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EFFECTS OF LOW CONCENTRATIONS OF ROTENONE UPON MITOHORMESIS IN SH-SY5Y CELLS

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□ The mitochondrial toxin rotenone exerts cytotoxicity *via* overproduction of reactive oxygen species (ROS) and depolarization of the mitochondrial membrane. We investigated the effects of rotenone (12.5, 25, 50, 100 nmol/L) on mitochondrial biogenesis and the potential roles of ROS production in SH-SY5Y cells. Mitochondrial biogenesis was assessed by counting the number of mitochondria, determining protein expression of peroxisome proliferator-activated receptor γ coactivator α (PGC1-α) and its regulator, SIRT1, and oxygen consumption. ROS production and levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were also determined. Compared with controls, rotenone (12.5 nmol/L) significantly increased the quantity of mitochondria and amount of oxygen consumption, whereas rotenone at >12.5 nmol/L decreased the quantity of mitochondria and amount of oxygen consumption. GSH contents and GSH/GSSG were also significantly enhanced by rotenone at 12.5 nmol/L and decreased by rotenone at >12.5 nmol/L. Except for ROS production and SIRT1 protein expression, all concentration–response relationships showed a typical inverted-U shape. ROS production was continually increased in cells treated with rotenone. These data indicate that low concentrations of rotenone can induce mitohormesis, which may be attributed to ROS production.

Key words: mitohormesis; rotenone; mitochondria; PGC1-α

1. INTRODUCTION

Rotenone is a highly toxic, naturally occurring botanical pesticide. It was mainly used in organic farming and in the management of nuisance fish (Hatcher *et al.*, 2008). It is extremely hydrophobic and hence readily crosses biologic membranes, apparently without a specific transport system, and acts by inhibiting mitochondrial complex I (Esteve-Rudd *et al.*,

2011). Thus, rotenone is recognized as a mitotoxin. However, Ichikawa and colleagues found that pretreatment with low-level rotenone could inhibit lipid peroxidation and alleviate the intestinal mucosal inflammation induced by ischemia–reperfusion in rats (Ichikawa *et al.*, 2004). This finding implied that, as a mitotoxin, rotenone may produce different cellular effects at low levels from those at high levels. Hence, exploring the potential underlying mechanisms of protection exerted by pretreatment with rotenone is important.

Mitochondria are the major sites for energy production in cells. When cells and tissues demand increased energy requirements, the primary response is to produce new mitochondria (Sano and Fukuda, 2008). In general, mitochondrial biogenesis is regulated by changing energy requirements and physiological conditions. Recent evidence has provided insight into the mechanisms underlying the interaction between mitochondria-derived reactive oxygen species (ROS) signaling and mitochondrial biogenesis (Piantadosi and Suliman, 2006; Piantadosi *et al.*, 2008). There is increasing evidence to suggest that ROS may be 'double-edged swords'. That is, ROS overproduction is toxic to cells, but they also play an important part in the cell signaling involved in the antioxidant defense network. The polyunsaturated fatty acids in membrane lipids are particularly vulnerable to ROS attack, and they undergo peroxidation. These products of lipid peroxidation form protein adducts and DNA adducts and have biphasic properties, i.e., high doses cause overt toxicity whereas low doses interact with genetic signaling systems that upregulate gene expression to counteract stressor challenges and re-establish homeostasis (Sano and Fukuda, 2008). This adaptive stress-protective mechanism resulting from the induction of ROS-related stress emanating from mitochondria has been defined as 'mitochondrial hormesis' or 'mitohormesis' (Tapia, 2006).

Besides ROS, peroxisome proliferator-activated receptor γ coactivator α (PGC1α) controls the biogenesis and function of mitochondria (Finck and Kelly, 2006), and thereby plays an important part in brain-energy homeostasis and neurodegenerative diseases (Cui LB *et al.*, 2006; St-Pierre *et al.*, 2006). Furthermore, PGC1α activity was shown to be regulated by Sirt1, a member of the silent information regulator 2 family of proteins (sirtuins) (Lagouge *et al.*, 2006; Nemoto *et al.*, 2005). Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent deacetylases regulating mitochondria-involved survival and longevity (Grubisha *et al.*, 2005). However, data about rotenone-induced mitohormesis as well as the potential roles of PGC1α and Sirt1 are lacking.

Evaluating the mitochondrial effects of low-level rotenone and the potential underlying mechanisms are important because they may provide more evidence for risk assessment and aid in the discovery of new agents for the prevention and treatment of neurodegenerative diseases.

2. MATERIALS AND METHODS

2.1. Materials

Rotenone, antimycin and cccp were purchased from Sigma–Aldrich (St. Louis, MO, USA). Minimum essential medium (MEM), Ham's F-12 Nutrient Mixture (F12 medium), non-essential amino acids and trypsin were obtained from Gibco BRL (Rockville, MD, USA). The fluorescent probe MitoTracker Red FM was purchased from Molecular Probes (Eugene, OR, USA). Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials (Hangzhou, China). DCFH-DA and lysis buffer were purchased from Biyotime (Nantong, China). β-actin primary antibody was from Abcam (Cambridge, MA, USA). PGC-1α and SIRT1 primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All secondary antibodies were from Boster Biological Technology (Wuhan, China). All other reagents were from commercial suppliers and of standard biochemical quality.

2.2. Culture of SH-SY5Y Cells

Human neuroblastoma SH-SY5Y cells (a gift from Dr. Zunji Ke, Chinese Academy of Sciences, Beijing China) were maintained in MEM/F12 medium, supplemented with 1% non-essential amino acids (Gibco) and 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in 5% CO_2. Cells were subcultured once every 3 days. Before experiments, SH-SY5Y cells were plated at $\approx 3 \times 10^4$ viable cells per well in six-well plates. Cells were treated with rotenone for 48 h after seeding. The choice of concentrations used (12.5–100 nmol/L) was based on our previous experiments (Xiong *et al.*, 2009). Rotenone was prepared by dissolving in dimethyl sulfoxide (DMSO). The final concentration of DMSO was <0.1%, which was shown to have no toxic effect on the cells.

2.4. Mitochondrial mass

MitoTracker Red FM binds to lipids in mitochondrial membranes regardless of the membrane potential or oxidant status of mitochondria. Thus, fluorescence intensity was used to measure the number of mitochondria present (Pendergrass *et al.*, 2004). To determine the numbers of mitochondria, a suspension of cells in free serum medium was loaded with 200 nmol/L MitoTracker Red FM for 30 min at 37°C. Measurements of fluorescence intensity were made at an excitation wavelength of 581 nm and emission wavelength of 644 nm using a fluorescence spectrometer (Molecular Devices Corporation, Sunnyvale, CA, USA). Amounts were determined by comparing the intensity of the fluorescence signal produced by 1×10^4 cells.

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2.5. Reduced glutathione (GSH) content

The reduced GSH content and total glutathione /its oxidized form (GSSG)in cells was determined using Ellman's reagent, i.e., 5,5-dithiobis-(2-nitrobenzoic acid) (Sedlak and Lindsay, 1968). Briefly, cells were lysed using cell lysis buffer and lysates centrifuged at $12,000 \times g$ at $4°C$ for 5 min. GSH content and total glutathione / GSSG in the supernatant was then estimated using Ellman's reagent. Spectrophotometric absorbance was determined at 420 nm and 405 nm using a GSH or T-GSH/GSSG kit (Nanjing Jiancheng Bioengineering Company, Nanjing, China). The reduced GSH results were expressed as milligrams of GSH per milligram of protein. To calculate the GSH/GSSG ratio, GSH (reduced form) was obtained by subtracting the $2 \times GSSG$ values from the total glutathione values.

2.6. Oxygen consumption

SH-SY5Y cells were harvested and resuspended in MEM/F12 medium. MEM/F12 medium was equilibrated at $37°C$ with atmospheric oxygen. Oxygen consumption was measured with a Clark-type oxygen electrode (Hansatech, King's Lynn, UK) fitted in a 2 mL thermojacketed sample chamber ($37°C$) under constant stirring. Results were expressed as nanomoles of O_2 per minute per cell.

2.7 ROS levels

ROS levels were measured using the ROS-specific probe $5',6'$-chloromethyl-$2',7'$-dichlorodihydrofluorescein diacetate (H_2DCFDA; Beyotime Institute of Biotechnology, Nantong, China). After diffusion into cells, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into $2',7'$-dichlorofluorescin (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 488 nm and 525 nm, respectively. Cells in free serum medium were loaded with freshly prepared 5 μmol/L H_2DCFDA for 30 min and washed thrice with phosphate-buffered saline (PBS). Fluorescence intensity representing ROS levels of live cells was measured using a fluorescence spectrometer. Results were expressed as fluorescence intensity per 1×10^4 cells.

2.8. Western blot analyses

After treatment, 1×10^6 cells were collected and subjected to western blot analyses. Cell proteins were extracted and quantified with a BCA Protein Quantitative Analysis Kit (Beyotime Institute of Biotechnology). After addition of sample loading buffer, protein samples underwent sodi-

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um dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 8% gels, and were subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Each membrane was incubated in fresh blocking buffer (0.1% Tween 20 in Tris-buffered saline, pH 7.4, containing 5% non-fat dried milk) at room temperature for 30 min and then probed with anti-PGC-1α antibody, anti-Sirt1 antibody or anti-β-actin antibody in blocking buffer at 4°C overnight. Membranes were washed thrice for 5-min each using PBST (PBS and 0.1% Tween 20). Membranes were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h. Immunoreactive proteins were visualized using the chemiluminescent reagent ECL according to manufacturer instructions. Quantity One software was used to quantify protein band intensities in digital images of the blot.

2.9. Statistical analyses

Differences were tested by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test as a *post-hoc* test. $P < 0.05$ was considered significant. All of the experiments were done in triplicate and repeated three or four times.

3. RESULTS

3.1 Effects of rotenone on mitochondrial biogenesis

We first investigated the effects of rotenone on the number of mitochondria in SH-SY5Y cells using MitoTracker Red FM. Alterations in the mitochondrial mass when SH-SY5Y cells were exposed to rotenone (12.5–100 nmol/L) for 24 h are shown in Fig. 1. When rotenone (25 nmol/L) was exposed to cells, the mitochondrial mass increased significantly ($p < 0.05$) compared with that of controls. When the concentrations of rotenone increased, the numbers of mitochondria decreased.

3.2 Effects of rotenone on mitochondrial respiration

Oxygen consumption is a critical readout of mitochondrial function(Gusdon *et al.*, 2012). The CCCP induced maximal respiratory capacity were increased in SH-SY5Y cells(Fig 2). Quantification of mitochondrial respiration in SH-SY5Y cells treated with various concentrations of rotenone is shown in Fig 2. In the concentration range 12.5–25 nmol/L, the oxygen consumption of cells increased by 33.8–32.5% compared with that in control cells ($p < 0.05$). The increased oxygen consumption can be attenuated by 20 μmol/L antimycin. This suggested that cellular mitochondrial metabolism was enhanced in response to low concentrations of rotenone.
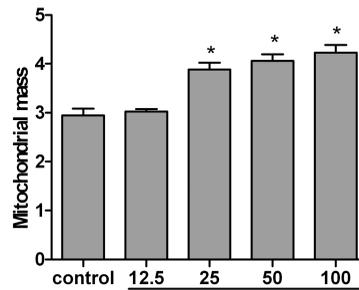
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FIG. 1. Rotenone stimulates mitochondria generation. Changes in the mitochondrial stimulation of SH-SY5Y cells treated with rotenone at the indicated concentration for 24 h were analyzed by fluorescence spectrometry. Data are means ± SD, n = 5. *P<0.05 *vs.* the control group.
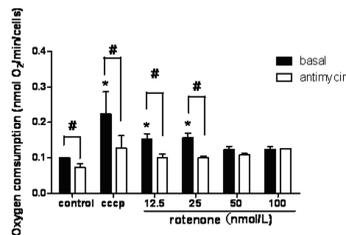

FIG. 2. Oxygen consumption in SH-SY5Y cells treated with rotenone. Quantification of the rate of oxygen consumption of SH-SY5Y cells treated with rotenone or 10 µmol/L cccp for 24 h. Antimycin induced oxygen consumption were measured by treatment with 20 µmol/L antimycin after detected the basal. Data are means ± SD, n = 5. *P<0.05 *vs.* the control group.

3.3 Effects of rotenone on protein expressions of PGC-1α and SIRT1

To test whether the mitochondrial biogenesis and improvement in mitochondrial function are closely related to mediated by SIRT1 and PGC-1α, we next investigated rotenone impact on its expression. Expression of PGC-1α and SIRT1 increased at low concentrations of rotenone (12.5 and 25 nmol/L) (Fig. 3). SIRT1 demonstrated a ~ 20% increase compared with control group, while PGC-1α showed ~26% increase. However, at higher concentrations (50 and 100 nmol/L), expression of SIRT1 still increased (~ 40% compared with control group)but that of PGC-1α decreased to normal level.

3.5 Effects of rotenone on ROS production

To elucidate whether ROS can initiate mitohormesis, cells were treated with 12.5–100 nmol/L rotenone for 24 h, and then measured by the fluorescent probe DCFH-DA, which is freely permeative to the cell membrane. Low doses of rotenone led to an increase in ROS levels. Figure 4 shows that 12.5-100 nmol/L rotenone present a 20% - 43% increase in ROS levels over control SH-SY5Y cells. ROS levels increased significantly
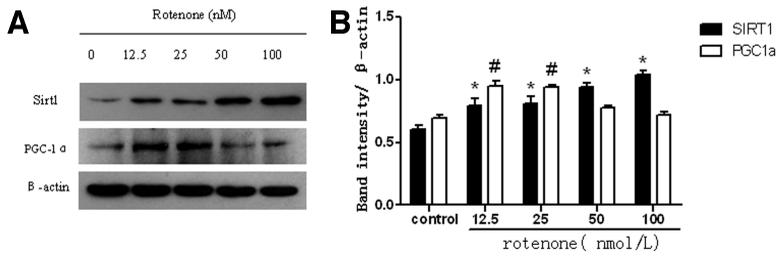
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FIG. 3. Effects of rotenone on expression of PGC1α and SIRT1. (A) SH-SY5Y cells were incubated with various concentrations of rotenone for 24 h. Levels of PGC1α and SIRT1 were detected by western blot. (B) Protein band intensities were normalized to β-actin. Data are means ± SD, n = 3. *P<0.05 *vs.* the control group.
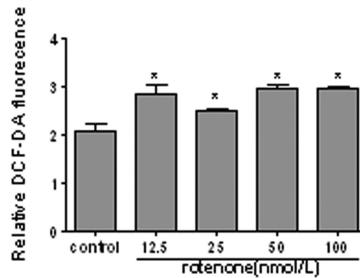

FIG. 4. ROS generation in SH-SY5Y cells after treatment with 12.5–100 nmol/L rotenone for 24 h. Values are means ± SD (n = 3) of fluorescence intensity. Data represent means ± SD, n = 5. *P<0.05 *vs.* the control group.

compared with the control group (p<0.05), and ROS levels increased in a dose-dependent manner.

3.4. Effects of rotenone on GSH contents

Oxidative stress is related to both an increase in ROS production and a decrease in the antioxidant content. Compared with the control group, GSH and GSH/GSSG ratio levels were significantly increased at a low concentration of rotenone (12.5 nmol/L) which is of great relevance with respect to oxidative stress, whereas higher levels of rotenone suppressed GSH contents (Fig. 5).

4. DISCUSSION

The main finding of the present study was that low doses of rotenone enhanced mitohormesis in SH-SY5Y cells *via* regulation of the interaction between PGC1α and SIRT1. These results provide a bio-energetic basis for the protection of rotenone preconditioning against lipid peroxidation and intestinal mucosal inflammation induced by ischemia–reperfusion in rats (Ichikawa *et al.*, 2004). Combined with the mitohormesis produced by another mitotoxin and mitochondrial uncoupler, 2,4-dinitro-
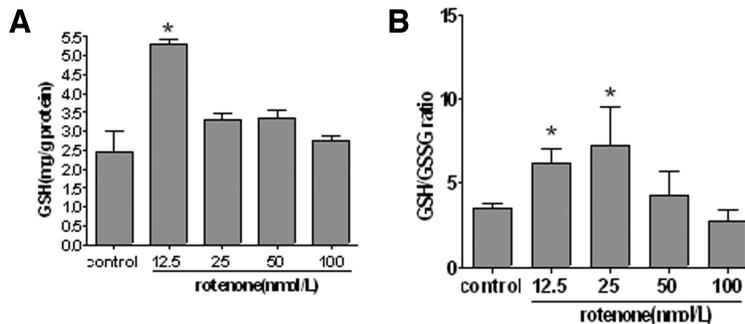
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FIG. 5. Effects of rotenone on the activities of (A) GSH and (B) GSH /GSSG in SH-SY5Y cells. Exponentially growing cells were treated with the indicated amount of rotenone for 24 h. *p<0.05 as compared with the control group. Data are means ± SD, n = 5. *P<0.05 *vs.* the control group.

phenol (Caldeira da Silva *et al.*, 2008), we may imply that the mitohormesis of low-level mitotoxins may be a general phenomenon.

Mitohormesis is based on the assumption that an increase in mitochondrial activity and the subsequent increase in ROS production leads to activation of the cellular defense system in response to low levels of deprivation of metabolic substrates (Ristow and Zarse, 2010; Ristow and Schmeisser, 2011; Schulz *et al.*, 2007; Schwartz and Sack, 2008; Tapia, 2006). Mitotoxins such as 2,4-dinitrophenol have been found to induce mitohormesis which can extend human lifespans by promoting: enhanced rates of tissue respiration; increased levels of glucose, triglyceride and insulin in serum; decreased levels of ROS; decreased oxidation of DNA and protein in tissues; reduced body weight (Caldeira da Silva *et al.*, 2008).

We identified induction of mitohormesis by treatment of cells with a low dose of rotenone (12.5 nmol/L) for 24 h, and oxygen consumption was also enhanced. This stimulation of mitohormesis and oxygen consumption was different to results in other studies, in which a decrease in mitochondrial mass and respiration were observed (Koopman *et al.*, 2006; Yang *et al.*, 2010). These differences might be due to different selected dosages and treatment duration, which may activate or inactivate signal pathways by regulating mitohormesis.

From the viewpoint of bio-energetics, PGC-1α appears to act as a 'master regulator' by integrating and coordinating the activity of multiple transcription factors such as nuclear respiratory factors (NRF-1, -2), peroxisome proliferator activated receptor-α (PPARα) and mitochondrial transcription factor A (mtTFA) in mitohormesis and energy metabolism (Puigserver *et al.*, 1998). Moreover, PGC-1α was shown to mediate mitohormesis *via* cyclic guanosine monophosphate (cGMP)-mediated upregulation of transcriptional factors (Brown, 2007). Increases in cGMP levels can lead to expression of the deacetylase SIRT1 (Nisoli *et al.*, 2005). SIRT1 is a NAD^{+}-dependent deacetylase that can deacetylate PGC-1α and

thereby increase its activity (Nisoli and Carruba, 2006). SIRT1 activation by 3,5,4,9-trihydroxystilbene (resveratrol) has recently been implicated in the mitohormesis induced by this polyphenol in cultures of liver and muscle cells (Baur *et al.*, 2006; Lagouge *et al.*, 2006). It appears that SIRT1 functions as a signaling mediator that links mitochondrial activity and transcriptional regulation at the nucleus, leading to the expression of genes involved in mitohormesis through activation of PGC-1α. The present study showed that rotenone activated PGC-1α and SIRT1 in different manners. The former showed biphasic effects and the latter demonstrated monophasic effects, which may imply complex interactions between PGC-1α and SIRT1 in mitohormesis. This finding was consistent with a study that showed that higher overexpression of SIRT1 caused significant mitochondrial abnormalities (i.e., abnormal morphology of mitochondria and abnormal gene expression related to mitochondria) whereas lower overexpression of SIRT1 showed the opposite effect (Kawashima *et al.*, 2011). Low overexpression of SIRT1 may induce mitohormesis *via* deacetylate PGC-1α, but higher SIRT1 overexpression may induce hyperdeacetylation of histone proteins and repress PGC-1α transcription, which will subsequently decrease the mass and function of mitochondria.

Mitohormesis theory posits that ROS production by mitochondria enhances stress resistance, thereby extending human lifespans (Schulz *et al.*, 2007). Deoxyglucose, which are capable of inducing mitochondrial ROS formation can generate lifespan extension, whereas pretreatment of cells with the antioxidant N-acetyl cysteine (NAC) was shown to significantly reduce the induction of ROS and, in parallel, completely abolish increases in stress resistance. These findings suggest that increased formation of ROS is required for the hormetic induction of stress resistance. Consistent with this hypothesis, the present study showed that a low dose of rotenone (12.5 nmol/L) can increase ROS production, which will activate mitohormesis-mediated stress resistance through upregulation of ROS defense enzymes. A mechanistic link between increased respiration, elevated production of ROS, and adaptive induction of ROS defense systems has been suggested. ROS have been traditionally viewed as damage-causing entities. However, they are equally important for normal cellular functioning, and have been noted as mediators of cellular metabolism (Cadenas, 2004). It is highly likely that increased ROS levels activate an array of sensors and mediators, which in turn orchestrate the multifaceted response to mitohormesis, ultimately leading to promotion of cellular homeostasis.

In total, the present study suggested that low concentrations of rotenone may induce mitohormesis *via* upregulation of the production and defense capacities of ROS. Moderate increases in ROS levels may lead to increased expression of SIRT1, which then deacetylates PGC-1α, and subsequently stimulates mitohormesis. If there is overproduction of

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ROS, high expression of SIRT1 may repress PGC-1α transcription, leading to damage to the mass and function of mitochondria. The present study demonstrated that application of mitotoxins may be an alternative intervention to maintain physiological and metabolic homeostasis, and to prolong lifespans in mammals.

5. ACKNOWLEDGMENTS

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