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Combined R-α–lipoic acid and acetyl-L-carnitine exerts efficient preventative effects in a cellular model of Parkinson's disease

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Abstract

Mitochondrial dysfunction and oxidative damage are highly involved in the pathogenesis of Parkinson's disease (PD). Some mitochondrial antioxidants/nutrients that can improve mitochondrial function and/or attenuate oxidative damage have been implicated in PD therapy. However, few studies have evaluated the preventative effects of a combination of mitochondrial antioxidants/nutrients against PD, and even fewer have sought to optimize the doses of the combined agents. The present study examined the preventative effects of two mitochondrial antioxidant/nutrients, R-α–lipoic acid (LA) and acetyl-L-carnitine (ALC), in a chronic rotenone-induced cellular model of PD. We demonstrated that 4-week pretreatment with LA and/or ALC effectively protected SK-N-MC human neuroblastoma cells against rotenone-induced mitochondrial dysfunction, oxidative damage and accumulation of α-synuclein and ubiquitin. Most notably, we found that when combined, LA and ALC worked at 100–1000-fold lower concentrations than they did individually. We also found that pretreatment with combined LA and ALC increased mitochondrial biogenesis and decreased production of reactive oxygen species through the up-regulation of the peroxisome proliferator-activated receptor-γ coactivator 1α as a possible underlying mechanism. This study provides important evidence that combining mitochondrial antioxidant/nutrients at optimal doses might be an effective and safe prevention strategy for PD.

Keywords: lipoic acid • acetyl-L-carnitine • mitochondrial dysfunction • oxidative damage • Parkinson's disease

Introduction

Increasing evidence indicates that mitochondrial dysfunction and oxidative stress play vital roles in the pathogenesis of sporadic and familial forms of Parkinson's disease (PD) [1, 2]. Mitochondrial antioxidants/nutrients [3, 4] that can improve mitochondrial function and protect mitochondria against oxidative damage have been shown to exert neuroprotective effects against PD in cellular and animal models as well as in clinical trials [5]. For example, in the mouse model of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Coenzyme Q10 (CoQ10) attenuated the generation of oxidants, elevated striatal dopamine levels, and increased the number of striatal mitochondria, the synthesis rate of ATP, and the activity of striatal complex I [6]. Also, a clinical study demonstrated that CoQ10 administration to PD patients attenuated the development of disability [7]. However, few studies evaluate the preventative effects of a combination of mitochondrial antioxidants/nutrients against PD, and even fewer seek to optimize the doses of the combined agents.

The mitochondria have a critical function in the maintenance of cellular energy stores. The peroxisome proliferators-activated receptor gamma co-activator 1α (PGC-1α) is a key regulator of mitochondrial biogenesis and respiration. It is rapidly induced by cold exposure, short-term exercise, and fasting; physiologic conditions known to increase the demand on mitochondria to produce heat or ATP. Recently, it has been reported that PGC-1α null mice have excessive

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oxidative damage and greater dopaminergic cell death in response to the neurotoxin MPTP whereas elevated PGC-1α expression protects neural cells from oxidative stress [8]. Another study suggests that down-regulation of PGC-1α expression leads to mitochondrial dysfunction and neurodegeneration in patients with Huntington's disease (HD) [9]. This evidence provides a connection between impaired energy metabolism and neurodegeneration in PD and HD and suggests that stimulating mitochondrial biogenesis by PGC-1α might be a promising strategy for the prevention of PD. However, no studies have been carried out on this mechanism underlying the beneficial effects of anti-PD drugs/agents.

We address three questions in this paper: first, whether a combination of mitochondrial antioxidants/nutrients has an advantage over individual application in preventing PD; second, if it is necessary to optimize the doses of the combined agents; and third, whether the stimulation of mitochondrial biogenesis by PGC-1α is a possible underlying mechanism for their preventative effects. We selected LA and ALC for study. LA is a disulfide compound that is found naturally in mitochondria as a coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase [10]. LA is also a powerful antioxidant and a potent inducer of the phase 2 antioxidant enzyme that raises cellular antioxidant defence [4, 11]. ALC is an acetyl derivative of L-carnitine, which facilitates the entry and exit of essential fatty acids from the mitochondria. Studies suggest it has a direct or indirect antioxidant activity [12]. Though ALC has proved to protect against MPTP-induced toxicity in murine neuroblastoma cells [13] and monkeys [14], and LA has been implicated in the treatment of PD in both PC-12 cells [15] and mouse models [16], the combination of LA and ALC has not yet been tested in PD models. In the present study, we employed a chronic PD model based on treating SK-N-MC human neuroblastoma cells with 5 nM rotenone for 4 weeks and showed that 4-week pretreatment of the cells with LA and/or ALC potently inhibited the rotenone-induced mitochondrial dysfunction, oxidative damage, and accumulation of α-synuclein and ubiquitin. Most significantly, we found that, when combined, LA and ALC worked at 100–1000-fold lower concentrations than they did individually. We also suggest that the link between increased mitochondrial biogenesis and decreased production of ROS through the up-regulation of PGC-1α might be a possible underlying mechanism for their preventative effects.

Materials and methods

Materials

ADP (high purity, ATP-free) and rotenone were purchased from Sigma (St. Louis, MO, USA); carboxy-H_2-DCFDA, JC-1, MitoTraker, Hoechst 33342, and TRITC-conjugated anti-mouse IgG from Molecular Probes (Eugene, OR, USA); Oxyblot protein oxidation detection kit from Chemicon International Inc. (Temecula, CA, USA); monoclonal anti-8-hydroxyguanine (8-oxo-dG) antibodies from R&D Systems, Inc. (Shanghai, China); BCA protein assay reagent kit from Pierce (Rockford, IL, USA); primary mouse anti-α-synuclein from Lab Vision (Fremont, CA, USA) or from Calbiochem (Darmstadt, Germany); polyclonal rabbit antibody against ubiquitin from DAKO (Carpinteria, CA, USA); monoclonal mouse antibody against complex I subunit (α subcomplex, 9) from Molecular Probes, Invitrogen (Carlsbad, CA, USA); polyclonal rabbit antibody against PGC-1α from Santa Cruz Biotechnology Inc. (San Diego, CA, USA); monoclonal mouse antibody against tubulin from Sigma (St. Louis, MO, USA); IQTM SYBER green supermix reagent from Bio-Rad (Hercules, CA, USA); fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and Rhodamine (TRITC)-conjugated goat anti-rabbit IgG from Sino-American Biotech Co. (China); Western blotting luminol reagent from Santa Cruz Biotechnology, foetal bovine serum from Hyclone (Logan, UT, USA); penicillin and streptomycin from Invitrogen (Carlsbad, CA, USA); GSH assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); and ALC from Sigma-Tau, Germany. R-α-lipoic acid tris salt was a gift from Dr. K. Wessel (Viatris, Germany).

Cell culture and treatments

SK-N-MC human neuroblastoma cell culture and rotenone treatments were carried out following Sherer *et al.*'s protocol [17]. For routine culture, cells were grown in 24-well dishes, fed three times per week, and passaged approximately twice a week when reaching confluence. Cells were pretreated with or without LA and/or ALC for 1–4 weeks, then followed by 5 nM rotenone treatment without LA and ALC (Fig. 1–4) or detected immediately without rotenone treatment (Fig. 5 and 6). Solvents were used for parallel control. The rotenone was added to the culture every 3 days when the medium was changed.

Assays for mitochondrial function

Assay for mitochondrial complex I activity

Mitochondria were isolated by differential centrifugation of cell homogenates. Complex I activity was assayed kinetically by measuring the Vmax reduction of 2,6-dichlorophenol-indophenol (DCPIP) by spectrophotometer (Specter MAX 190, Molecular Devices, Sunnyvale, CA, USA) [18]. In brief, 12.5 μg mitochondrial protein was incubated with Tris-HCl (pH 8.1), 0.2 mM NaN_3, 1 μM antimycin A, 0.1% fat-free BSA, 50 μM CoQ1 and 64 μM DCPIP at 30°C for 5 min. in order to deplete the endogenous substrates of DCPIP, and 0.2 mM NADH was finally added to initiate the reaction.

Assay for mitochondrial membrane potential (MMP) using JC-1

Determination of MMP was carried out using the fluorescent dye JC-1 [19] and analysed by laser confocal microscopy using LSM510META (Zeiss, Germany) and a dual-wavelength/double-beam recording microplate reader (Flex Station 384, Molecular Devices, USA).

Assay for ATP synthesis rate

ATP synthesis rate was assayed by a bioluminescent assay kit (Sigma).

MitoTraker staining for viable mitochondria

Viable mitochondria were examined with MitoTracker staining [20]. The Fluorescence density was measured with Flow Cytometry (FACSAria TM, BD Company) at Ex 488 nm and Em 530 nm.

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Assays for antioxidant defence system and oxidative damage

Assay for GSH levels

GSH was determined using an assay based on a thiol-specific reagent, dithionitrobenzoic acid (DTNB) and measured spectrophotometrically at 412 nm.

Detection of oxidant generation

Oxidant generation was detected using 2′,7′-dichlorodihydrofluorescein (DCFH) [21]. Fluorescence was determined by a flow cytometry apparatus (FACS-SCAN, Becton-Dickinson, Franklin Lakes, NJ, USA) at Ex 488 nm and Em 510–540 nm.

Detection of protein carbonyls

Protein carbonyls were assayed with the Oxyblot protein oxidation detection kit using Western blotting.

Oxidative DNA damage

Oxidative DNA damage was assessed using monoclonal anti-8-hydroxyguanine (8-oxo-dG) antibodies. Fluorescence images were captured on a LSM510META laser scanning microscope (Zeiss, Germany). Fluorescence intensity was quantified from a minimum of 200 cells. DNA damage, *i.e.* single and double strand breaks, was also assayed with the Comet assay [22].

Determination of α-synuclein and ubiquitin levels

Distribution of α-synuclein and ubiquitin was determined by immunofluorescence labelling [17]. The immunofluorescence was observed with a LSM510META (Zeiss, Germany) confocal microscope equipped with a 63×/1.4 HC×PlanAPO oil immersion objective. α-Synuclein was excited with an argon laser (488-nm line), ubiquitin with a helium neon laser (543 nm) and DNA with a UV laser (364 nm).

Assays for mitochondrial biogenesis

Measurement of mitochondrial DNA

Quantitative PCR was performed with an Mx3000P Real-Time PCR system (Stratagene) using the following primers: mitochondrial D-loop forward, 5'–AATCTACCATCCTCCGTG-3' and reverse 5'-GACTAATGATTCTTCACCGT. The human 18SrRNA gene served as the endogenous reference gene [23].

Western blotting analysis of complex I and PGC-1α protein expression

Protein expression of complex I and PGC-1α was detected using Western blotting with primary antibodies directed against PGC-1α (1:1000) or complex I (1:2000) and with horseradish peroxidase-conjugated secondary antibody.

Statistical analysis

One-way ANOVA followed by Turkey/ Kramer *post hoc* tests were used in Figures 1–3, 5E and 6B; while two-way ANOVA followed by Tukey/Kramer *post hoc* tests were applied in Figure 5A, D and 6C. Significance was set at $P < 0.05$. Values shown represent mean ± S.E.M.

Results

Four-week pretreatment with LA and/or ALC counteracted the rotenone-induced mitochondrial dysfunction

Much evidence suggests a major role for mitochondrial dysfunction in the pathogenesis of PD, in particular, defects in mitochondrial complex I of the respiratory chain [1, 2]. As depicted in Figure 1A, SK-N-MC cells treated with 5 nM rotenone alone for 4 weeks exhibited a ~30% loss of complex I activity (the maximum velocity, Vmax), compared with control cells without any additional treatments. The pretreatment with combined LA and ALC at the same concentration (0.1 μM) for 4 weeks significantly counteracted the rotenone-induced decrements in complex I activity ($P < 0.05$). In contrast, though pretreatments with individual LA at 10 μM and 100 μM, ALC at 100 μM, and combined LA+ALC at 1 μM each also attenuated the loss of complex I activity induced by rotenone, no significant effects were observed.

The mitochondrial respiratory chain produces energy that is stored in the form of mitochondrial membrane potential (MMP). This energy is then able to drive the synthesis of ATP. Therefore, diminished mitochondrial function will lead to decreased MMP and a fall in ATP synthesis [24]. We determined MMP using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 can selectively enter into mitochondria and reversibly change colour from green to red as the MMP increases. As shown in Figure 1B (quantitative analysis of JC-1 by fluorescence plate reader) and 1D (JC-1 under confocal microscopy), compared to control, chronic rotenone exposure caused a decrease in the ratio of red to green fluorescence intensity, which reflected a fall in MMP. The 4-week pretreatments with combined LA+ALC with both at a 0.1 μM concentration and with both at a 1 μM concentration significantly prevented the rotenone-induced reduction in MMP ($P < 0.01$). In contrast, pretreatments with LA or ALC alone showed preventative effects at much higher concentrations (10 μM for LA and 100 μM for ALC) ($P < 0.01$ and $P < 0.05$, respectively).

We then examined the rate of ATP synthesis (Fig. 1C). In accordance with JC-1 results, rotenone significantly impaired the ability of the cells to make ATP, compared to control ($P < 0.01$). Pretreatments with LA at 10 μM and ALC at 100 μM, as well as combined LA+ALC with both at a 0.1 μM concentration and both at a 1 μM concentration all significantly prevented the rotenone-induced decrease in ATP synthesis rate ($P < 0.01$, $P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively).

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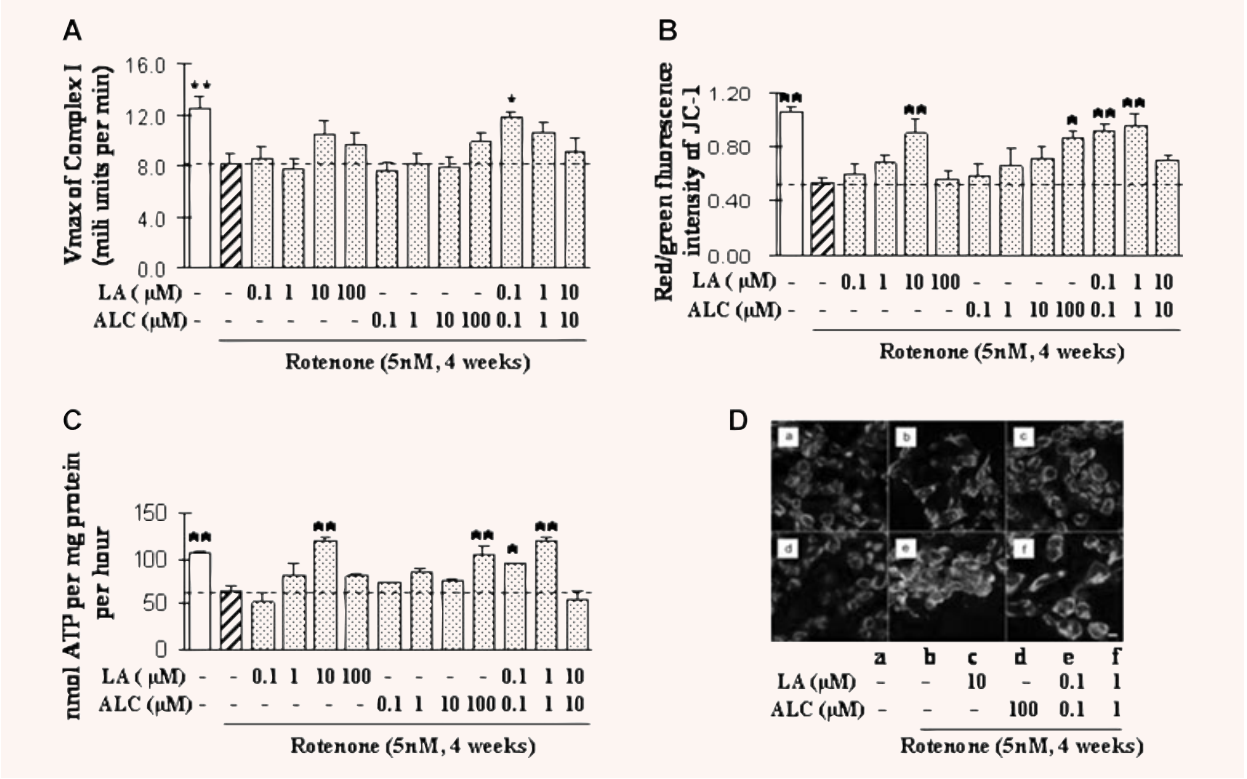

Fig. 1 Pretreatments with LA and/or ALC counteracted mitochondrial dysfunction caused by rotenone. (**A**) Vmax of complex I. The mitochondrial complex I activity was measured kinetically by following the reduction of dichlorophenol-indophenol (DCPIP) and the Vmax was determined. Each experiment was performed in triplicate wells. Data represent means ± S.E.M. of three independent experiments. $^{*}P < 0.05$ *versus* rotenone group. (**B**) Quantitative analysis of MMP with JC-1 staining. Each experiment was performed in triplicate wells. Data represent means ± S.E.M. of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* rotenone group. (**C**) ATP synthesis rate. Each experiment was performed in triplicate wells. Data represent means ± S.E.M. of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* rotenone group. (**D**) Representative confocal microscopic images of cells stained with JC-1 (bar, 10 μm).

Four-week pretreatment with LA and/or ALC attenuated the rotenone-induced GSH reduction and oxidative protein and DNA damage

Mitochondrial dysfunction is one of main sources of oxidative stress. Post-mortem studies have consistently implicated oxidative damage in the pathogenesis of PD [25–27]. We examined the production of reduced glutathione (GSH), an important endogenous antioxidant, and oxidative damage to protein and DNA. As indicated in Figure 2A, chronic rotenone exposure induced a significant decrease in GSH level compared to control ($P < 0.01$). Pretreatment with individual LA at 10 μM and 100 μM, ALC at 100 μM, and combined LA+ALC with both at 0.1 μM and both at 1 μM all profoundly prevented the GSH reduction ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively).

The reduction in GSH raised the possibility of oxidative damage. Protein carbonyls, an index of oxidative protein damage, were detected using the DNPH reaction followed by Western blotting analysis of insoluble protein fractions [28]. As represented in Figure 2B, chronic rotenone exposure resulted in elevated protein carbonyls, compared to control. Pretreatment with individual ALC at 100 μM, and combined LA+ALC at 0.1 μM and 1 μM markedly inhibited the elevation of protein carbonyls.

The above-mentioned results consistently indicate that the individual LA showed preventative effects at 10 μM, individual ALC at 100 μM, and combined LA+ALC at 0.1 μM each and 1 μM each. Thus, we choose these four groups plus control and rotenone-alone treated cells in most of the following experiments.

To assess oxidative DNA damage, we used two methods: immunostaining with antibodies to 8-oxo-dG, a marker of oxidative DNA damage [17], and the comet assay, a single cell-based technique that allows the researcher to detect and quantify DNA damage [22]. As shown in Figure 3, rotenone-alone treated cells showed increased 8-oxo-dG immunoreactivity (Fig. 3A) and a much higher tail moment value (Fig. 3B), compared to control cells ($P < 0.001$

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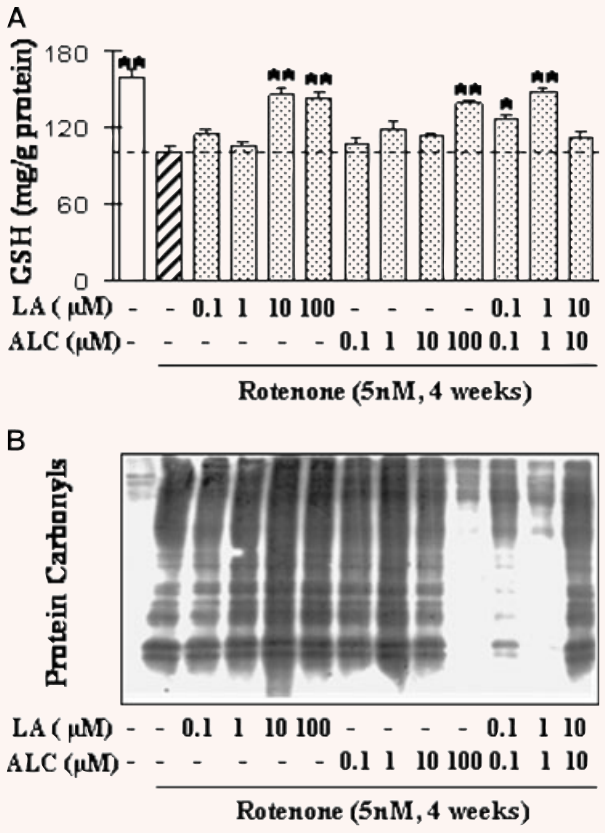

Fig. 2 Pretreatments with LA or/and ALC attenuated rotenone-induced GSH reduction and oxidative protein damage. (**A**) GSH levels were determined using a GSH assay kit and measured spectrophotometrically at 412 nm. Each experiment was performed in triplicate wells. Data represent means ± S.E.M. of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* rotenone group. (**B**) Protein carbonyls were assayed with the Oxyblot protein oxidation detection kit and tested with Western blotting. Images were representative of three independent experiments.

for both). Pretreatment with individual LA at 10 μM, ALC at 100 μM, and combined LA+ALC at 0.1 μM each and at 1 μM each dramatically reduced the 8-oxo-dG immunoreactivity and the tail moment value, indicating that they prevented the oxidative DNA damage caused by rotenone ($P < 0.001$ for all cases except $P < 0.01$ for the effect of 10 μM LA on 8-oxo-dG immnunoreactivity).

Four-week pretreatment with LA and/or ALC prevented rotenone-induced accumulation of α-synuclein and ubiquitin

PD is marked by the accumulation of α-synuclein- and ubiquitin-positive cytoplasmic inclusions known as Lewy bodies. Distribution of α-synuclein and ubiquitin was evaluated by immunofluorescence followed by laser-scanning confocal microscopy. As shown in Figure 4, chronic rotenone exposure resulted in elevated cytoplasmic α-synuclein and ubiquitin accumulation. Pretreatments with individual LA at 10 μM, individual ALC at 100 μM, and combined LA+ALC at 0.1 μM each and at 1 μM each significantly inhibited the accumulation of α-synuclein and ubiquitin.

LA and/or ALC stimulated mitochondrial biogenesis, up-regulated the expression of PGC-1 α and reduced the production of ROS during the 4-week pretreatment

Since LA and ALC were withdrawn after a 4-week pretreatment, the question arose as to whether they actively enhanced the antioxidant defence systems for cells to be resistant to the ensuing 4-week rotenone challenge. Defective mitochondrial function and impaired energy metabolism are highly implicated in the pathogenesis of PD [8]. Thus, the stimulation of mitochondrial biogenesis might be a promising strategy for PD prevention. We evaluated three aspects of mitochondrial biogenesis: number of viable mitochondria, mitochondrial DNA (mtDNA) and the protein expression of complex I, the most important mitochondrial protein involved in PD pathogenesis.

MitoTracker fluorescence was used to assess viable mitochondria. As depicted in Figure 5A, during the 4-week pretreatment, LA and/or ALC increased viable mitochondria in a time-dependent manner though a significant result was only observed in cells pretreated with LA alone at 10 μM for 4 weeks compared to control ($P < 0.05$). Representative flow cytometry histograms for week 4 are shown in Figure 5B.

The D-loop is known as the major site of transcription initiation on both the heavy and light strands of mtDNA. We evaluated the D-loop using real-time PCR with 18SrRNA, a nuclear-encoded gene, as control. As shown in Figure 5E, compared to control, the ratio of mitochondria D-loop/18SrRNA was significantly increased by the pretreatments with combined LA+ALC at 0.1 μM each for 4 weeks ($P < 0.05$), suggesting that this pretreatment caused a marked increase in the number mtDNA copies. Cells pretreated with individual LA at 10 μM, individual ALC at 100 μM, and combined LA+ALC at 1 μM each also showed an elevated ratio of mitochondria D-loop/18SrRNA, but this was not statistically significant.

In accordance with the mtDNA results, compared with control, pretreatments with combined LA+ALC at 0.1 μM each significantly enhanced the protein expression of complex I at weeks 3 and 4, but not at weeks 1 and 2 (Figure 5C and D), revealing a consistent time-dependent effect. No significant results were observed for cells pretreated with individual LA at 10 μM, individual ALC at 100 μM or combined LA+ALC at 1 μM each.

To confirm the beneficial effects of LA and ALC, we further assessed the protein expression of PGC-1α, the central regulator

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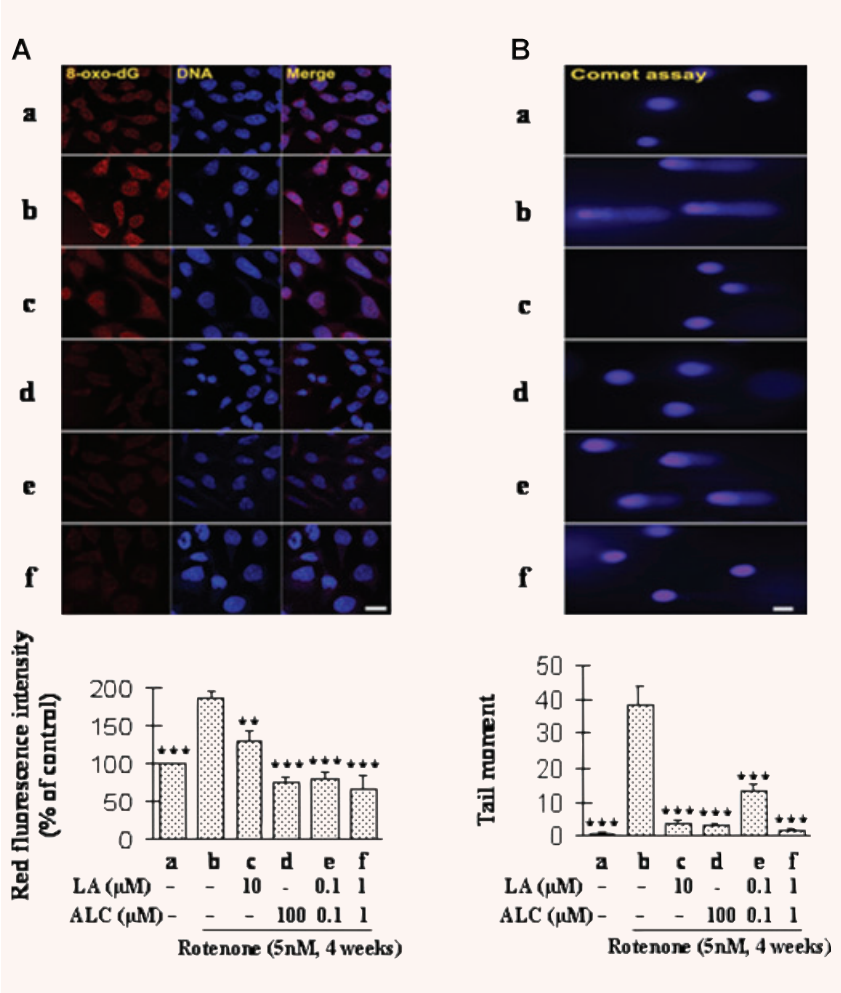

Fig. 3 Pretreatments with LA and/or ALC inhibited rotenone-induced oxidative DNA damage. (**A**) Confocal microscopic images of oxidative DNA damage determined using monoclonal anti-8-oxo-dG antibodies (upper panel, bar, 10 μm) with quantitative analytical results (lower panel). Data are means ± S.E.M. of 3 independent experiments and at least 200 cells were counted in each experiment; (**B**) Microscopic images for comet assay (upper panel, bar, 10 μm). Tail moment, an index of DNA damage, was calculated as the product of DNA fraction in tail and tail length (lower panel). Data are means ± S.E.M. of 3 independent experiments and at least 50 cells were counted in each experiment. $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* rotenone group.

of mitochondrial biogenesis (8, 9). As shown in Figure 6A (Western blotting image) and Figure 6B (the quantitative results), although all cells subjected to pretreatments for 4 weeks showed an increase in the protein level of PGC-1α compared to control, significant up-regulation of PGC-1α protein expression was only observed in cells pretreated with combined LA+ALC at 0.1 μM each ($P < 0.05$), which was consistent with the mtDNA and complex I results.

The maintenance of low ROS levels is critical for normal cellular functions. We determined the level of intracellular ROS by measuring the oxidation of DCFH-DA. As shown in Figure 6C, cells with all pretreatments displayed a time-dependent decrease in ROS levels compared with control. Also, similar to the results of mtDNA, complex I, and PGC-1α, only cells pretreated with combined LA+ALC at 0.1 μM each and at 1 μM each for 4 weeks showed significant decrements in ROS levels ($P < 0.01$ and $P < 0.05$, respectively). Representative flow cytometry histograms for week 4 are shown in Figure 6D. These results not only demonstrate that during the 4-week pretreatment, LA and/or ALC enhanced the antioxidant defence system of the cells by up-regulating the expression of PGC-1α, thereby stimulating mitochondrial biogenesis and reducing the production of ROS, but the results also confirmed the efficiency of the combination of mitochondrial nutrients.

Discussion

We propose that, in order to prevent PD with high levels of efficacy and safety, mitochondrial nutrients should be combined, and they should be combined at optimal doses by targeting key features of the disease, *i.e.* the various aetiologic and pathogenetic factors and development characteristics. Pathogenetic factors involved in PD are not affected to the same degree in each

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patient, and it remains unclear which are primary, which are secondary and how they interact in leading to the degenerative process [1, 26]. Therefore, combined agents that focus on the various aetiologic and pathogenetic factors would be extremely helpful. A combination of mitochondrial nutrients may effectively complement one another in defending the system against mitochondrial dysfunction and oxidative damage [3]. We have demonstrated in previous studies that in aged rats, LA and ALC were more efficient in combination than individually in ameliorating mitochondrial decay with age, inhibiting oxidative damage, and improving cognitive function and ambulatory activity [29–31]. This evidence strongly supports the general concept of determining optimal combinations of mitochondrial nutrients for better neuroprotective effects.

The development of PD is a lengthy process [32, 33]; therefore, its prevention is a long-term task. It has even been suggested that prevention for those who are at high risk for PD should begin during childhood [27]. Therefore, safety as well as efficacy should be emphasized. Because 75–80% of all side effects are dose-related, the lowest effective doses of drugs, even dietary supplements, are desirable [34]. We have selected the chronic rotenone-induced cellular model of PD because in this model, damage develops over a protracted time course and features of PD pathogenesis, such as modest systemic complex I dysfunction (a ~25% loss of activity), oxidative damage, and α-synuclein accumulation are reproduced [17]. In contrast, most of the other MPTP and rotenone-induced cellular and animal models are acute, and the concentrations of rotenone or MPTP used are more than sufficient to completely inhibit complex I [17, 35].

Our present study is the first to provide important clues as to the types and optimal doses of the combined mitochondrial antioxidants/nutrients in preventing PD. We have demonstrated that, when combined, 4-week pretreatment of LA and ALC exerted preventative effects at 100–1000-fold lower concentrations than they did individually in a chronic rotenone-induced cellular model of PD. These effects included (*i*) preventing mitochondrial dysfunction (decrease in complex I activity, MMP and ATP production), (*ii*) attenuating the decrease of GSH, (*iii*) reducing oxidative protein and DNA damage and (*iv*) inhibiting the accumulation of α-synuclein and ubiquitin. The major difference in the effective dose between the combined and individual treatments not only reveals the efficiency of the combined agents, but also consolidates the notion of optimizing doses of multiple mitochondrial antioxidants/nutrients to effectively and safely prevent PD. Though marked differences exist among cellular models, animal models and clinical trials in terms of dose application, the significance of these results could be extended to animal models as well as clinical trials.

We also showed that when LA and ALC were combined at 0.1 μM each or at 1 μM each, they displayed significant preventative effects. However, when they were combined at 10 μM each, no protective effects were observed, and when combined at 10 and 100 μM or at 100 μM each, negative effects occurred (data not shown), though the best doses for LA and ALC alone are 10 and 100 μM, respectively. These data suggested that simply combining multiple agents at each one's optimal dosage might be ineffective or counterproductive, again illustrating the necessity of determining optimal doses of the combined agents.

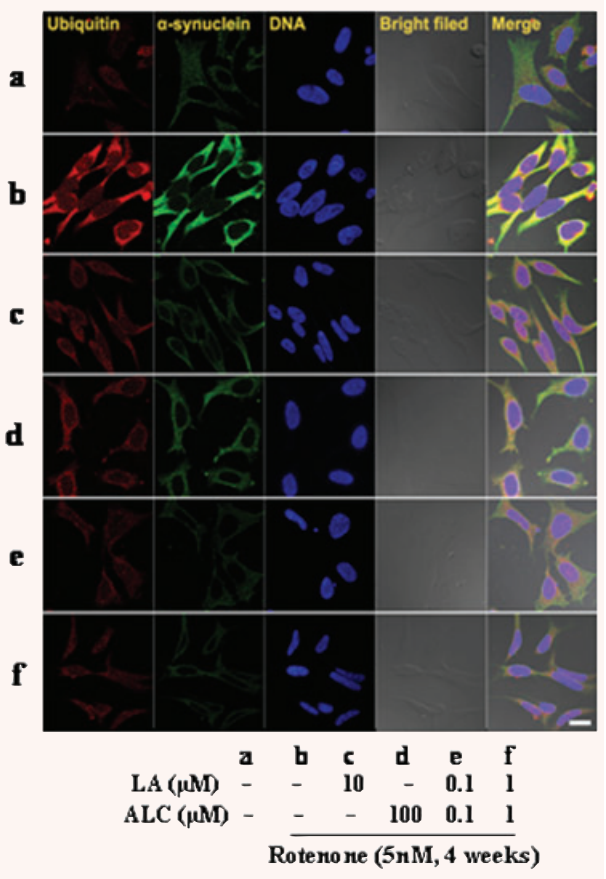

Fig. 4 Pretreatments with LA or/and ALC prevented rotenone-induced accumulation of α-synuclein and ubiquitin Distribution of α-synuclein and ubiquitin were determined by immunofluorescence labelling and confocal microscopy (bar, 10 μm). Images shown are representative of three independent experiments.

Our study is also revolutionary in demonstrating that LA and/or ALC promoted the expression of PGC-1α and time-dependently promoted mitochondrial biogenesis while decreasing ROS levels during the 4-week pretreatment. The prolonged increase in mitochondrial biogenesis could, in principle, cause an increase in the production of ROS, since mitochondrial electron transport chain was the main producer of ROS in most cells. Yet, the paradox found in the present study can be resolved by the novel role recently reported for PGC-1α. It was found that PGC-1α possesses dual activities – stimulating mitochondrial electron transport while susppressing ROS, and may serve as an adaptive

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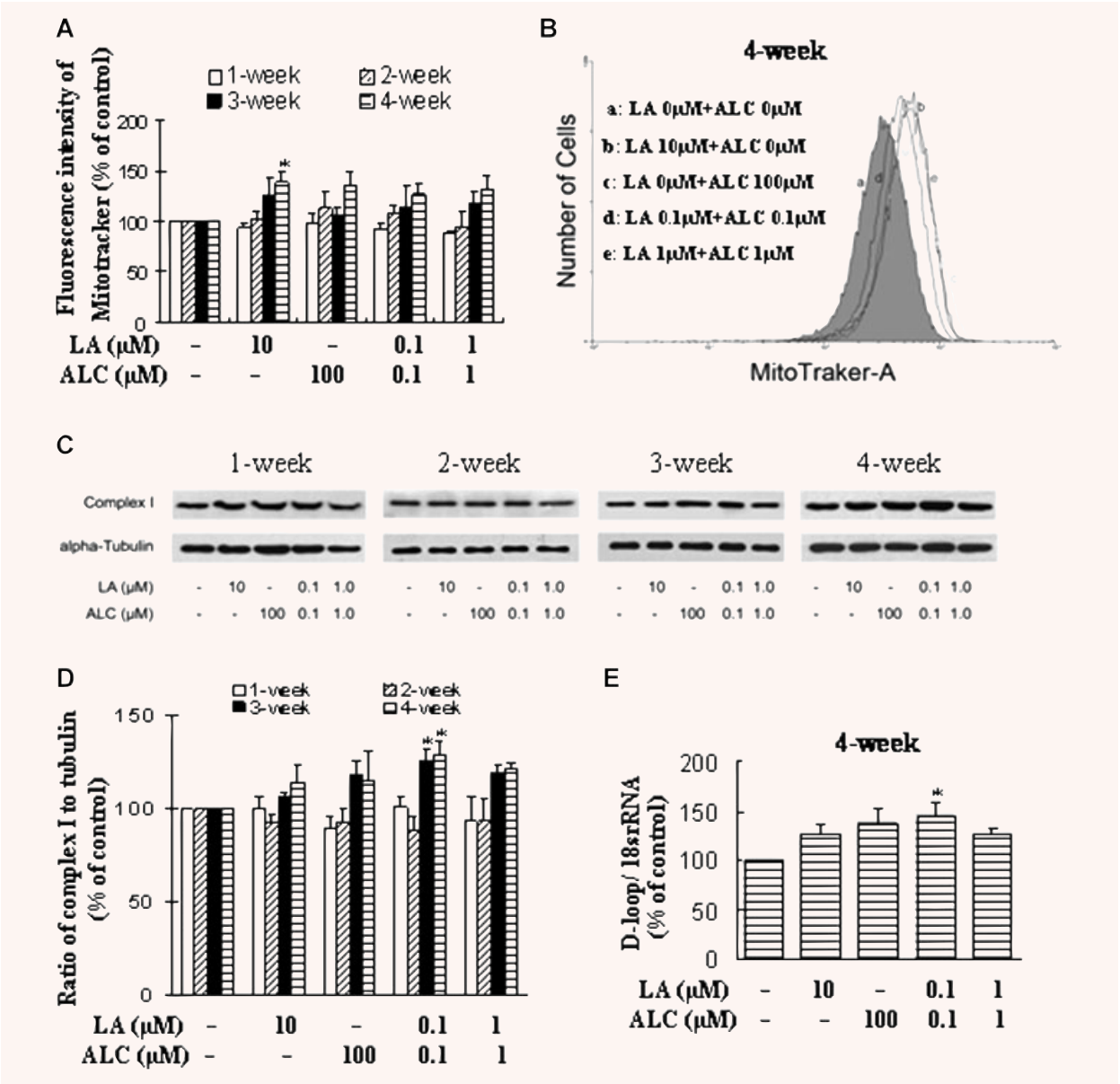

Fig. 5 Pretreatments with LA and/or ALC stimulated mitochondrial biogenesis (viable mitochondria, protein expression of complex I, and mitochondrial DNA (mtDNA)). Cells were pretreated with or without LA and/or ALC for 1–4 weeks, and evaluated immediately without rotenone challenge. Cells that were pretreated with only solvents but neither LA nor ALC were considered as control. (**A**) MitoTracker Green (MTG) was used to label viable mitochondria and assessed by flow cytometry. MTG fluorescence intensity of cells was expressed as a percentage of control. Data are means ± S.E.M. of 3 independent experiments $^*P < 0.05$ *versus* control group. (**B**) Representative flow cytometry histograms of MTG for week 4. (**C**) Representative Western blotting images for complex I protein expression in whole cell homogerates using complex I antibody (Molecular Probes, Catalog #A21344). (**D**) Quantitative results for the analysis of protein expression of complex I by Western blotting in whole cell homogentes. Results were expressed as the relative intensity of complex I to α-tubulin as a percentage of control. Data are means ± S.E.M. of 3 independent experiments $^*P < 0.05$ *versus* control group. (**E**) Expression of mitochondrial DNA for week 4 was measured by real-time PCR. Quantitative values tabulated for D-loop/18sRNA ratio are expressed as a percentage of control. Data are means ± S.E.M. of 3 independent experiments $^*P < 0.05$ *versus* control group.

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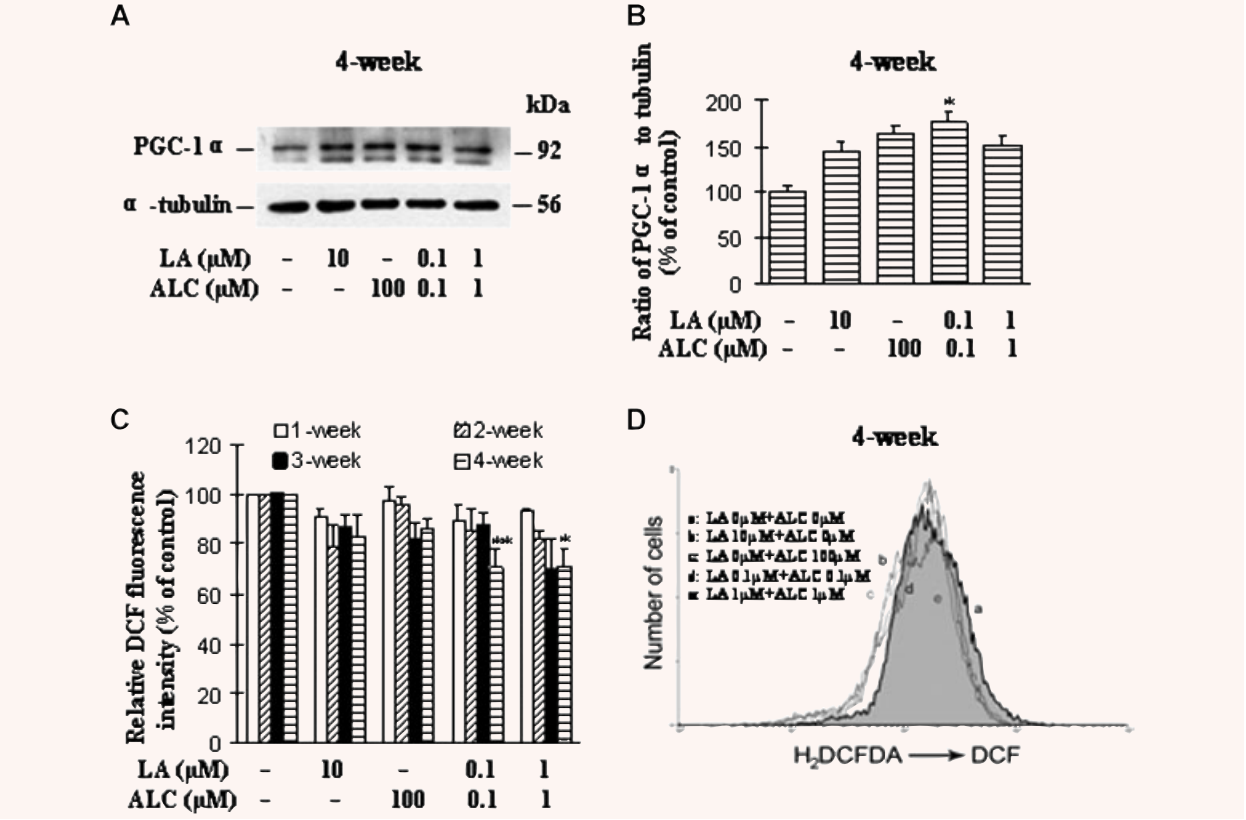

Fig. 6 Pretreatments with LA and/or ALC up-regulated the protein expression of PGC-1 α and reduced the production of ROS Cells were pretreated with or without LA and/or ALC for 1–4 weeks, and evaluated immediately without rotenone challenge. Cells that were pretreated with only solvents but neither LA nor ALC were considered as control. (**A**) Representative Western blotting images for PGC-1α protein expression for week 4. (**B**) Quantitative results for the analysis of protein expression of PGC-1α by Western blotting for week 4. Results were expressed as the relative intensity of PGC-1α to α-tubulin as a percentage of control. Data are means ± S.E.M. of 3 independent experiments $^{*}P < 0.05$ *versus* control group. (**C**) The intracellular levels of ROS were measured using flow cytometric analysis of DCF fluorescence. DCF fluorescence intensity of cells was expressed as a percentage of control. Data are means ± S.E.M. of 3 independent experiments $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* control group. (**D**) Representative flow cytometrographs of DCF assay for week 4.

set-point regulator, capable of providing an accurate balance between metabolic requirements and cytotoxic protection from the ensuing consequences of increased ROS activation [8]. These dual activities make PGC-1α an almost ideal target protein to control or limit the damage associated with defective mitochondrial function in neurodegenerative diseases. It is not yet possible to conclude that linking promotion of mitochondrial biogenesis tightly to reduced ROS production through the up-regulation of PGC-1α is the sole cause of the protective effects of LA and ALC. However, the fact that after the removal of LA and ALC, the cells still resisted the ensuing 4-week rotenone insults makes it extremely likely that this linkage is at least an important contributing factor.

In conclusion, our present study provides important and novel insights into the types and optimal doses of the combined mitochondrial nutrients in PD prevention. We demonstrated that combined LA and ALC exhibited preventative effects at 100–1000-fold lower concentrations than they did individually in a chronic cellular model of PD. We also provide evidence that the link between promotion of mitochondrial biogenesis and reduced production of ROS through the up-regulation of PGC-1α might be a possible underlying mechanism for their protective effects. Though only two of the mitochondrial nutrients were examined in our present study, we hypothesize that more mitochondrial nutrients could be combined to achieve even better effects. The preventative effects of LA and ALC on PD need to be further confirmed in animal models and clinical trials.

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