Synergistic anti-Parkinsonism activity of high doses of B vitamins in a chronic cellular model

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Abstract

We propose that elevation of mitochondrial enzyme cofactors may prevent or ameliorate neurodegenerative diseases by improving mitochondrial function. In the present study, we investigated the effects of high doses of B vitamins, the precursors of mitochondrial enzyme cofactors, on mitochondrial dysfunction, oxidative stress, and Parkinsonism in a 4-week long rotenone treatment-induced cellular model of Parkinson’s disease (PD). Pretreatment with B vitamins (also 4 weeks) prevented rotenone-induced: (1) mitochondrial dysfunction, including reduced mitochondrial membrane potential and activities of complex I; (2) oxidative stress, including increase in reactive oxygen species, oxidative DNA damage and protein oxidation, and (3) Parkinsonism parameters, including accumulation of α-synuclein and poly-ubiquitin. The optimum doses were found around 2.5- and 5-fold of that in normal MEM medium. The 4-week pretreatment was chosen based on time-dependent experiments that pretreatments longer than 2 weeks resulted in a decrease in oxidants, an increase in oxygen consumption, and up-regulation of complex I activity and PGC-1α expression. Individual B vitamins at the same doses did not show a similar effect suggesting that these B vitamins work synergistically. These results suggest that administration of high doses of B vitamins sufficient to elevate mitochondrial enzyme cofactors may be effective in preventing PD by reducing oxidative stress and improving mitochondrial function.

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Keywords: Mitochondrial membrane potential; Complex I; Reactive oxygen species; Oxidative DNA damage; Protein carbonyl

1. Introduction

Mitochondrial dysfunction plays an important role in aging and age-associated neurodegenerative diseases such as Parkinson’s disease (Beckman and Ames, 1998; de Grey, 1999; Shigenaga et al., 1994). The mitochondrial dysfunction appears due to oxidation of mitochondrial RNA/DNA, protein, and membrane lipids (Hagen et al., 1997; Liu et al., 2002a). Oxidation deforms many enzymes, thereby decreasing their affinity for their substrates or coenzymes and their activities. Mechanisms of protein loss of function due to chemical modifications include protein oxidation, addition of aldehydes produced from lipid peroxidation and, in the case of membrane proteins, decreases in fluidity of oxidized membranes. This oxidative decay is particularly acute in mitochondria. Thus, feeding high levels of several mitochondrial cofactors and substrates of mitochondrial enzymes may reverse some of the decay of aging because high levels of substrates/cofactors could bind to enzymes to protect enzyme active sites from oxidant attack and stimulate activity of partially oxidized damaged enzymes (Ames et al., 2002).

Several lines of evidence show that PD is associated with mitochondrial dysfunction: (1) the striatum of PD patients...
displays a defect in complex I of the mitochondrial electron transport chain, i.e., reduced NADH-CoQ reductase activity (Ebadi et al., 2001; Parker and Swerdlow, 1998); (2) MPTP, which causes a PD-like syndrome in humans and rodents, acts by inhibiting mitochondrial complex I (Ebadi et al., 2001; Langston, 1989); (3) similar to MPTP, rotenone and 6-hydroxydopamine (6-OHDA) cause a PD-like syndrome in animals by inhibiting complex I activity and increasing oxidative damage (Betarbet et al., 2002; Glinka et al., 1998). Poon et al. (2005) showed that proteins associated with impaired energy metabolism and mitochondria are particularly prone to oxidative stress associated with A30P-mutant α-synuclein. Veda et al. observed in C. elegans that expressing α-synuclein, deleting Parkin, or knocking down DJ-1 or Parkin produces similar patterns of pharmacological vulnerability and rescue suggesting that diverse PD-related genetic modifications disrupt mitochondrial function. They raise the possibility that mitochondrial disruption is a pathway shared in common by many types of familial PD (Ved et al., 2005). The major physiologically and pathologically relevant oxidant-generating site in mitochondria is found to be limited to the flavin mononucleotide group of complex I, not at the ubiquinone of complex III, as commonly believed, which further emphasizes the importance of ameliorating complex I defects in PD (Liu et al., 2002c).

There are several in vitro models of Parkinson’s disease; however, most of them are “acute” models because cells are treated with high doses of dopamine-related neuronal toxins such as MPP+ (the metabolite of MPTP) for a short period (several hours or 1–2 days). These models may not exactly recapture the characteristics of PD, a chronic neurodegenerative disease. Sherer et al. (2002) have developed a “chronic” model with a low dose (5 nM) of rotenone exposure for 4 weeks to human neuroblastoma SK-N-MC cells. The rotenone exposure causes complex I enzyme dysfunction, ATP depletion, an increase in the level of α-synuclein and poly-ubiquitin protein, and also an increase in oxidative damage, including loss of GSH, increased oxidative DNA damage and protein carbonyls (Sherer et al., 2002). These observations suggest that this chronic rotenone cellular model recapitulates the mechanisms that may be central to PD pathogenesis, and thus can be used efficiently for screening anti-PD drugs.

The peroxisome proliferators-activated receptor gamma co-activator 1α (PGC-1α) is a key regulator of mitochondrial biogenesis and respiration; mitochondrial biogenesis by up-regulation of the PGC-1α pathway has been suggested as a strategy for preventing and reversing various diseases, including obesity and diabetes (Choo et al., 2006; Flachs et al., 2005; McCarty, 2005). It has been reported that PGC-1α null mice have excessive oxidative damage and greater dopaminergic cell death in response to the neurotoxin MPTP whereas elevated PGC-1α expression protects neural cells from oxidative stress (St-Pierre et al., 2006). On the other hand, down-regulation of PGC-1α expression leads to mitochondrial dysfunction and neurodegeneration in patients with Huntington’s disease (Cui et al., 2006). More recently, we have demonstrated that R-α-lipoic acid and acetyl-L-carnitine, which stimulate PGC-1α in 3T3-L1 adipocytes (Shen et al., 2008), also protect SK-N-MC neuroblastoma cells from rotenone-induced damage and stimulate PGC-1α expression and mitochondrial function (Zhang et al., 2008). These results suggest that stimulating mitochondrial biogenesis by PGC-1α might be a promising strategy for the prevention of PD.

No treatments can cure PD or slow down its progress, and most of them can only alleviate the symptoms. Therefore, the best strategy is to prevent the onset of PD. Among the possible preventive strategies, nutritional bioenergetics approaches to enhance mitochondrial function and reduce oxidative damage appear promising (Beal, 2003). The most studied and promising nutrient is coenzyme Q₁₀ (CoQ) (Liu and Ames, 2005). CoQ has been shown to attenuate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity in mice (Ebadi et al., 2001) and slows the progressive deterioration of function in PD patients (Ebadi et al., 2001; Shults et al., 2002). B vitamins are nutrients without toxicity at quite large doses and can be administered for a long time in humans (Ames et al., 2002). It has been proposed that high-dose vitamin B therapy may stimulate variant enzymes with decreased coenzyme binding affinity (increased $K_m$) associated with genetic disease, polymorphisms, and aging (Ames et al., 2002; Ames et al., 2006). However, the ability to adequately measure the effects and mechanisms of B vitamins on motor function in animals is greatly limited and the effects on mitochondrial function and oxidative stress in cellular systems have not been well studied. We hypothesized that B vitamins as antioxidants and enzyme cofactors could inhibit oxidative damage and improve mitochondrial function, and thus, protect neurodegeneration and improve motor function in PD. In this study, we have investigated the effects of long-term (4 weeks) pre-treatment of high doses of B vitamins on improving mitochondrial biogenesis and function, reducing oxidative stress, and ameliorating Parkinsonism in the chronic rotenone cellular model.

2. Materials and methods

2.1. Materials

Carboxy-H₂DCFDA, JC-1, and TRITC-conjugated antimouse IgG were purchased from Molecular Probes (Eugene, OR); Oxysterol protein oxidation detection kit from Chemicon International Inc. (Temecula, CA); monoclonal anti-8-hydroxyguanine (8-oxo-dG) antibodies from Trevigen (Gaithersburg, MD), BCA protein assay reagent kit from PIERCE (Rockford, IL); primary mouse anti-α-synuclein from Lab Vision (Fremont, CA) or from Calbiochem (Darmstadt, Germany); polyclonal rabbit antibody against ubiquitin from DAKO (Carpinteria, CA); polyclonal rabbit antibody against PGC-1α from Santa Cruz Biotechnology Inc. (San
2.2. Cell culture and treatments

SK-N-MC neuroblastoma cells were cultured in minimum essential medium (MEM) with Earle’s salts containing 5 mM glucose, 2 mM l-glutamine, 1 mM sodium pyruvate, nonessential amino acid, 10% fetal bovine serum, 50 U/ml penicillin and streptomycin as described (Sherer et al., 2002). The base levels of B vitamins in the MEM were 1 mg/L for B1, B3, B6, B5 and B11, and 0.1 mg/L for B2 and B7. Cells were exposed to the various concentrations of B vitamin combinations (B1, B2, B3, B5, B6, B7 and B11) for 4 weeks. For 1X group, the B vitamins are the same as the base levels in the MEM except an addition of 0.1 mg/L biotin (B7); For 2.5X, 5X and 10X groups, the B vitamins were 2.5, 5 and 10 times of that in 1X group. The cells were treated from 1 to 4 weeks and the effects on inhibiting ROS and stimulating complex I activity were monitored weekly. After triplicate washes with MEM, the cells were supplemented with or without 5 nM rotenone for 4 weeks (Sherer et al., 2002). Cells were incubated with cell lysis buffer at 20°C for 30 min. Cells were scraped, and the cellular lysate was centrifuged at 800×g for 15 min. The supernatant was mixed with 200×g for 25 min, and the mitochondrial pellet was washed once with the isotonic buffer. Complex I activity was measured kinetically by following the decrease in the absorbance due to 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm for 3 min. The NADH ubiquinone oxidoreductase activity was standardized and expressed as a percentage of complex I activity level in the rotenone-treated control cells.

2.4. Detection of oxygen consumption

Following pre-treatment with the vitamin B combination for the indicated time, 1×10^6 cells were trypsinized and mixed with 200 μL MEM medium with 10% FBS in a BD™ Oxygen Biosensor System 96-well plate. The increase in fluorescence in 2 h was recorded, while only the increase in ratio of fluorescence during the first 1000 s was used to reflect cell oxygen consumption, adjusted by the corresponding protein level.

2.5. Assay for mitochondrial complex I activity

Mitochondria were isolated by differential centrifugation of SK-N-MC cell homogenates (Humphries and Szweda, 1998). In brief, 3×10^7 cells were washed in PBS, resuspended in an appropriate isotonic buffer (0.25 M sucrose, 5 mM Tris–HCl, pH 7.5, and 1 mM EDTA) and homogenized using a glass teflon homogenizer. Unbroken cells and nuclei were pelleted by centrifugation at 800×g for 15 min. Supernatants were centrifuged at 10,000×g for 25 min, and the mitochondrial pellet was washed once with the isotonic buffer. Complex I activity was measured kinetically by following the decrease in the absorbance due to 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm for 3 min. The NADH ubiquinone oxidoreductase activity was measured by spectrophotometer (Specter MAX 190, Molecular Devices, Sunnyvale, CA). The complex I activity was standardized and expressed as a percentage of complex I activity level in the rotenone-treated control cells.

2.6. Detection of protein carbonyls

For determination of protein carbonyls, cells were grown on 100 mm plates. Proteins were extracted for Western blotting as described previously (Sherer et al., 2002) with modifications. Cells were incubated with cell lysis buffer at 4°C for 30 min. Cells were scraped, and the cellular lysate was centrifuged at 12,000×g for 5 min. The supernatant was collected for protein carbonyls detection. Protein carbonyls were assayed with the Oxyblot protein oxidation detection kit.

2.7. Oxidative DNA damage

Oxidative DNA damage was determined using monoclonal anti-8-hydroxydeoxyguanine (8-oxo-dG) antibody (1:300), according to the manufacturer’s protocol. Fluorescence images were captured on a LSM510META laser scanning microscope (Zeiss, Germany).

Determination of MMP was carried out using the dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide) (Smiley et al., 1991). To stain cells with JC-1, 5×10^4 cells were cultured in 96-well dishes (blk/crlbtm, Costar), incubated with 10 μg/mL of JC-1 for 15 min at 37°C, washed twice with PBS, and analyzed by a dual-wavelength/double-beam recording spectrophotometer (Flex Station II384, Molecular Devices, Sunnyvale, CA) (Tirosh et al., 2000). For microscopic observation of JC-1 staining, cells were cultured on glass sheets, with the same staining as above. Fluorescent images were captured on a LSM510META laser scanning microscope (Zeiss, Germany).
and Durand, 2005; Tice et al., 2000). Images were analyzed using Image-Pro Plus 5.0 software. Fifty images were randomly selected from each sample, and the comet tail moment (a product of the DNA fraction in tail and tail length) was measured as an index of oxidative DNA damage.

2.8. Detection of ROS

Cells were grown in 6-well plates until confluent. Hank’s buffer (500 μl) containing 10 μM carboxy-H2DCFDA and 2 mM sodium pyruvate was added to the plate and the cells were incubated at 37°C for 30 min. After washing three times with PBS, ROS levels were analyzed by flow cytometry (FACSRIA™, Becton Dickinson) (Shamoto-Nagai et al., 2003).

2.9. Determination of α-synuclein, ubiquitin and PGC-1α levels

Levels of α-synuclein, ubiquitin and PGC-1α were determined by Western blotting. Soluble and insoluble proteins were prepared in the same way as for determining protein carbonyls described above. The soluble proteins were evaluated by Coomassie blue staining for equal loading. Equal proteins were subjected to 15% SDS-PAGE and transferred onto nitrocellulose membranes, blocked with 5% BSA in TBS-T (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature with rocking. The membranes were incubated overnight at 4°C with a primary mouse anti-α-synuclein (1:2000) or polyclonal rabbit antibody against ubiquitin (1:500) or polyclonal rabbit antibody against PGC-1α (1:1000) in blocking solution. After washing with TBS-T six times for 5 min each, membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (goat anti-mouse 1:4000 for α-synuclein, 1:1000 goat anti-rabbit for ubiquitin and PGC-1α (Dako Cytomation, Denmark) in blocking solution at room temperature for 1–2 h. Chemiluminescence signals were visualized using Western blotting luminol reagent and exposed to film.

2.10. Statistical analysis

Results are expressed as means ± S.E.M. or S.D., and statistical comparisons between groups were performed using one-way ANOVA or generalized linear model (GLM) repeated measures or multivariate measures followed by post hoc comparison using Tukey/Kramer HSD, or LSD tests in SPSS.

3. Results

3.1. Effect on MMP

The MMP was examined both qualitatively (JC-1 distribution under confocal microscopy, Fig. 1A) and quantitatively (JC-1 quantitative analysis by fluorescence plate reader, Fig. 1B). Compared with untreated cells, chronic rotenone exposure caused a fluorescence emission shift from red to green as a decrease in the intensity ratio of red to green fluorescence, indicating mitochondrial depolarization. Pretreatment with B vitamins at 2.5X and 5X prevented the rotenone-induced decrease in MMP significantly.

3.2. Effect on complex I activity

As shown in Fig. 2, chronic rotenone exposure resulted in ∼40% loss of complex I activity, compared to that in untreated cells. Consistent with the MMP results, pretreatment with B vitamins prevented the rotenone-induced decrease in complex I activity, with the optimum protection at 2.5X and 5X B vitamins (Fig. 2).

3.3. Effect on protein oxidation

Protein carbonyl levels, an index of oxidative protein damage, were detected using the DNPH reaction followed by Western blots of the soluble protein fraction. As shown in Fig. 3, chronic rotenone exposure resulted in elevated protein carbonyls, compared to control. The optimum combinations (2.5X and 5X) of B vitamins showed significant inhibition of the rotenone-induced increase in protein carbonyls.

3.4. Effect on oxidative DNA damage

Cells were stained with antibody against 8-oxo-dG, a marker of oxidative DNA damage (red). The same cells were also labeled with DAPI for nuclear morphology. As shown in Fig. 4A, rotenone-treated cells showed increased...
8-oxo-dG immunoreactivity. Many cells with oxidative DNA damage showed fragmented nuclear morphology, a characteristic of apoptosis. Vitamin B combinations at 2.5X and 5X markedly reduced the 8-oxo-dG immunoreactivity. The rotenone-induced DNA damage and the protection of B vitamins were further confirmed with the Comet assay (Fig. 4B). Rotenone-treatment markedly induced the long tail of nuclei, while B vitamins at optimum doses of 2.5X and 5X showed a protective effect on rotenone-induced long tails while the doses of 1X and 10X showed no effect (data not shown).

3.5. Effect on generation of ROS

Since oxidative damage to proteins and nucleic acids and the reduction of antioxidant levels are caused by increases in ROS, we then tested whether the rotenone-induced mitochondrial dysfunction and oxidative damage were accompanied by an increase in ROS, using DCF staining and flow cytometry. As shown clearly in Fig. 5, rotenone-induced a significant increase in ROS and the B vitamins at 2.5X and 5X significantly prevented the rotenone-induced ROS increase (Fig. 5). Either lower or higher doses of B vitamins (1X and 10X) did not have a significant effect on ROS (data not shown).

3.6. Effect on expression of α-synuclein and poly-ubiquitin

The hallmark of PD is the accumulation of α-synuclein- and poly-ubiquitin-positive cytoplasmic inclusions known as Lewy bodies. The Western blot image and quantitative results are shown in Fig. 6A and B for α-synuclein and Fig. 6C for poly-ubiquitin, respectively. Compared to control, chronic rotenone exposure significantly increased the soluble protein level of α-synuclein, while pretreatment with vitamin B combinations at 2.5X and 5X, showed an inhibition of rotenone-induced increase in α-synuclein. Similar results were also observed in poly-ubiquitin protein levels.
Fig. 4. Dose-dependent protective effects of B vitamins on rotenone-induced increase in oxidative DNA damage. (A) Confocal microscopic images of oxidative DNA damage determined using monoclonal anti-8-hydroxydeoxyguanine (8-oxo-dG) antibodies (bar, 10 \( \mu \)m). Representative images of Comet assays showing the DNA fractioning (bar, 10 \( \mu \)m). (B) Quantitative results of the comet tail moment, a product of DNA fraction in tail and tail length was measured as an index of DNA damage. Values are means ± SEM of over 50 cells in each group of four experiments. One-way ANOVA revealed a significant main effect of B vitamins, \( F(3, 182) = 78.277, p < 0.001 \). Tukey/Kramer post hoc tests found there was a significant decrease at control, 2.5X and 5X group, compared to rotenone group, \( p < 0.001, p < 0.001, \) and \( p < 0.001 \).

Fig. 5. Dose-dependent protective effects of B vitamins on rotenone-induced increase in ROS levels assayed by flow cytometry. (A) Representative flow cytometry graph showing the DCF staining. The leftward shift in the curve represents a decrease in oxidant level. (B) Quantitative results of ROS levels, expressed as percentage of the fluorescence intensity over untreated control cells. Values are means ± S.E.M of four experiments. One-way ANOVA revealed a significant main effect of vitamin B, \( F(3, 11) = 4.73, p = 0.023 \). LSD post hoc tests found there was a significant decrease in ROS level after B vitamin treatment for the control, 2.5X and 5X groups compared to rotenone group, \( p = 0.008, p = 0.016 \) and \( p = 0.012 \).

3.7. Time-dependent effects of B vitamins on ROS levels, complex I activity, oxygen consumption and PGC-1\( \alpha \)

To find the optimum time period for the pretreatment with B vitamins, we have tested the effects of pretreatment for 1, 2, 3, and 4 weeks on ROS levels and mitochondrial complex I activities. One week treatment did not cause any change in either ROS levels or complex I activity; however, the pretreatments from week 2 to 4 significantly inhibited ROS generation (Fig. 7A) and also increased complex I activity (Fig. 7B). For oxygen consumption, even 1 week pretreatment resulted in a significant increase; the oxygen consumption reached the highest position at the second week; the oxygen consumption was kept at a relative high level in the following 2 weeks (Fig. 7C). Therefore, we have focused on the time at week 4 in our subsequent experiments.

To identify whether mitochondrial biogenesis is involved in the B vitamin pretreatment, we detected the level of PGC-1\( \alpha \), a transcriptional co-activator for regulating mitochondrial biogenesis (St-Pierre et al., 2006). We found that 4-week pretreatment of B vitamins resulted in an over-expression of PGC-1\( \alpha \), especially in the 2.5X and 5X groups (Fig. 7D).

In order to see whether these effects are due to a synergistic action from the combination of these B vitamins, we tested the effects of the individual B vitamins on MMP and ROS. Unlike the B vitamin combinations, individual B vitamins at 2.5X, 5X, and 10X doses did not significantly increase mitochondrial membrane potential or decrease ROS levels at weeks 1, 2, 3, and 4, respectively (Supplementary data Fig. 1).

4. Discussion

The effects of B vitamins on PD have not been well studied. Alisky (2005) reported that niacin improved rigidity and
bradykinesia in a PD patient but also caused unacceptable nightmares and skin rash. Some researchers have proposed that higher dietary intakes of folate, vitamin B12, and vitamin B6 (cofactors in homocysteine metabolism) may decrease PD risk by decreasing plasma homocysteine. The Rotterdam Study in Netherlands (a prospective, population-based cohort study of people aged 55 years and older) evaluated the association between dietary intake of folate, vitamin B12, and vitamin B6 and the risk of incident PD among 5289 participants and concluded that dietary vitamin B6 may decrease PD risk, probably through antioxidant effects unrelated to homocysteine metabolism and through its role in dopamine synthesis (Aruoma et al., 2006).

We have hypothesized that B vitamins act as antioxidants and mitochondrial enzyme cofactors to produce protective effects in PD cellular and animal models. Our results on the protective effects on the rotenone-induced decrease in mitochondrial membrane potential and the activities of mitochondrial complex I provide supportive evidence to this proposal, though much work should be directed to study the specific enzymes and their kinetics. In addition, it seems likely that aldehydes generated by lipid peroxidation in mitochondria are involved in the decline in function of many mitochondrial enzymes and that a fractional loss of activity due to a decrease in binding affinity to substrates and coenzymes might be ameliorated in part by elevating the levels of substrates and coenzymes (Ames et al., 2006; Liu and Ames, 2005; Liu et al., 2002b). The increased levels of B vitamins and cofactors may protect the enzymes by interfering with the covalent bonding of the aldehydes to enzymes.

Mitochondria are the sources and also the targets of ROS. Studies, using different model systems, demonstrate the involvement of oxidative damage in rotenone toxicity and support the evaluation of antioxidant therapies for PD (Sherer et al., 2003). The most studied antioxidant example is CoQ (Frei et al., 1990). In MPTP-treated mice, CoQ attenuates MPTP-neurotoxicity, elevates striatal dopamine levels, decreases oxidants, and increases the number of striatal mitochondria, the synthesis of ATP, and the activity of striatal complex I (Beal et al., 1998; Ebadi et al., 2001). A clinical study demonstrated that CoQ administration to PD patients attenuated disability development, is safe, and is well tolerated at dosages of up to 1200 mg/d. Thus, it appears that CoQ slows the progressive deterioration of function in PD (Shults et al., 2002). Therefore, reducing ROS and oxidative damage is essential to protect mitochondria and ameliorate PD. The rotenone model demonstrates a significant increase in ROS, protein oxidation and oxidative DNA damage (Sherer et al., 2002). Pretreatment with B vitamins markedly reduced the rotenone-induced increase in ROS, protein oxidation, and oxidative DNA damage. B vitamins and the cofactors derived from them are shown to participate in redox reactions. Hu et al. (1995) showed that thiamine, nicotinic acid, pantothenate, pyridoxine and pyridoxal inhibited hydroxyl radical-induced deoxyribose oxidation with a second order rate constant comparable to or higher than that for mannitol, a known hydroxyl radical scavenger; in addition, pyridoxal...
has a potent ability to bind and deactivate iron. A high dose of nicotinic acid raises NAD/NADP levels in both mitochondria and cytoplasm (Ames et al., 2002). NAD(P)H acts as a hydro- 
gen anion donor in a variety of enzymatic processes, such as the reduction of GSSG to GSH. Therefore, NADPH has been suggested to also act as an indirectly-operating antioxidant to maintain the antioxidant power of glutathione. Kirsch and De Groot (2001) have proposed that NAD(P)H may also act as a directly-operating antioxidant, which limits the action of freely diffusing radicals by scavenging the attacking, oxidizing radical and re-reducing oxidized biomolecules. Based on this proposal, we suggest that the riboflavin derived cofactors FMNH2 and FADH2 might also act as directly-operating antioxidants. Though the direct antioxidant activity of FMNH2 and FADH2 needs further study, it is possible that these cofactors are more efficient at scavenging free radicals and protecting and repairing mitochondrial dysfunction because they are located in mitochondria where the ROS are generated.

The most important biomarker for PD is the accumulation of α-synuclein and ubiquitin. It has been suggested that complex I dysfunction plays a role in regulating α-synuclein levels and elevated α-synuclein expression causes oxidative damage. The chronic rotenone cellular PD model captures...
these pathologies of PD and shows a marked increase in α-synuclein and ubiquitin accumulation (Betarbet et al., 2005; Sherer et al., 2002). In our results, it is clear that pretreatment with B vitamins significantly reduced the rotenone-induced increase in α-synuclein and ubiquitin accumulation; in part, consistent with and due to their reduction of ROS and oxidative damage and their improvement of mitochondrial complex I activity.

In order to understand the protective mechanisms of B vitamins, we have performed a control experiment with treatments of the B vitamins by themselves without rotenone. As shown in Fig. 7, the treatments longer than 2 weeks significantly decreased ROS levels, increased mitochondrial function (complex I activity and oxygen consumption). From these results, it is clear that B vitamins not only protected against the toxic effects of rotenone, but also directly improved mitochondrial and cellular function.

It is known that PGC-1α stimulates mitochondrial electron transport and also suppresses ROS (St-Pierre et al., 2006), therefore, PGC-1α may be an ideal target to control or limit the damage-associated with defective mitochondrial function in neurodegenerative diseases. The 4-week pretreatment also caused an increase in PGC-1α protein level. This PGC-1α stimulation of B vitamin mix is consistent with our work with lipoic acid and acetyl-l-carnitine in SK-N-MC neuroblastoma cells (Zhang et al., 2008) and suggests that one possible mechanism for the protection by B vitamins is the stimulation of mitochondrial biogenesis. This possible mechanism warrants further study.

The rationale of using a mixture rather than a single compound is based on the fact that there is no single initiating factor for dopamine neuron death in idiopathic PD and no consensus as to the mechanisms contributing to the ultimate degeneration of neurons in PD. Thus, putative neuroprotective drugs for PD focusing on a single etiological factor or a single mechanism are unlikely to demonstrate unequivocal neuroprotective effects in the clinic (Anderson et al., 2006), B vitamins and their derived cofactors have various functions and could target various etiological factors and mechanisms of cell death. Therefore, we have proposed that combinations of B vitamins or other nutrients/antioxidants may have additive or synergistic effects. For example, supplementation with combinations of vitamin B6, biotin, and pantethenic acid showed additive effects on extending the mean lifespan of Drosophila (Gardner, 1948). Feeding ApoE-deficient mice (a neurodegeneration model for AD) a diet with vitamin E, ginkgo biloba, pycnogenol, and ascorbyl palmitate resulted in a significant increase in lifespan and a marked reduction of inclusion body histopathology in the hippocampus, and a significant reduction in DNA fragmentation levels (Veurink et al., 2003). A mixture (vitamin E, phosphatidyl choline, and pyruvate) is shown to be more effective than a single antioxidant in preventing beta-amyloid toxicity in cell culture (Shea et al., 2003) and in buffering neuronal degeneration and oxidative stress in cultured cortical neurons and central nervous tissue of apolipoprotein E-deficient mice (Shea et al., 2002). Beal’s group has shown, in a transgenic mouse model of Huntington’s disease, that ramacemide and CoQ showed additive effects on survival, behavior, and weight loss, and that a combination of four different agents (a transglutaminase inhibitor, a nitric oxide synthase inhibitor, ramacemide, and CoQ) showed better protective effects, with a survival increase of up to 46% (Beal, 2003). However, few studies have examined the effects of mitochondrial nutrient combinations in PD except our recent work with R-α-lipoic acid and acetyl-l-carnitine (Zhang et al., 2008). In the study, we demonstrated that 4-week pretreatment with R-α-lipoic acid and/or acetyl-l-carnitine effectively protected SK-N-MC human neuroblastoma cells against rotenone-induced mitochondrial dysfunction, oxidative damage, and accumulation of α-synuclein and ubiquitin, and that when combined, R-α-lipoic acid and acetyl-l-carnitine worked at 100- to 1000-fold lower concentrations than they did individually. Considering the synergistic or additive effects of these mitochondrial nutrients in aging and other diseases (Liu and Ames, 2005), a combination of B vitamins may be more effective for PD prevention and amelioration than the individual B vitamins. As shown clearly in our experiments, it is the combinations of B vitamins, and not the individual B vitamins at doses of 2.5X, 5X and 10X, that showed protective effects on mitochondrial dysfunction and oxidative damage in our chronic cellular model.

In conclusion, we have demonstrated that a 4-week pretreatment with high doses of B vitamins in SK-N-MC cells inhibited 4-week rotenone treatment-induced toxicity, as indicated by amelioration of mitochondrial dysfunction, oxidative damage and hallmarks of Parkinsonism. The protective effects of B vitamins could possibly be attributed to their direct and indirect antioxidant activities and their action as cofactors/substrates of mitochondrial enzymes. It appears that using a mixture of neuroprotective agents with various activities to target various initiating factors and mechanisms of neurodegeneration in PD is likely to demonstrate unequivocal neuroprotective effects in the clinic. However, the protective effects in the cellular system may suggest that more studies should be done to optimize the levels of B vitamins in the current cell culture medium.

Disclosure statement
All authors declare that no any actual or potential conflicts of interest that could inappropriately influence (bias) this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2008.05.031.

References


