α-lipoic acid protects dopaminergic neurons against MPP⁺-induced apoptosis by attenuating reactive oxygen species formation

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Abstract. Reactive oxygen species (ROS) elicited by oxidative stress are widely recognized as a major initiator in the degeneration of dopaminergic neurons distinctive of Parkinson's disease (PD). The interaction of ROS with mitochondria triggers sequential events in the mitochondrial cell death pathway, which is thought to be responsible for ROS-mediated neurodegeneration in PD. α-lipoic acid (LA) is a pleiotropic compound with potential pharmacotherapeutic value against a range of pathophysiological insults. Its protective actions against oxidative damage by scavenging ROS and reducing production of free radicals have been reported in various in vitro and in vivo systems. This study analyzed the ability of LA to protect PC12 neuronal cells from toxicity of 1-methyl-4-phenylpyridinium (MPP⁺), the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is known to kill dopaminergic neurons selectively and to cause severe parkinsonism-like symptoms in humans and primates. Our results demonstrate that the apoptosis of PC12 cells elicited by MPP⁺ could be significantly prevented by pretreatment with LA for 1 h. In addition, LA inhibits intracellular ROS levels and the mitochondrial transmembrane permeability, the key players in the pathogenesis of PD, thereby protecting dopaminergic neuronal cells against oxidative damage.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the selective and progressive loss of dopaminergic neurons in the substantia nigra, leading to a depletion of the dopamine neurotransmitter in the striatum. Although the underlying mechanisms by which nigrostrial dopaminergic neuron degeneration are not completely understood, accumulating data suggest that reactive oxygen species (ROS) induced by oxidative stress play a crucial role in dopaminergic cell death in PD (1,2). PC12 cells treated with 1-methyl-4-phenylpyridinium (MPP⁺) provide a reliable in vitro model for insight into the pathogenesis of PD. PC12 cells are a rat pheochromocytoma cell line which has been shown to produce dopamine (3), and possesses a dopaminergic uptake system (4), indicating that these cells can be used as a cell culture model for studying the dopaminergic neuron degeneration. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin which selectively kills dopaminergic neurons and causes irreversible parkinsonism-like symptoms in humans and primates (5-8). MPTP is a lipophilic molecule and can rapidly cross the blood-brain barrier. Once it crosses the barrier, it is oxidized in the brain to its toxic metabolite MPP⁺ by type B monoamine oxidase (9), and finally causes dopaminergic neuron death. The exact mechanism for this toxic action of MPP⁺, although not fully understood, appears to involve ROS generation, which initiates the downstream apoptotic pathway. MPP⁺ is taken up by dopaminergic neurons via DA transporter and accumulates in mitochondria where it causes excessive ROS formation by inhibiting the respiration complex I (10). The interaction of ROS and mitochondria increases the permeability in mitochondria transmembrane which results in cytochrome c release, followed by caspase-3 activation, the crucial contributor to the neuronal cell death in PD (11).

Currently, the main therapeutic approaches for treating PD are limited to the replacement of dopamine in the brain either by levodopa or by dopamine agonists. Although these drugs can relieve the symptoms of PD, the degeneration of dopaminergic neurons is not prevented and still slowly progresses. Promising new concepts in the therapy of PD
should prevent or reverse the progressive dopaminergic neuron degeneration. Since neurodegenerative diseases are mainly mediated by ROS generation, attenuation of ROS levels may result in neuroprotection in these disorders. LA, also known as 1,2-dithiole-3-pentanone acid or thioctic acid, is a pleiotropic compound with potential pharmacotherapeutic value against a range of pathophysiological insults. Evidence has shown that LA has effective antioxidative activities by scavenging ROS (12,13) and inhibits free radical formation by chelating various metal types (14), indicating that it can exert beneficial effects on various disorders correlated with oxidative stress including PD. LA is also reported to have anti-inflammatory properties and to increase intracellular glutathione (GSH) formation in a range of cell types and tissues (15-17), which may be beneficial in neurodegenerative conditions. Notably, LA serving as an effective antioxidant has been in common clinical use for several diseases that are associated with increased production of free radicals (13,18-22), and has been administered in moderate doses with no evidence of serious side-effects (23,24). These properties of LA may be beneficial in PD, a neurodegenerative disease in which increasing formation of ROS is involved. Therefore, in the present study, we investigated whether LA attenuated MPP⁺-induced oxidative damage in PC12 cells, which may provide potential effective strategies for PD treatment.

Materials and methods

Drugs and chemicals. All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless noted otherwise.

PC12 cell cultures. PC12 cells, obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), were maintained in high glucose DMEM supplemented with 10% serum, 4.00 mM L-Glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin (Gibco, Grand Island, NY, USA). The cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C, and culture medium was changed every 3-4 days. The cells were seeded at a density of 30,000 cells/cm².

Cell viability assay. Cell viability was evaluated by using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is a colorimetric assay for measuring the activity of mitochondrial dehydrogenases that convert MTT into blue formazan crystals. Briefly, after plating at the density of 30,000 cells/cm² on 96-well plates for 24 h, PC12 cells were exposed to different concentrations of MPP⁺ (100-1,000 µM) for 24, 48 and 72 h. The wells were supplemented with MTT solution (5 mg/ml) and incubated for 4 h, and the culture medium was then removed and dimethyl sulfoxide was added to each well to solubilize the formazan into a colored solution. The absorbance of colored solution was measured at 570 nm using a microplate reader (BioTek Epoch; BioTek Instruments, Inc., USA). Results were expressed as the percentage of the absorbance of vehicle-treated control culture wells. Following time- and dose-response studies, the conditions of 500 µM MPP⁺ for 72 h were chosen in all experiments.

To assess the neuroprotective effects of LA on MPP⁺-induced toxicity in PC12 cells, the cells were treated with different concentrations of LA (0.001-1,000 µM), 1 h prior to the addition of 500 µM MPP⁺. Cell viability was assessed 72 h later by measuring the absorbance of colored solution. Based on these results, we used 0.01 µM LA in all subsequent experiments.

Nuclear staining assay. Nuclear change induced by MPP⁺ was evaluated using acridine orange/ethidium bromide staining (AO/EB). Cells at the concentration of 30,000 cells/cm² were plated on 6-well plates and incubated in DMEM medium at 37°C. After 1 h of pretreatment with LA (0.01 µM), MPP⁺ (500 µM) was added for 72 h. The cells were washed three times and resuspended in PBS. Then, the samples were stained with AO/EB (final concentration 1 µg/ml) and at least 200 cells were randomly observed under a fluorescence microscope (IX71; Olympus, Japan). Viable cells with intact structures stained only with AO showed bright green nuclear staining, the early apoptotic cells were bright green and the later apoptotic cells were red-orange with condensed chromatin. The number of apoptotic cells is expressed as a percentage of total cells counted.

Flow cytometric analysis of apoptosis. The membrane and nuclear events during apoptosis were analyzed by flow cytometry using FITC-Annexin V and propidium iodide (PI) staining. Following treatment, PC12 cells were harvested and centrifuged for 10 min at room temperature at 1,000 x g. Cells were washed with PBS and resuspended in binding buffer, and then 5 µl Annexin V-FITC (20 µg/ml) and 5 µl PI (50 µg/ml) were added. After incubating in the dark for 15 min, the samples were analyzed by flow cytometry (Becton-Dickinson). The assay was performed with a two-color analysis of FITC-labeled Annexin V binding and the uptake of PI. Living cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-, Q4), late apoptotic cells (Annexin V+/PI+, Q2), and necrotic cells (Annexin V-/PI+, Q1) were distinguished. Therefore, the total apoptotic proportion included the percentage of cells with fluorescence for Annexin V+/PI- and Annexin V+/PI+.

ROS activity assay. The production of ROS in the PC12 cells was measured by DCFH-DA assay based on the ROS-dependent oxidation of DCFH-DA to 2',7'-dichlorofluorescein (DCF). The cells were seeded at 3x10⁴ cells/well in 6-well tissue culture plates. After 1 h of pretreatment with LA, MPP⁺ was added for 72 h. The cells were then incubated in BSA-free DMEM with DCFH-DA at a final concentration of 20 µM for 30 min at 37°C. DCFH-DA crosses the cell membrane and is hydrolyzed by intracellular esterases to non-fluorescent DCF. It is then trapped in the cells and becomes fluorescent following oxidation to DCF by ROS. The intensity of fluorescence was recorded using a flow cytometer (Becton-Dickinson). The excitation wavelength was 485 nm and the reading was performed at 530 nm.

Measurement of mitochondrial transmembrane potential. Mitochondrial transmembrane potential was measured by flow cytometry with mitochondrial dye 3,3'-dihexyloxocarbocyanine iodide [DiOC₆(3)]. DiOC₆(3) is lipophilic fluorescent stain that becomes highly fluorescent when incorporated into membranes. The levels of mitochondria depolarization repre-
sent membrane permeabilization. The cells at a concentration of 30,000 cells/cm² were cultured in 24-well plates for 24 h, followed by the treatment of 0.01 µM LA prior to the addition of 500 µM MPP⁺ 1 h. After 72 h of incubation, 1 ml of serum-free culture medium containing DiOC₆(3) was added to each well with the final concentration of 1 µM and the cells were cultured in a humidified incubator for 15 min. For the detection of mitochondrial transmembrane potential, cells were collected and centrifuged at 1,000 x g for 5 min, and the cell pellets were resuspended in PBS containing 0.5 mM EDTA. Thereafter, cells were analyzed by flow cytometry using the FL1 flow cytometer detection channels.

**Western blot analysis.** Following treatment, PC12 cells were collected and extracted in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS 30 mM Tris, 1% protease inhibitor and 1% nuclease mix. The protein concentration was determined with the 2-D Quant kit (Amersham Biosciences, Uppsala, Sweden). Equal amounts of protein were loaded onto a 12% SDS-polyacrylamide gel. Following electrophoretic separation, the polyacrylamide gels were transferred to PVDF transfer membrane (Amersham Biosciences), and western blotted using rabbit anti-Bax, anti-Bcl-2. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies were used as the secondary antibodies.

**Evaluation of caspase-3 activity.** Caspase-3 activity was determined using an ApoAlert® caspase-3 assay kit according to the manufacturer’s instructions. Following treatment, the cells were collected and centrifuged at 400 x g for 10 min. The cell pellet was lysed in 50 µl of lysis buffer. After incubating for 10 min on ice, the cells were centrifuged at 10,000 x g for 3 min at 4°C. The supernatant was collected and the reaction mixture containing dithiothreitol and caspase-3 substrate (N-Acetyl-Asp-Glu-Val-Asp-p-nitroanilide) was added. Samples were incubated for an additional 1 h at 37°C. Absorbance of the chromophore p-nitroanilide produced was measured at 405 nm. The standard curves were obtained from the absorbance of p-nitroanilide standard reagent diluted with cell lysis buffer (up to 20 nM). One unit of the enzyme was defined as the activity producing 1 nmol of p-nitroanilide.

**Statistical analysis.** Data are expressed as the means ± SEM. Statistical analysis was performed by one-way analysis of variance, followed by Dunnett’s multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**The neurotoxicity of MPP⁺ in PC12 cells.** To determine the toxicity of MPP⁺ in PC12 cells, cell viability was assessed using MTT, a mitochondrial dye, which can be converted into a blue formazan product by mitochondrial dehydrogenases, thereby partially detecting the levels of metabolically active cells. PC12 cells were treated with different concentrations of MPP⁺ for different periods of time. The measurements revealed that the treatment of the cells for 72 h with 500 µM MPP⁺ caused ~48% cell viability loss (Fig. 1A). The higher concentrations of MPP⁺ resulted in significant and irreversible neurotoxicity, which would prevent neuroprotection measurement. Thus, the cells exposed to 500 µM MPP⁺ for 72 h were selected as optimal conditions to measure neuroprotection of LA against the toxicity of MPP⁺.

**LA reduces MPP⁺-induced cell viability loss.** The ability of LA to prevent the cytotoxicity of MPP⁺ in PC12 cells was measured by the MTT assay. The measurements revealed significant decrease in cell viability in PC12 cells following exposure to 500 µM MPP⁺ for 72 h, but not in LA used alone. In the presence of 500 µM MPP⁺, 0.01 µM LA showed a significant protective effect on the cells (Fig. 1B).

**LA exerts anti-apoptotic effects against the cytotoxicity of MPP⁺ in PC12 cells.** To determine whether LA protects PC12 cells from MPP⁺-induced apoptosis, we undertook AO/EB assay and FITC-Annexin V and PI staining. Apoptosis is a major type of cell death, characterized by a series of nuclear morphological changes. These changes can be detected by AO/EB staining. The fluorescence microscopic analysis results are shown in Fig. 2A, and three types of cells can be recognized under fluorescence microscopy: live cells (green), early apoptotic cells (bright green with condensed chromatin), and later apoptotic cells (red-orange with condensed chromatin).
Following administration of 0.01 µM LA, the proportion of apoptotic cells induced by MPP⁺ treatment was significantly reduced, indicating that LA significantly prevents MPP⁺-induced apoptosis in PC12 cells.

FITC-Annexin V and PI staining were analyzed by flow cytometry, and the total apoptotic cells included early apoptotic cells (Annexin V⁺/PI⁻, Q4) and late apoptotic cells (Annexin V⁺/PI⁺, Q2). The results showed that LA administered alone did not significantly induce changes in the number of apoptotic cells, while the administration of MPP⁺ markedly increased their number in comparison with control cells (Fig. 2B). This increase was strongly prevented when LA was administered 1 h prior to MPP⁺, confirming the anti-apoptotic activities of LA against the toxicity of MPP⁺.

**LA inhibits the formation of ROS.** To investigate the effects of LA on ROS levels in MPP⁺-induced PC12 cells, we evaluated the levels of ROS by flow cytometry with DCFH-DA, a stable compound, which can easily diffuse into cells and is converted into DCFH by intracellular esterase. DCFH is then trapped within cells and oxidized to highly fluorescent DCF by ROS, thereby the intensity of fluorescence produced by DCF may reflect an intracellular oxidative state. Our results showed that the administration of LA alone, compared with the control group, did not elicit DCF fluorescence intensity change. Exposure of PC12 cells to MPP⁺ alone induced a significant increase of DCF fluorescence levels, which was prevented by pre-treatment with LA, indicating a preventive role of LA in ROS formation in PC12 cells elicited by MPP⁺ (Fig. 3).
LA prevents MPP⁺-induced mitochondrial transmembrane potential discharge. Mitochondrial membrane potential was assessed by measuring the response to the mitochondrial dye DiOC₆(3), which is converted into highly green fluorescence by the incorporation into mitochondrial membranes, thereby allowing qualitative assessment of the mitochondrial membrane potential. When MPP⁺ was administered alone, we observed the increased percentage of cells with depolarized mitochondria in comparison with control cells. We also observed a marked reduction in the number of cells with depolarized mitochondria when LA was added prior to MPP⁺ and LA alone did not induce the change (Fig. 4). These results demonstrate that the mitochondrial membrane permeability induced by MPP⁺ is restored by the administration of LA.

LA modulates Bax and Bcl-2 protein expression. To examine the effects of LA on Bax and Bcl-2 protein levels induced by MPP⁺ in PC12 cells, western blotting was performed in the cells untreated, or treated with 500 µM MPP⁺ alone, or treated with 500 µM MPP⁺ in the presence of 0.01 µM LA. When MPP⁺ was administered alone, we observed a significant increase in the Bax/Bcl-2 ratio compared with control cells. We also observed a marked reduction in the Bax/Bcl-2 ratio when LA was added prior to MPP⁺ and LA alone did not induce the change (Fig. 5).

LA reduces caspase-3 activity. To investigate the effects of LA on caspase-3 activity, ELISA was performed in PC12 cells. The cells were pretreated with LA for 1 h and then exposed to MPP⁺ or not for 72 h. Caspase-3 activity was determined using an ApoAlert caspase-3 assay kit. The results demonstrated that PC12 cells treated with 500 µM MPP⁺ showed a significant increase in caspase-3 activity, but not in LA used alone. Pretreatment of LA significantly suppressed the MPP⁺-induced caspase-3 activity (Fig. 6), suggesting a protective role of LA against the MPP⁺ toxicity in PC12 cells.

Discussion

The present investigation indicates for the first time that LA, a naturally occurring dithiol compound, can exert protective effects against apoptotic cell death elicited by MPP⁺ in PC12 cells, a reliable model for PD in which dopaminergic neurons present a slow and progressive loss. LA effectively inhibits MPP⁺-induced ROS formation, mitochondrial potential discharge, caspase-3 activation, and cytotoxic cell death in dopaminergic neurons.

Oxidative stress is a central event in a range of pathological conditions. Mitochondria are generally considered to be the primary source of ROS within most mammalian cells (25-28), and the generation of ROS is mainly correlated with the inhibition of complex I (25,29-31). This production of ROS activates the mitochondrial cell death pathway by oxidative damage to mitochondria and/or by transferring redox signal from the organelle to the nucleus. Such a pathway appears to underlie the pathological processes of PD, in which the inhibition of the mitochondrial complex I elicited by the neuron toxicant increases the formation of ROS that cause the mitochondrial...
dysfunction (32,33), and finally cause cell death. MPTP, a well known neurotoxin, has been widely used to study the pathogenesis of PD. Its toxic metabolite MPP⁺ is thought to selectively kill dopaminergic neurons and to elicit severe parkinsonism-like symptoms in humans and primates (5-8). ROS serving as a key mediator are responsible for MPP⁺-induced oxidative damage in dopaminergic neuronal cells. This contribution of ROS may be related to mitochondrial membrane potential discharge, cytochrome c release and caspase-3 activation, which together contribute to cell death. LA, also known as 1,2-dithiolane-3-pentanoic acid, can be synthesized enzymatically in the mitochondria from octanoic acid and absorbed from dietary sources including animal and plant foods (34). LA functioning as an essential cofactor for mitochondrial α-ketoacid dehydrogenases plays a critical role in mitochondrial energy metabolism. Its protective actions against oxidative stress and toxin insults have been reported in vitro and in vivo studies (35-38). We investigated the anti-apoptotic activity of LA in PC12 cells treated with MPP⁺, a reliable cellular model of PD. These results showed that the number of apoptotic cells significantly decreased when LA was administered 1 h prior to MPP⁺, and the activation of caspase-3 was also prevented by the administration of LA. Moreover, in PC12 cells treated by MPP⁺ we established that LA inhibited the formation of ROS, an oxidative damage inducer and redox signal transducer in a range of pathogeneses (27,39). Our studies demonstrated that when MPP⁺ was administered, the fluorescence density of DCF significantly increased, indicating high ROS levels. By contrast, the treatment with LA prior to MPP⁺ administration indicated low ROS levels. These results indicate that the neuroprotective actions of LA may be mediated by the inhibition of ROS formation, which reverses the neurodegenerative processes. Previous evidence suggested that mitochondria play a key role in neurodegenerative diseases, including Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) (40-43). Mitochondrial damage mediated by ROS have been recognized as a central event in dopaminergic neuron degeneration (44,45). Maintenance of mitochondrial membrane potential is essential for living cells; its loss has numerous consequences for the cells, including apoptosis (46). In agreement with these reports, in the present study, mitochondrial transmembrane potential was implicated in PD. We also observed a marked reduction in the number of cells with depolarized mitochondrial when LA was administered, supporting a neuroprotective role of LA in the neurodegenerative diseases mediated by the mitochondrial cell death pathway. Similar protection of LA by inhibiting ROS production and mitochondrial membrane depolarization has previously been reported in other cell types such as β cells (47).

Bax and Bcl-2 are key members of the Bcl-2 protein family which plays a central role in the mitochondrial cell death pathway. Bax increases the mitochondrial membrane permeabilization by inducing the mitochondrial transmembrane pore formation (48), leading to the pro-apoptotic molecule release. By contrast, Bcl-2 preserves mitochondrial membrane integrity, thereby inhibiting the release of apoptogenic molecules such as cytochrome c (49). The ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 has been reported to be strongly correlated with apoptosis in various pathophysiological conditions (50). In this study, the administration of MPP⁺ significantly increased the Bax/Bcl-2 ratio, which was significantly prevented when LA was administered 1 h prior to MPP⁺, further supporting the protective role of LA against the toxicity of MPP⁺ in PC12 cells.

The protection of neuron cells from apoptosis has been underlined in the therapeutic strategy in neurodegenerative disorders. Studies in vitro and in vivo provide considerable evidence for the neuroprotective activities of certain agents, such as EPO or estrogens. However, the adverse effects associated with these agents should be underlined in some pathological conditions. Treatment with EPO, for example, increases red blood cell count and platelet aggregation, the key contributors to congestive heart failure and ischemic diseases, respectively. LA serving as a necessary mitochondrial cofactor, is ubiquitous in living organisms including neurons. Owing to its powerful antioxidative activities, LA has been used in several disorders to prevent oxidative stress (51,52). Preclinical and clinical data demonstrate that LA is bioavailable and safe in moderate doses (51). It was administered intravenously in doses of 600 mg/day for three weeks with no serious side-effects (23). Oral doses of 1,800 mg LA (600 mg t.i.d.) for six months did not elicit significant adverse effects (24). In addition to directly scavenging ROS, LA is reported to increase intracellular glutathione (GSH) that is an abundant natural thiol antioxidant and co-substrate for detoxification enzymes in a range of cell types and tissues (15-17), further supporting the antioxidative activities of LA with potential clinical value against oxidative damage. Evidence has also shown that exogenous LA can easily cross the blood-brain barrier (53,54), suggesting it can exert antioxidative effects in the neuronal system.

LA with strong antioxidative activities and clinical safety record may be an efficient agent in protecting the dopaminergic neurons against oxidative damage. However, the majority of biological actions of LA have yet to be fully elucidated, thus, significantly more research is required to understand these actions and the underlying mechanisms in the potential of LA to improve neurodegenerative disorders.

In conclusion, our results demonstrate the marked protective activities of LA against MPP⁺-induced apoptosis in PC12 cells. The exact mechanism for this action, although incompletely understood, appears to relate to inhibiting ROS formation and stabilizing mitochondrial membrane, thereby preventing the downstream pro-apoptotic events including cytochrome c release and caspase-3 activation. Further studies are required to elucidate the precise cellular and molecular mechanisms by which LA protects dopaminergic neurons from degeneration, which may lead to potential advancement in the treatment of PD with a neuroprotective strategy.

References


