (10 μg/mL).

The scavenging effect of boldine on hydroxyl radicals was assayed with 2-deoxy-D-ribose degradation produced by Fe^{3+}, EDTA, H_2O_2, and ascorbate as in a previous report [32]. The 50 μM Fe^{3+}, 50 μM EDTA, 500 μM H_2O_2, and 100 μM ascorbate-induced deoxyribose degradation was inhibited significantly by a 10 mM concentration of the hydroxyl radical scavengers DMSO and mannitol. Boldine (10–100 μM) decreased the iron and EDTA-mediated deoxyribose degradation.

The oxidation products of dopamine in the substantia nigra may polymerize to form neuromelanin, which appears to exert a toxic effect on nigrostriatal neurons [34,35,40]. The present study examined the effect of boldine on melanin formation due to the oxidation of dopamine. When dopamine was suspended in the reaction mixture containing mitochondria for 2 hr, the black color of melanin was seen. The oxidation of dopamine (5 mM) in the presence of mitochondria produced 45.6 μg of melanin in a 2 hr-incubation. Boldine (10 and 100 μM) and 10 μg/mL of SOD or catalase significantly decreased the formation of melanin in dopamine-treated mitochondria (Fig. 9).

3.6. Effect of boldine on dopamine cytotoxicity in PC12 cells

The neuroprotective effect of boldine was explored in dopamine-induced cells by measuring loss of viability, including that caused by apoptosis, in PC12 cells. Dopa-

Table 1
Effect of boldine on mitochondrial thiol oxidation

Compound	Concentration (μM)	Concentration + DA (% protection)	Concentration + 6-OHDA (% protection)	Without catecholamines (nmol thiols/mg of protein)
Boldine	5	17.6 ± 1.8	15.9 ± 1.9	34.72 ± 0.40
Boldine	10	20.7 ± 1.2	37.1 ± 3.2	33.87 ± 0.77
Boldine	50	21.6 ± 2.7	40.1 ± 3.0	33.13 ± 0.69
Boldine	100	30.4 ± 2.2	46.3 ± 2.1	33.35 ± 0.80

Brain mitochondria (1 mg of protein/mL) were treated with 100 μM dopamine (or 6-OHDA) in the presence of boldine for 1 hr at 37°. Dopamine and 6-OHDA oxidized 9.54 ± 0.23 and 8.82 ± 0.26 nmol thiols/mg protein ($N = 5$), respectively. Data are means ± SEM, $N = 5$, and represent percent protection against catecholamines and nanomoles of thiols/mg of protein.

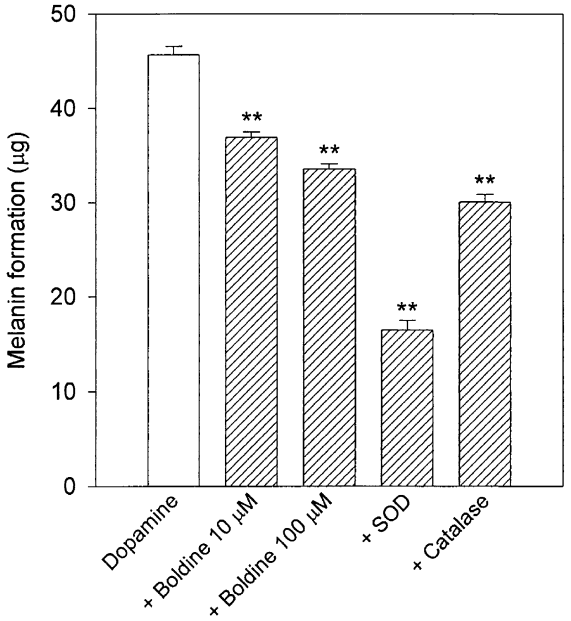

Fig. 9. Effect of boldine on melanin formation from dopamine. Brain mitochondria (1 mg of protein/mL) were treated with 5 mM dopamine in the presence or absence of various concentrations of boldine and 10 μg/mL of antioxidant enzymes for 2 hr. The melanin formed was measured at 405 nm and expressed as micrograms. Data represent means ± SEM, $N = 3$. (**) $P < 0.01$: significantly different from dopamine alone.

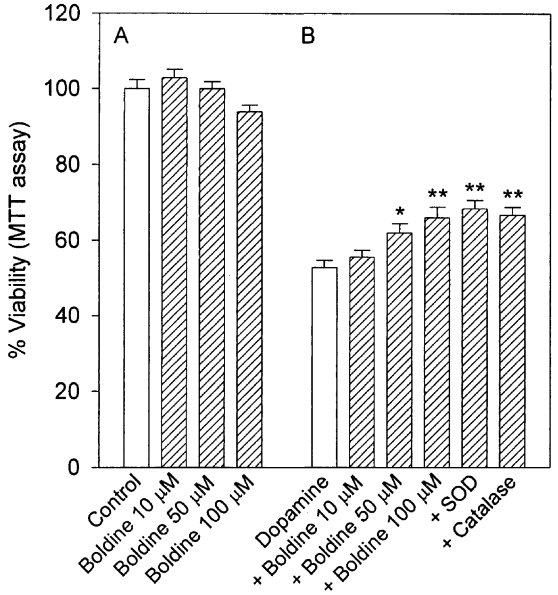

Fig. 10. Effect of boldine on dopamine-induced loss in cell viability. PC12 cells (3×10^5/200 μL) were treated without (A) or with (B) 200 μM dopamine in the presence of various concentrations of boldine or 10 μg/mL of antioxidant enzymes for 24 hr. Data represent means ± SEM, $N = 5$. (*) $P < 0.05$ and (**) $P < 0.01$: significantly different from dopamine alone.

mine treatment for 24 hr at 250 μM has been found to cause about 50% viability loss in PC12 cells, as measured by trypan blue exclusion and thymidine uptake [34]. Rat embryonic forebrain neurons incubated with 250 μM dopamine for 2 hr showed a characteristic apoptotic morphology [41]. Incubation of PC12 cells with dopamine (100 μM) for 24 hr induced death in about 20% of their population in this study (data not shown). Therefore, to obtain a detectable effect of boldine, we used 200 μM dopamine, which causes an easily measurable viability loss in PC12 cells. As shown in Fig. 10, when PC12 cells were treated with 200 μM dopamine for 24 hr, cell viability decreased to 53% in the MTT assay. Boldine (10–100 μM) and 10 μg/mL of antioxidant enzymes attenuated the dopamine-induced viability loss in PC12 cells. We examined the toxic effect of boldine on PC12 cells. Incubation of PC12 cells with 100 μM boldine for 24 hr induced a cell viability loss of about 6%, which was not significantly different from the control.

Exposure of PC12 cells to 25 μM 6-OHDA results in apoptosis, whereas 50 μM induces both apoptosis and necrosis [42]. In the present experiment, 50 μM dopamine was used to induce apoptosis in PC12 cells. Apoptosis in PC12 cells was analyzed by measuring the activity of caspases, which are found to participate in programmed cell death [42,43]. Treatment of PC12 cells with 50 μM dopamine for 24 hr showed an increase in caspase-3 activity. The dopamine-induced increase in caspase-3 activity in PC12 cells was decreased by the addition of 10 μg/mL of SOD or catalase. Fig. 11 shows that boldine attenuated the dopamine-induced caspase-3 activation in PC12 cells in a concentration-dependent manner, and the compound showed a 45% inhibition at 100 μM. Boldine alone did not show a significant effect on caspase-3 activity in PC12 cells.

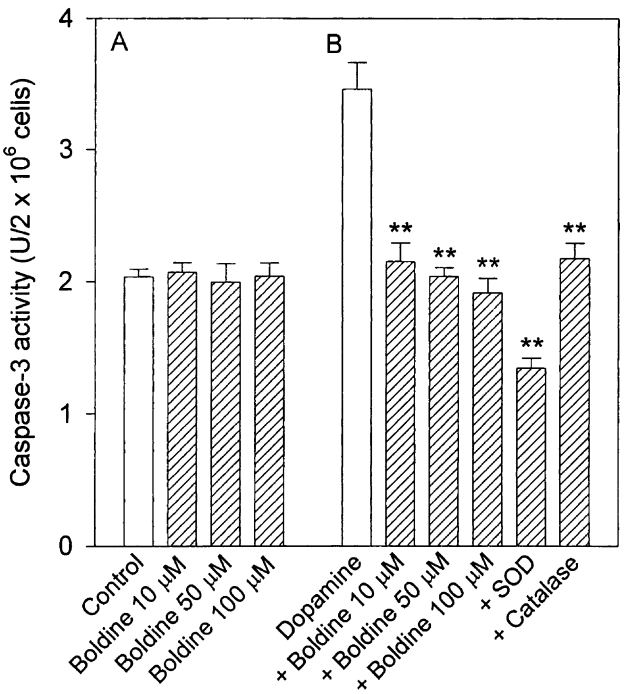

Fig. 11. Effect of boldine on the dopamine-induced increase in caspase-3 activity. PC12 cells (2×10^6 per well) were treated without (A) or with (B) 50 μM dopamine in the presence of various concentrations of boldine or 10 μg/mL of antioxidant enzymes for 24 hr. Data represent means ± SEM, $N = 4$. (**) $P < 0.01$: significantly different from dopamine alone.

4. Discussion

The membrane permeability change in brain mitochondria due to catecholamine oxidation products was determined by examining the swelling and membrane potential change. Our previous report [10] and the present study show that brain mitochondria pretreated with dopamine (100 and 200 μM) for 10 min attenuated Ca^{2+}-induced mitochondrial swelling. Like dopamine, Ca^{2+}-induced swelling in rat liver mitochondria has been found to be inhibited by nitric oxide [44]. A similar finding was observed in mitochondria pretreated with 100 μM 6-OHDA. The inhibitory effects of SOD and catalase indicate that the effect of catecholamines on mitochondrial swelling is accomplished by oxidative attack of reactive oxygen species. Dopamine at 50 μM showed a significant cytotoxic effect on neuroblastoma SH-SY5Y cells and PC12 cells, causing increased lipid peroxidation and leakage of cytosolic lactic dehydrogenase [35,45]. The oxidation of dopamine and 6-OHDA induces free radical formation, inhibition of the mitochondrial respiratory chain, and opening of the mitochondrial transition pore, which is inhibited by oxidant scavengers [8,10,13]. These findings suggest the involvement of oxidative stress in the disruption of cell membrane integrity and the formation of membrane permeability transition. Therefore, the depressant effect of catecholamine oxidation products on Ca^{2+}-induced mitochondrial swelling may be associated with the oxidative modification of Ca^{2+} transport rather than a protective action on the cell membrane.

The effect of catecholamine oxidation on the membrane potential formation in brain mitochondria was examined. Succinate-induced polarization in brain mitochondria was attenuated by treatment of catecholamines. The preincubation of mitochondria with dopamine or 6-OHDA for 10 min showed a membrane potential shape consisting of an initial slow onset of shallow polarization and subsequent depolarization. A similar finding has been found in brain mitochondria pretreated with iron and ascorbate [10]. These findings indicate that the oxidation products of catecholamines cause alteration of the mitochondrial membrane potential. Compared with 6-OHDA, the effect of dopamine oxidation on mitochondria may be caused more by hydrogen peroxide than by superoxide anions.

Selegiline has been suggested to have a beneficial effect in the treatment of Parkinson's disease by inhibiting monoamine oxidase in the dopaminergic neurons [46]. The use of some antioxidants has been postulated in the treatment of disease. In this respect, the present study explored the protective effect of boldine, which has been reported to have anti-inflammatory and antioxidant activities, on oxidative damage to brain mitochondria and PC12 cells. Boldine decreased alteration of Ca^{2+} and succinate-induced mitochondrial swelling and the formation of membrane potential induced by preincubation with catecholamines. Boldine showed a protective effect on the membrane permeability transition in mitochondria comparable to that of antioxidant enzymes. Boldine alone at the given concentrations did not affect mitochondrial membrane permeability. The findings suggest that boldine could exert a protective effect on the neuronal damage caused by catecholamine oxidation.

Although there is some controversy, opening of the membrane permeability pore in mitochondria has been hypothesized to induce the release of cytochrome *c*. Cyclosporin A prevents the membrane permeability transition induced by tumor necrosis factor-α and blocks the cytochrome *c* release, caspase-3 activation, and apoptosis in hepatocytes [47,48]. In this respect, we examined the protective effect of boldine on the alteration of mitochondrial permeability with a cytochrome *c* release assay. Incubation of dopamine or 6-OHDA induced the release of cytochrome *c* from brain mitochondria. The inhibitory effect of antioxidant enzymes indicates the involvement of reactive oxygen species in cytochrome *c* release. The depressant effect of boldine on the release of cytochrome *c* appears to support, in part, the action on membrane permeability transition.

Inhibition of the mitochondrial respiratory chain is suggested to play a part in the degeneration of nigrostriatal dopaminergic neurons. It has been shown that tyramine exerts a similar effect on both mitochondrial respiration and MTT reduction [27]. Therefore, the present study examined the effect of catecholamines on the respiratory chain in brain mitochondria with an electron flow assay. Like the mitochondrial membrane permeability, the catecholamine-induced depression of the electron flow was reduced by the addition of antioxidant enzymes. Although boldine (100 μM) alone inhibited the electron flow, the compound did not affect the dopamine- or 6-OHDA-induced depression of electron flow. This finding shows that the effect of boldine on the brain mitochondrial damage induced by catecholamine oxidation may not be associated with the action on the respiratory chain.

The mitochondrial matrix DTNB reductase has been shown to be a thioredoxin reductase that reduces glutathione disulfide to glutathione [49]. The membrane permeability of mitochondria may be modulated by the redox potentials of dithiols and pyridine nucleotides [29,39]. The activity of thioredoxin reductase is also affected significantly by the redox state of thiols. Therefore, mitochondrial thioredoxin reductase may participate in the regulation of mitochondrial membrane permeability. In this respect, the inhibitory effect of boldine on membrane permeability transition was elucidated by measuring thioredoxin reductase activity and thiol oxidation in mitochondria. Dopamine and 6-OHDA decreased thioredoxin reductase activity and induced thiol oxidation in mitochondria. The decrease in thioredoxin reductase activity and the thiol oxidation in catecholamine-treated brain mitochondria may be mediated by reactive oxygen species.

We observed that boldine attenuated the catecholamine-induced decrease in the enzyme activity and the increase in thiol oxidation. The indicated concentrations of boldine alone did not induce thiol oxidation in mitochondria. Therefore, boldine may exert a protective effect on the neurotoxin-induced damage of mitochondria through the maintenance of reduced thiols.

The oxidation of dopamine and 6-OHDA produces reactive species and quinones. Boldine has been shown to decrease iron-mediated oxidative tissue damage through its antioxidant activity. Therefore, we examined the effect of boldine on the decomposition of hydrogen peroxide and hydroxyl radicals and on melanin formation. Free radical scavengers are found to decrease the age-related increase in protein carbonyl content in mouse synaptic mitochondria [50] and to protect dopaminergic cell lines from apoptosis induced by inhibitors of complex I at the mitochondrial respiratory chain [12]. Boldine at the indicated concentrations exerted a decomposing effect on hydrogen peroxide, a precursor of highly reactive species, and attenuated the formation of melanin derived from dopamine oxidation. The 2-deoxy-D-ribose degradation induced by Fe^{3+}, EDTA, H_2O_2, and ascorbate is used as a sensitive detection method to assay hydroxyl radicals and is decreased significantly by the addition of the hydroxyl radical scavengers mannitol, DMSO, and sodium formate [32,33]. A similar finding was also observed in the present study. The inhibitory effect of boldine on deoxyribose degradation suggests a scavenging action on hydroxyl radicals. The decomposing and inhibitory effect of boldine on reactive species and melanin formation may provide a protective action on brain mitochondria against the oxidative attack of catecholamines.

Dopamine, 6-OHDA, and MPP^+ have been shown to induce apoptosis and necrosis in PC12 cells and cultured dopaminergic neurons [34,42,43]. The toxic effect of dopamine on neuronal cells is found to be inhibited by thiol compounds and some antioxidants [34,41] and potentiated by mitochondrial function inhibitors [51]. The neuroprotective effect of boldine was examined with the dopamine-induced cell viability loss, including apoptosis, in PC12 cells. Boldine significantly decreased dopamine-induced death in PC12 cells and showed a protective effect against the cytotoxic effect of dopamine comparable to that of antioxidant enzymes. Boldine alone showed little cytotoxicity, which was statistically insignificant. This finding also suggests a protective effect of boldine on dopamine oxidation-induced neuronal cell damage.

In conclusion, boldine may attenuate the dopamine- and 6-OHDA-induced oxidative damage of brain mitochondria and decrease the dopamine-induced death of PC12 cells through a scavenging action on reactive oxygen species and inhibition of melanin formation and thiol oxidation. The boldine alkaloids appear to show a protective effect against the neuronal damage associated with catecholamine oxidation.

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