BIOCHEMICAL PHARMACOLOGY 76 (2008) 128–138

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GSK3β and endoplasmic reticulum stress mediate rotenone-induced death of SK-N-MC neuroblastoma cells

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ARTICLE INFO

Article history:
Received 13 February 2008
Accepted 22 April 2008

Keywords:
Neurodegeneration
Oxidative stress
Parkinson's disease
GSK3β
ER stress

ABSTRACT

Rotenone, an environmental toxin that inhibits mitochondrial complex I, has been used to induce experimental Parkinsonism in animals and cell cultures. We investigated the mechanism underlying rotenone-induced death of SK-N-MC neuroblastoma cells. Rotenone-induced cell death preceded intracellular accumulation of reactive oxygen species, and antioxidants failed to protect cells, indicating that oxidative stress was minimally involved in rotenone-induced death of SK-N-MC cells. Glycogen synthase kinase 3β (GSK3β), a multifunctional serine/threonine kinase, has been implicated in the pathogenesis of neurodegeneration. We showed that rotenone activated GSK3β by enhancing its phosphorylation at tyrosine 216 while inhibiting phosphorylation at serine 9. Inhibitors of GSK3β and dominant negative (kinase deficient) GSK3β partially protected SK-N-MC cells against rotenone cytotoxicity. Rotenone also induced endoplasmic reticulum (ER) stress which was evident by an increase in phosphorylation of PERK, PKR, and eIF2α as well as the expression of GRP78. Rotenone had a modest effect on the expression of CHOP. An eIF2α siRNA significantly reduced rotenone cytotoxicity. ER stress was experimentally induced by tunicamycin and thapsigargin, but tunicamycin/thapsigargin did not activate GSK3β in SK-N-MC cells. Down-regulation of eIF2α also offered partial protection against rotenone cytotoxicity. Combined treatment of GSK3β inhibitors and eIF2α siRNA provided much greater protection than either treatment alone. Taken together, the results suggest that GSK3β activation and ER stress contribute separately to rotenone cytotoxicity.

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Abbreviations: Carboxy-H_2DCFDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; CHOP, growth arrest and DNA-damage inducible protein/C/EBP homologous protein; eIF2α, α subunit of the eukaryotic initiation factor-2; ER, endoplasmic reticulum; dsRNA, double-stranded RNA; IRE1, inositol requiring enzyme 1; GRP78, the 78-kD family member of glucose-regulated proteins; GSH-MEE, glutathione monoethyl ester; GSK3β, glycogen synthase kinase 3β; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffered saline; PD, Parkinson's disease; PERK, pancreatic endoplasmic reticulum kinase/PKR-like endoplasmic reticulum kinase; PKR, double-stranded RNA-activated protein kinase; ROS, reactive oxygen species; SK-N-MC, SK-N-MC neuroblastoma cells; siRNA, small interfering RNA; TPBS, Tween phosphate buffered saline; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.04.010

1. Introduction

Rotenone, a widely used pesticide, is a well-characterized, high-affinity inhibitor of mitochondrial complex I, one of the five enzyme complexes of the inner mitochondrial membrane that regulates oxidative phosphorylation [1,2]. Rotenone has been used to experimentally model the pathogenesis of Parkinson's disease (PD) [3,12].

PD is the second most common neurodegenerative disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra and accumulation of cytoplasmic inclusions (Lewy bodies), resulting in resting tremors, rigidity, slowness of movement and postural instability [4,5]. The majority of PD is sporadic and the cause of its pathogenesis is unclear. Epidemiological studies reveal that exposure to pesticides or herbicides is a risk factor for PD pathogenesis [6,7]. The systemic dysfunction in complex I is associated with the occurrence of Parkinsonism [8,9]. Therefore, chemicals targeting the mitochondrial complex I are introduced to model PD pathogenesis [10]. Rotenone exposure to rats produces selective degeneration of nigrostriatal dopaminergic neurons, Lewy body-like inclusions and specific behavioral characters of PD such as abnormal postures and slowness of movement [3].

It is suggested that rotenone neurotoxicity may be mediated by complex I inhibition and oxidative stress [11]. In animal models, the brain rotenone concentration is around 20–30 nM [9,12]. At these concentrations, however, rotenone does not significantly impact mitochondrial respiration, suggesting that bioenergetic (ATP) deficit is not the main cause for rotenone neurotoxicity [12]. On the other hand, oxidative stress is considered a major mediator since rotenone-induced neurodegeneration is accompanied by oxidative damage to protein and DNA [13]. In cell cultures, rotenone-induced intracellular accumulation of reactive oxygen species (ROS) is detected after exposure periods of 24 h or longer [13,14]. However, rotenone cytotoxicity is evident before significant ROS accumulation. Therefore, mechanisms other than ROS accumulation must be operative.

GSK3β is a multifunctional kinase; more than 40 proteins are substrates of GSK3β, including transcription factors, cell cycle/survival regulators and oncogenic/proto-oncogenic pro-

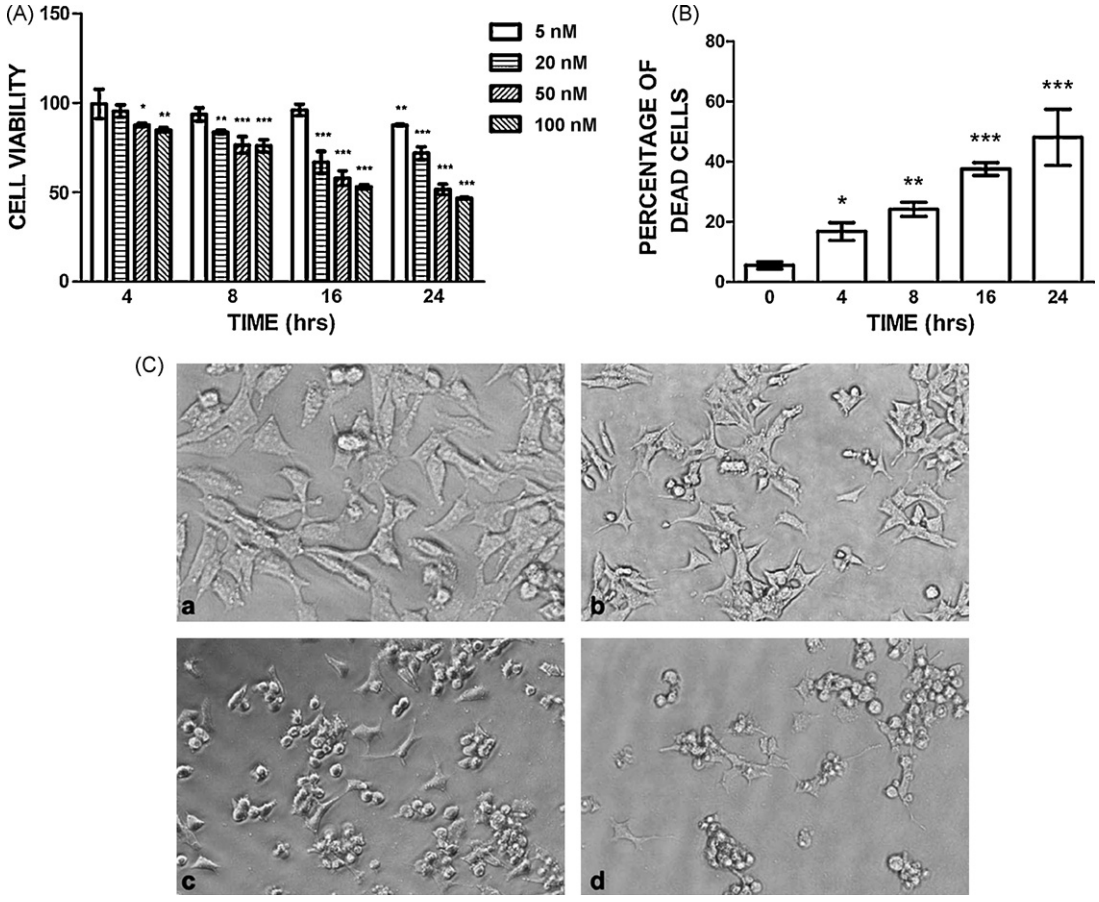

Fig. 1 – Concentration- and duration-dependent effect of rotenone on the viability of SK-N-MC cells. (A) SK-N-MC cells cultured in serum free medium were treated with rotenone (5–100 nM) for specified times. Cell viability was detected by MTT assay and rotenone-induced cell loss was expressed as a percentage of the control. (B) SK-N-MC cells cultured in serum free medium were treated with rotenone (100 nM) for specified times. Dead/dying cells were determined by trypan blue staining and expressed as a percentage of the control. Each data point is the mean of three experiments (means ± S.E.M.). (C) Images of SK-N-MC cells were captured at 0, 4, 16 and 24 h of rotenone (100 nM) treatment. (a) Control; (b) rotenone treatment for 4 h; (c) rotenone treatment for 16 h; (d) rotenone treatment for 24 h. *, ** and *, denote significant difference ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, respectively).**

teins [15]. GSK3β regulates neuronal survival and has been implicated in neurodegeneration [16–18]. Recently, endoplasmic reticulum (ER) stress has been suggested in various neurodegenerative processes, such as brain ischemia [19], Alzheimer's disease (AD) [20], Parkinson's disease (PD) [21,22], Huntington's disease (HD) [23] and amyotrophic lateral sclerosis [24]. ER regulates posttranslational protein processing and transport. Under certain circumstances, ER dysfunction leads to the accumulation of unfolded or misfolded proteins in the ER lumen and activates compensatory mechanism, which has been referred to as ER stress response or unfolded protein response (UPR) [25]. Several ER transmembrane proteins are identified as sensors of ER stress. These include pancreatic ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). PERK phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF2α), which attenuates the initiation of translation in response to ER stress. The activation of IRE1 and ATF6 signaling promotes pro-apoptotic transcription factor CHOP and the expression of ER-localized chaperones, such as GRP78 and GRP94, which facilitate the restoration of proper protein folding within the ER [25]. These protective responses result in an overall decrease in translation, enhanced protein degradation and increased levels of ER chaperones, which consequently increase the protein folding capacity of the ER. However, sustained ER stress ultimately leads to the cell death [25].

In this study, we examine the mechanisms of rotenone-induced death of SK-N-MC human neuroblastoma cells, a neuronal cell line displaying moderate dopamine-β-hydroxylase activity. We demonstrate that both GSK3β activation

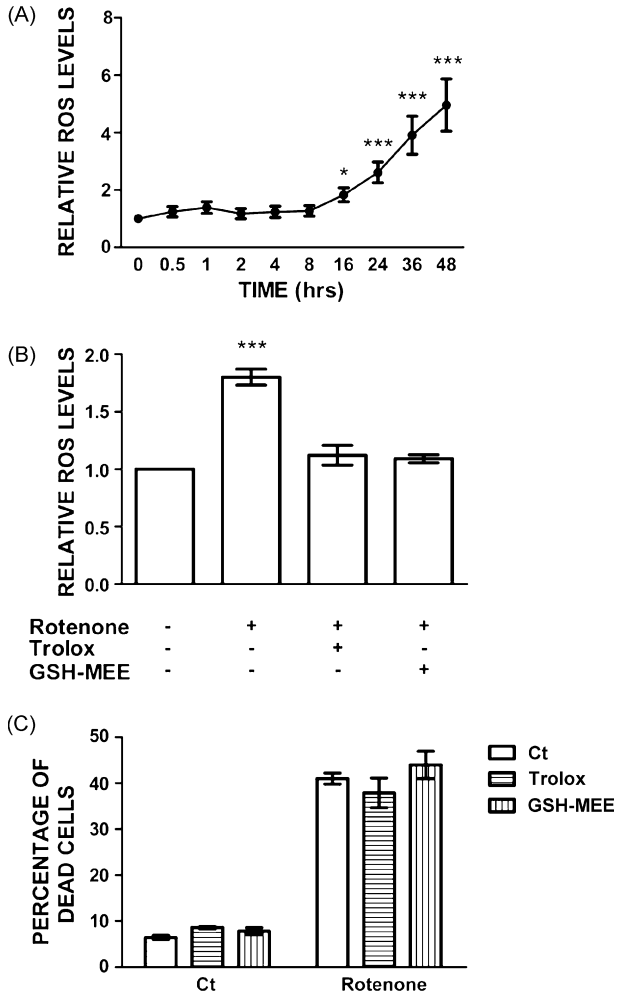

Fig. 2 – Rotenone-induced ROS production. (A) SK-N-MC cells were exposed to rotenone (100 nM) for specified times. After exposure, cells were labeled with carboxy-H_2DCFDA (10 μM) as described in Section 2. Relative ROS levels were determined by flow cytometry. Each data point represents the mean of three independent experiments. * and * denote significant difference from control group ($^{*}p < 0.05$; $^{***}p < 0.001$). (B) SK-N-MC cells were pretreated with Trolox (100 nM) or GSH-MEE (2 mM) for 3 h and then exposed to rotenone (100 nM) for 24 h. Intracellular ROS was detected as described above. *** denotes significant difference from control group ($^{***}p < 0.001$). (C) SK-N-MC cells were pretreated with Trolox (100 nM) or GSH-MEE (2 mM) for 3 h and then exposed to rotenone (100 nM) for 24 h. The percentage of dead/dying cells was determined by trypan blue assay. Each data point is the mean of three experiments (mean ± S.E.M.).**

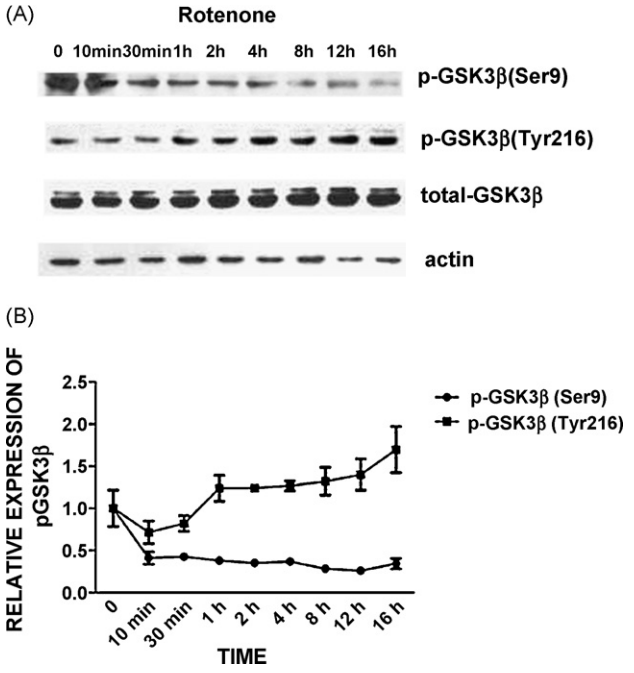

Fig. 3 – Effect of rotenone on GSK3β phosphorylation. (A) SK-N-MC cells cultured in serum free medium were exposed to rotenone (100 nM) for specified times. GSK3β expression and its phosphorylation at Serine 9 and Tyrosine 216 [p-GSK3β(Ser9) and p-GSK3β(Tyr216)] were determined by immunoblots. The same blots were stripped and probed with an anti-actin antibody. The experiment was replicated for 3–4 times. (B) The relative amounts of phosphorylated GSK3β were measured by densitometry and normalized to the expression of actin. Each data point (mean ± S.E.M.) is the mean of three independent experiments.

and ER stress, but not oxidative stress, contribute to rotenone cytotoxicity in SK-N-MC cells.

2. Materials and methods

2.1. Materials

Albumin from bovine serum (BSA), aprotinin, rotenone, lithium chloride, Nonidet P-40 (NP-40), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), thapsigargin, tris(hydroxymethyl) aminomethane, trypan blue, tunicamycin, and Tween-20 were purchased from Sigma Chemical Co. (St. Louis, MO). Glutathione monoethyl ester (GSH-MEE), inhibitors of GSK3β (TDZD-8; L803-mts), sodium dodecyl sulfate (SDS), and trolox (a cell-permeable, water-soluble derivative of vitamin E) were purchased from Calbiochem (La Jolla, CA). Phenylmethylsulfonyl fluoride was purchased from Amresco (Solon, Ohio). 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA) was purchased from Molecular Probes (Eugene, OR). The antibodies directed against actin and phospho-tau (Ser262) were purchased from Sigma Chemical Co. The antibodies directed against GSK3β, phospho-GSK3β (Ser9 and Tyr216), eIF2α, phospho-eIF2α (Ser51) and tubulin were purchased from Cell Signaling Technology, Inc. (Beverly, MA). PERK, phospho-PERK (Thr981), CHOP, and GRP78 antibodies, eIF2α small interfering (siRNA) and scrambled siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH antibody was obtained from Kangchen Biotechnology (Shanghai, China). Oligofectamine reagent was purchased from Invitrogen (Carlsbad, CA). Minimum essential medium (MEM), sodium pyruvate solution, and Penicillin–Streptomycin were purchased from GIBCO (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT).

2.2. Cell culture and determination of cell viability

Human neuroblastoma SK-N-MC cells obtained from ATCC, were grown in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), 5 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2.

Cell viability was determined by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and trypan blue assay as previously described [21,26].

2.3. Immunoblotting

Cells were lysed with RIPA buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% Nonidet P-40 (NP-40), 0.1% sodium dodecylsulfate (SDS), 0.5% deoxycholic acid sodium, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 3% aprotinin] on ice for 10 min, solubilized cells were centrifuged at 12,000 *g* for 10 min at 4 °C, and the supernatant was collected.

The protein samples were separated by electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk or BSA in 0.01 M PBS (pH 7.4) and 0.05% Tween-20 (TPBS) at room temperature for 1 h. The membranes were then probed with primary antibodies directed against target proteins overnight at 4 °C. The final dilutions for primary antibodies were: CHOP, 1:500; actin, p-eIF2α, total-eIF2α, p-PERK, p-PKR, total PKR, p-GSK3β and total-GSK3β, 1:1000; GAPDH and GRP78, 1:10,000. After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (The Jackson ImmunoResearch, Tucker, GA) diluted at 1:2500 in TPBS for 1 h at room temperature. The immune complexes were detected by the enhanced chemiluminescence method (Amersham; Arlington Hts. IL). The density of immunoblotting was quantified with the software of Quantity One (Bio-Rad Laboratories, Hercules, CA).

2.4. Stable cell transfection

SK-N-MC cells over-expressing kinase deficient (K85R) GSK3β (KD GSK3β) were established using a previously described method [27]. V5-tagged K85R GSK3β construct carried by vector pcDNA3 were generous gifts from Dr. Thilo Hagen (University Hospital Nottingham, Nottingham, UK). The GSK3β mutant functions as a dominant negative protein and inhibits GSK3β activity [27].

2.5. Small interfering RNA transfection

eIF2α small interfering RNA (siRNA) and its scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were transfected

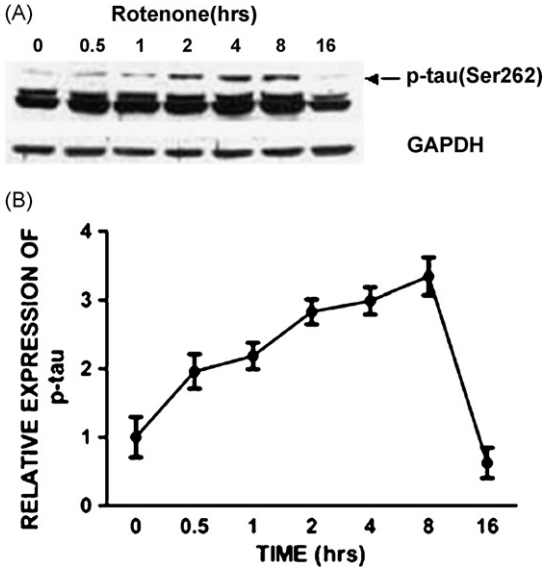

Fig. 4 – Effect of rotenone on tau phosphorylation. (A) SK-N-MC cells cultured in serum free medium were exposed to rotenone (100 nM) for specified times. Tau phosphorylation [p-tau (Ser262)] was determined by immunoblots. The same blots were stripped and probed with an anti-GAPDH antibody. (B) Relative amounts of p-tau (Ser262) were measured by densitometry and normalized to the expression of GAPDH. Each data point (mean ± S.E.M.) is the mean of three independent experiments.

into SK-N-MC cells with Oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. At 48 h after transfection, the cells were subjected to immunoblotting analysis for eIF2α expression.

2.6. Measurement of reactive oxygen species accumulation

Intracellular reactive oxygen species (ROS) levels were measured using the fluorescent dye carboxy-H_2DCFDA staining method as previously described [28]. We have demonstrated that this assay is sensitive in detecting intracellular ROS in neuronal cells [29]. Briefly, SK-N-MC were cultured in 35 mm dishes and treated with rotenone and antioxidants for the indicated times. After treatment, the cells were incubated with PBS containing carboxy-H_2DCFDA (10 μM) in the dark for 30 min at 37 °C and then collected in microtubes. Intracellular ROS levels (carboxy-H_2DCFDA signals) were measured with a flow cytometer (FACSAria^{TM}, BD Biosciences, San Jose, CA) at λ_{ex} of 488 nm and λ_{em} of 520 nm. Data acquisition and processing were performed using the Cellquest program (BD Biosciences, San Jose, CA).

2.7. Statistical analysis

The alterations were analyzed by ANOVA. Differences in which the p-value was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific post hoc comparisons between treatment groups were examined with Student–Newman–Keuls tests or Dunnett's T3 tests. The analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

3. Results

3.1. Reactive oxygen species (ROS) are not involved in rotenone-induced death of SK-N-MC cells

Rotenone caused a dose-dependent loss of SK-N-MC cells (Fig. 1A). Rotenone-induced cell loss occurred rapidly; at concentrations of 50 and 100 nM, it caused significant cell loss as early as 4 h of exposure and a maximal loss was observed by 16 h of exposure. Since SK-N-MC cells maintained in serum free medium had a minimal proliferation rate, rotenone-

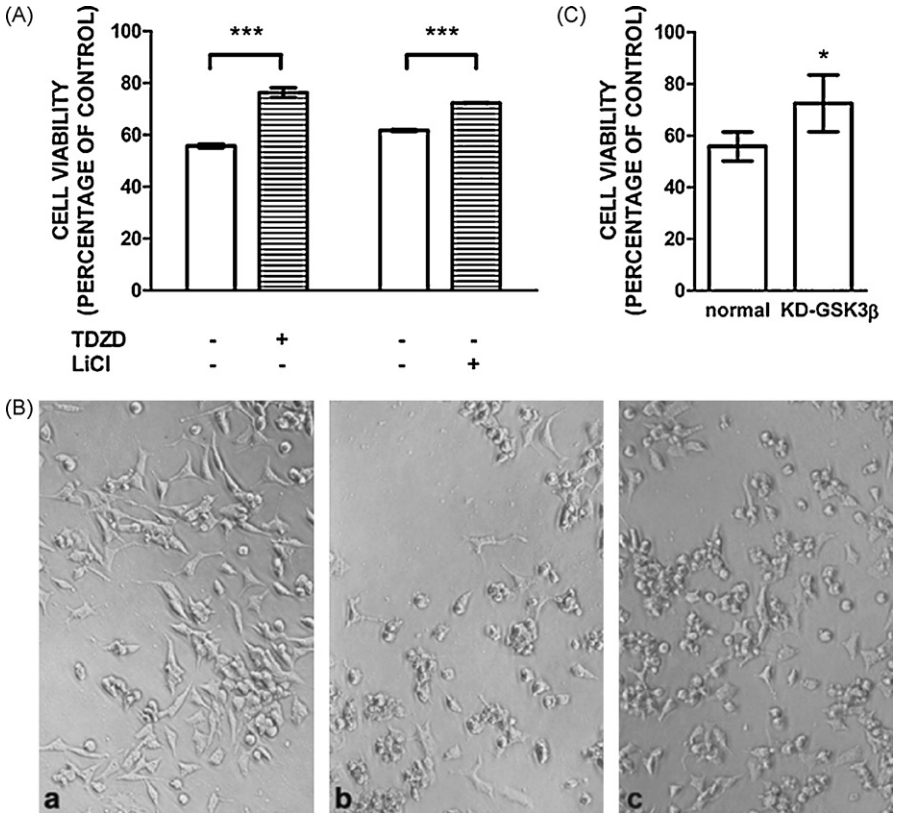

Fig. 5 – The effect of GSK3β inhibitors and dominant negative GSK3β mutant on rotenone cytotoxicity. (A) SK-N-MC cells were pretreated with GSK3β inhibitors, LiCl (20 mM) or TDZD (10 μM), for 1 h, and then exposed to rotenone (100 nM) for 24 h. Cell viability was determined by MTT assay and expressed as a percentage of the control. Each data point is the mean of three independent experiments (mean ± S.E.M.). * denotes significant difference ($^{***}p < 0.001$). (B) Image of cultured SK-N-MC cells. (a) Control cells; (b) cells treated with rotenone (100 nM) for 24 h; (c) cells pretreated with TDZD (10 μM) for 1 h, and then exposed to rotenone (100 nM) for 24 h. (C) SK-N-MC cells and SK-N-MC cells stably expressing a dominant negative GSK3β mutant were exposed to rotenone (100 nM) for 24 h. Cell viability was determined by MTT assay and expressed as a percentage of the control. Each data point is the mean of three independent experiments (mean ± S.E.M.). * denotes significant difference ($^{*}p < 0.05$).**

induced cell loss likely resulted from cell death; this was confirmed by trypan blue exclusion assay (Fig. 1B) and cell image (Fig. 1C) which showed that rotenone increased the percentage of dead/dying cells. Rotenone is reported to generate reactive oxygen species (ROS) and cause oxidative stress in neuronal cells [13]. Rotenone-induced intracellular accumulation of ROS is detected after exposure periods of 24 h or longer [13]. Consistent with these findings, our results indicated that rotenone did not produce a significant accumulation of ROS until 16 h of exposure and the production of ROS steadily increased thereafter (Fig. 2A). Antioxidants (Trolox and GSH-MEE) eliminated rotenone-induced ROS (Fig. 2B). However, these antioxidants failed to protect cells against rotenone cytotoxicity (Fig. 2C). Therefore, ROS was not involved in rotenone-induced death of SK-N-MC cells.

3.2. *Rotenone activates GSK3β in SK-N-MC cells*

GSK3β is a multifunctional protein and regulates neuronal survival. GSK3β is activated under various stress conditions; we, therefore, sought to determine whether rotenone activates GSK3β. The activity of GSK3β is regulated negatively by the phosphorylation of serine 9 (Ser9) and positively by the phosphorylation of tyrosine 216 (Tyr216) [15]. Our results demonstrated that rotenone caused dephosphorylation of GSK3β at serine 9 (Ser9) while it increased its phosphorylation at tyrosine 216 (Tyr216) without affecting the expression of total GSK3β (Fig. 3). Rotenone-induced activation of GSK3β was further confirmed by its effect on the phosphorylation of tau, a substrate of GSK3β; rotenone increased tau phosphorylation at Ser262 (Fig. 4). The profile of rotenone-induced tau phosphorylation was generally consistent with that of GSK3β activation within the 8 h of rotenone treatment. However, the level of tau phosphorylation reduced dramatically after 8 h of rotenone exposure; this may be due to cell death after prolonged rotenone exposure.

To determine whether GSK3β was involved in rotenone-induced death of SK-N-MC cells, we pretreated cells with selective GSK3β inhibitors (lithium or TDZD-8). As shown in Fig. 5A and B, both lithium and TDZD-8 significantly protected SK-N-MC cells against rotenone cytotoxicity. To further verify the role of GSK3β in rotenone-induced cell death, we generated a SK-N-MC cell line stably expressing a dominant negative (kinase deficient) GSK3β mutant (KD GSK3β). The KD GSK3β mutant has been previously demonstrated to specifically inhibit GSK3β activity [27]. Like GSK3β inhibitors, KD GSK3β also offered partial protection against rotenone cytotoxicity (Fig. 5C). Thus, GSK3β activation contributed to rotenone cytotoxicity in SK-N-MC cells.

3.3. *Rotenone induces ER stress in SK-N-MC*

GSK3β activation may caused by ER stress [18,21,30]. Sustained ER stress may result in neuronal death and has been implicated in neurodegeneration [20–23]. Therefore, we sought to determine whether rotenone caused ER stress in SK-N-MC cells. Our results indicated that rotenone induced the expression of several important indexes of ER stress, namely, p-PERK, GRP78 and p-eIF2α (Fig. 6). Rotenone also stimulated the phosphorylation of another eIF2α kinase,

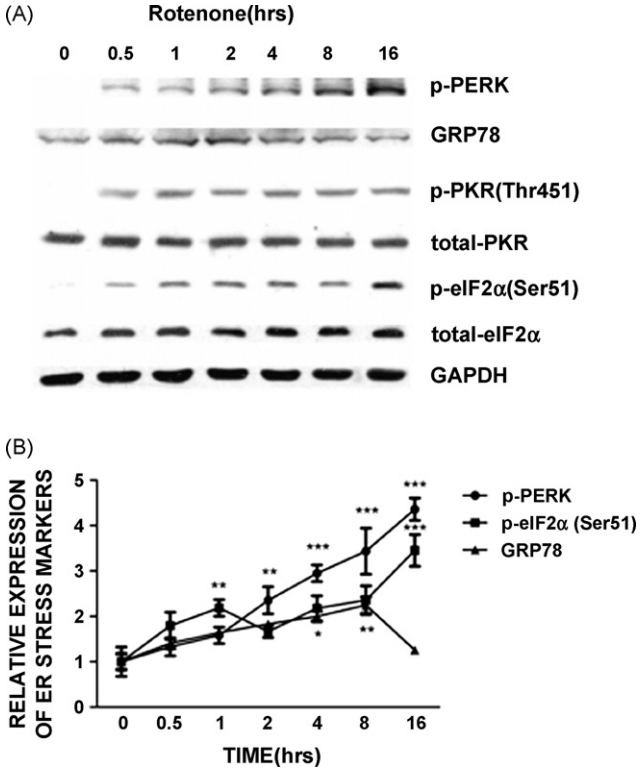

Fig. 6 – Rotenone evokes ER stress in SK-N-MC cells. (A) SK-N-MC cells were treated with rotenone (100 nM) for specified times. The expressions of p-PERK, GRP78, p-PKR and p-eIF2α were determined with immunoblotting. The same blots were stripped and probed with an anti-GAPDH antibody. The experiment was replicated three times. (B) The relative amount of the protein imaged on the films was measured by densitometry and normalized to the expression of GAPDH. Each data point is the mean of three independent experiments (mean ± S.E.M.). *, **, and * denote significant difference from control group ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).**

double-stranded RNA (dsRNA)-activated protein kinase (PKR). Rotenone had a modest effect on the expression of CHOP (data not shown). We next sought to determine whether ER stress caused death of SK-N-MC cells. Tunicamycin and thapsigargin are widely used agents to experimentally induce ER stress. Tunicamycin is an inhibitor of *N*-acetylglucosamine transferases, disrupting protein glycosylation [31]. Thapsigargin is an effective inhibitor of the Ca^{2+} ion pump proteins of intracellular membranes, perturbing intracellular calcium homeostasis [32]. Tunicamycin and thapsigargin-induced ER stress was confirmed by the up-regulation of p-PERK, GRP78, and p-eIF2α (Fig. 7A). Tunicamycin and thapsigargin had little effect on CHOP and p-PKR (data not shown). Similar to rotenone, tunicamycin and thapsigargin also caused death of SK-N-MC cells (Fig. 7B, right panel). Both CHOP and eIF2α are important mediators of cell death/survival [33,34]. Since CHOP was not induced in SK-N-MC cells, we sought to determine the role of eIF2α in ER stress-induced death of SK-N-MC cells. An eIF2α siRNA was introduced to knock down the expression of

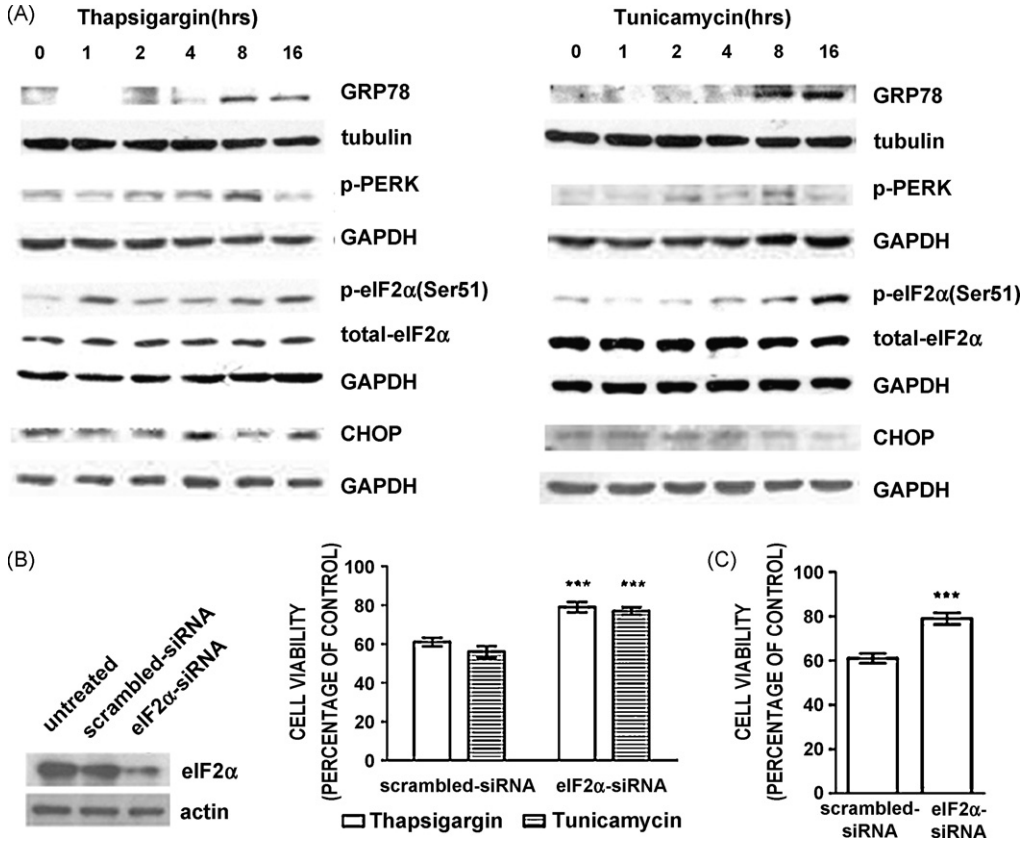

Fig. 7 – ER stress-induced death of SK-N-MC cells. (A) SK-N-MC cells cultured in serum free medium were exposed to thapsigargin (5 μM) or tunicamycin (6 μg/ml) for 24 h. The expression of GRP78, p-PERK, p-eIF2α, total-eIF2α, and CHOP were determined with immunoblotting. The same blots were stripped and probed with an anti-tubulin or anti-GAPDH antibody. The experiment was replicated three times. (B) SK-N-MC cells were transfected with a scrambled siRNA or eIF2α siRNA for 48 h. The expression of eIF2α was determined with immunoblotting (left panel). SK-N-MC cells were transfected with a scrambled siRNA or eIF2α siRNA for 48 h, and then exposed to thapsigargin (5 μM) or tunicamycin (6 μg/ml) for 24 h. Cell viability was determined by MTT assay and expressed as a percentage of the control (right panel). Each data point is the mean of three experiments (mean ± S.E.M.). * denotes significant difference (*** $p < 0.001$) from scrambled siRNA-treated groups. (C) SK-N-MC cells were transfected with a scrambled siRNA or eIF2α siRNA for 48 h, and then exposed to rotenone (100 nM) for 24 h. Cell viability was determined by MTT assay and expressed as a percentage of the control. Each data point is the mean of three experiments (mean ± S.E.M.). *** denotes significant difference (*** $p < 0.001$) from scrambled-siRNA-treated group.**

eIF2α (Fig. 7B, left panel). This eIF2α siRNA significantly diminished tunicamycin and thapsigargin-induced death of SK-N-MC cells (Fig. 7B, right panel), indicating that eIF2α was involved in ER stress-induced cell death. The eIF2α siRNA also partially protected SK-N-MC cells against rotenone cytotoxicity (Fig. 7C), suggesting that ER stress/eIF2α contributed to rotenone cytotoxicity. Tunicamycin and thapsigargin did not alter the phosphorylation of GSK3β at either Ser9 or Tyr216 in SK-N-MC cells (data not shown).

3.4. *eIF2α mediates the interaction between rotenone and ER stress inducers in SK-N-MC cells*

Since both GSK3β activation and ER stress contributed to rotenone cytotoxicity, we sought to determine their interaction. We showed that thapsigargin treatment enhanced rotenone-induced death of SK-N-MC cells. When administered together, thapsigargin and rotenone acted in synergy to produce greater loss of SK-N-MC cells (Fig. 8A). Paralleling their effect on cell survival, combined treatment of thapsigargin with rotenone caused much greater expression of p-eIF2α than either treatment alone (Fig. 8B). However, the additive cytotoxicity was not observed in rotenone and tunicamycin interaction. Since blocking either GSK3β or eIF2α pathways offered protection against rotenone cytotoxicity (Figs. 5 and 7), we sought to determine whether blocking both pathways simultaneously provided better protection. As shown in Fig. 9, combined treatment of SK-N-MC cells with GSK3β inhibitors plus eIF2α siRNA offered much greater protection compared with either treatment alone. The result indicates that GSK3β and eIF2α contribute separately to rotenone cytotoxicity.

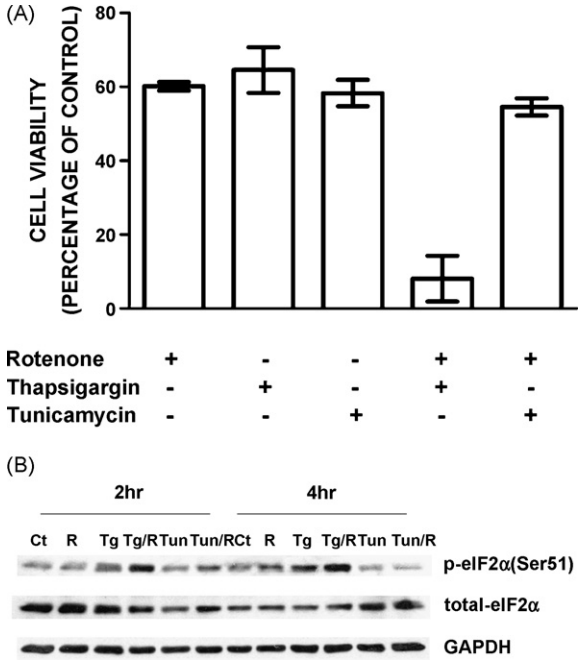

Fig. 8 – Interaction between rotenone and thapsigargin/tunicamycin. (A) SK-N-MC cells cultured in serum free medium were exposed to rotenone with/without thapsigargin/tunicamycin for 24 h. Cell viability was determined by MTT assay as described. Each data point is the mean of three independent experiments (mean ± S.E.M.). (B) SK-N-MC cells cultured in serum free medium were exposed to rotenone with/without thapsigargin/tunicamycin for specified times. Phosphorylation and expression of eIF2α were determined with immunoblotting. The same blots were stripped and probed with an anti-GAPDH antibody. The experiment was replicated three times. R, rotenone; Tg, thapsigargin; Tg/R, thapsigargin plus rotenone; Tun, tunicamycin; Tun/R, tunicamycin plus rotenone.

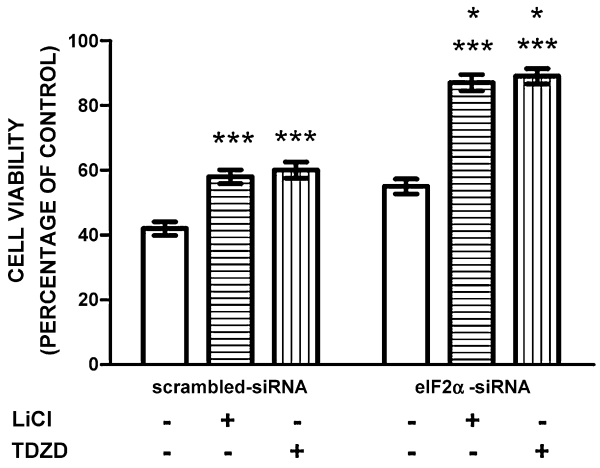

Fig. 9 – GSK3β inhibitor- and eIF2α siRNA-mediated protection against rotenone cytotoxicity. SK-N-MC cells were transfected with scrambled siRNA or eIF2α siRNA for 48 h. SK-N-MC cells were pretreated with GSK3β inhibitors, LiCl (20 mM) or TDZD (10 μM), for 1 h, and then exposed to rotenone (100 nM) for 24 h. Cell viability was determined by MTT assay and expressed as a percentage of the control. Each data point is the mean of three independent experiments (mean ± S.E.M.). * denotes significant difference from matched controls ($^{***}p < 0.001$); * denotes significant difference from scrambled siRNA-treated and LiCl- or TDZD-treated groups ($^{*}p < 0.05$).**

4. Discussion

Rotenone is a high-affinity inhibitor of complex I. Due to its high lipophilicity, rotenone easily crosses biological membranes independent of transporters [9]. Rotenone exposure to rodents causes selective degeneration of dopaminergic neurons in the substantia nigra, and is used to model PD pathogenesis. In these animal models, brain rotenone concentrations reach 20–30 nM [8]. Although rotenone at these concentrations partially inhibits complex I, it does not significantly impair respiration of brain mitochondria, produce ATP depletion and explain its effect on neurodegeneration [8,9,12]. Mechanisms other than a bioenergetic (ATP) deficit must be operative. Rotenone-induced neurodegeneration is accompanied by oxidative damage to protein and DNA [13]. In cell cultures, rotenone induces intracellular accumulation of ROS and antioxidants provided protection against rotenone neurotoxicity [8,9], suggesting that oxidative stress is involved in the action of rotenone.

Our results indicate that a dramatic increase in ROS accumulation is observed after exposure to rotenone exposure for 24 h (Fig. 2). The time frame is consistent with previous findings using similar concentrations of rotenone [13,14]. Rotenone-induced loss of SK-N-MC cells precedes the large-scale up-regulation of ROS; a significant loss is observed as early as 4 h of exposure, peaking at 16 h of exposure. Moreover, antioxidants do not to protect cells against rotenone cytotoxicity. Thus, oxidative stress cannot explain rotenone cytotoxicity in SK-N-MC cells.

GSK3β regulates neuron survival [15]. Cellular stress, such as ER stress, oxidative stress, heat shock and hyperosmotic stress, may modulate the activity of GSK3β [18,30,35]. We demonstrate that rotenone induces rapid activation of GSK3β (30 min after exposure). The activation is evident by up-regulation of p-GSK3β(Tyr216) and p-tau (Ser262) while down-regulation of p-GSK3β(Ser9) occurs. GSK3β activation well precedes cell death. Inhibition of GSK3β activation by specific inhibitors or the expression of dominant negative GSK3β mutant provides partial but significant protection against rotenone cytotoxicity. p-GSK3β(Tyr216) is mediated by alterations in intracellular calcium levels [36], activation of MEK1/2 [37] or Fyn, a member of the Src tyrosine family [38]. Rotenone is shown to disrupt intracellular calcium homeostasis [39]. Numerous kinases, such as Akt/protein kinase B (PKB), protein kinase C (PKC), ERK, p70 S6 kinase, p90Rsk and protein kinase A (PKA) can phosphorylate GSK3β at Ser9, and therefore, inactivate GSK3β [15]. p-GSK3β(Ser9) is also negatively regulated by mitochondrial protein phosphatase 2A (PP2A).

PP2A activity is reported to be modulated by mitochondrial dysfunction [40]. Our previous study demonstrates that PP2A is involved in 6-OHDA-induced dephosphorylation of GSK3β at Ser9 [21]. It is possible that rotenone may regulate p-GSK3β(Ser9) through PP2A. Regardless how rotenone alters phosphorylation, our evidence indicates that GSK3β is a mediator of rotenone-induced death of SK-N-MC. The mechanisms underlying cell death caused by GSK3β activation remain to be elucidated. Several transcription factors that regulate neuronal survival are known targets of GSK3β; these include nuclear factor of activated T cells (NFAT), heat shock factor-1 (HSF-1), cyclic AMP response element binding protein (CREB), activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) [15]. In addition, GSK3β may phosphorylate Bax and promote its mitochondrial localization which results in neuronal apoptosis [41].

Rotenone also induces ER stress in SK-N-MC cells. This is evident by rotenone-induced increase in phosphorylation on PERK, eIF2α and the expression of GRP78. Neither rotenone nor tunicamycin/thapsigargin induces significant change of CHOP expression in SK-N-MC cells. Apparently, the effect of rotenone is different from that of tunicamycin and thapsigargin; rotenone induces p-PKR while tunicamycin/thapsigargin do not. PKR is one of the eIF2α kinases that phosphorylate eIF2α; others are PERK, heme-regulated inhibitor (HRI) and general control nonderepressible 2 (GCN2) family members [42]. PKR is initially identified as an interferon-induced protein that is activated in virus-infected cells by double-stranded RNA (dsRNA) produced during the virus life cycle [43]. In addition to dsRNA, PKR can be activated by cytokines, growth factors, serum deprivation, disruption of intracellular Ca^{2+} homeostasis, bacterial products or physiochemical stress [44-47]. Strong eIF2α phosphorylation caused by rotenone exposure may result from the activation of both PKR and PERK. eIF2α activation causes the inhibition of protein synthesis and cell death [48–50], although some reports indicate that p-eIF2α may be protective [51,52]. We show that eIF2α siRNA provides significant protection against rotenone cytotoxicity as well as tunicamycin/thapsigargin-induced cell death, indicating that eIF2α activation promotes death of SK-N-MC cells. The role of eIF2α in cell death is further supported by the additive effect of rotenone and thapsigargin; rotenone and thapsigargin act in synergy to enhance cell death, as well as induction of p-eIF2α.

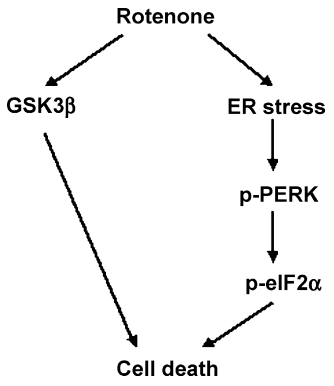

Fig. 10 – Potential mechanisms of rotenone-induced death of SK-N-MC cells. Rotenone activates GSK3β and induces ER stress, which results in the phosphorylation of eIF2α. Both GSK3β activation and p-eIF2α contribute to rotenone cytotoxicity.

It is interesting to note that only thapsigargin but not tunicamycin potentiates rotenone cytotoxicity. Thapsigargin and tunicamycin induce ER stress by different mechanisms; thapsigargin induces ER stress by disturbing the ER calcium, while tunicamycin evokes ER stress by disrupting the glycosylation of newly synthesized proteins. Rotenone also disrupts intracellular calcium homeostasis (our unpublished observation). Therefore, it is likely that the interaction between thapsigargin and rotenone results from their effect on intracellular calcium homeostasis. This remains to be elucidated. Rotenone-induced GSK3β activation and eIF2α phosphorylation occurred at about the same time. ER stress inducers, thapsigargin and tunicamycin did not cause significant activation of GSK3β in SK-N-MC cells. It is reported that thapsigargin induces dephosphorylation of GSK3β at Ser9 in SH-SY5Y neuroblasoma cells [18]. We do not observe a significant alteration of p-GSK3β in SK-N-MC cells following thapsigargin exposure. Therefore, we postulate that rotenone-induced GSK3β activation and eIF2α phosphorylation may be separate events and both pathways contribute to rotenone cytotoxicity (Fig. 10). In this scenario, blocking either pathway will only provide partial protection, and blocking both will offer greater protection. This is supported by results showing that combined treatment of SK-N-MC cells with GSK3β inhibitors plus eIF2α siRNA offered much greater protection than either treatment alone (Fig. 9).

Both GSK3β activation and ER stress have been implicated in the pathogenesis of neuodegeneration. We demonstrate that rotenone-induced death of SK-N-MC cells is mediated by GSK3β activation and ER stress. Although the mechanisms underlying rotenone cytotoxicity may vary depending on cell types being examined, our study provides an insight into the potential involvement of GSK3β and ER stress in the rotenone model of PD pathogenesis.

Acknowledgements

We would like to thank Ms. Kimberly A. Bower for reading this manuscript. This research was supported by grants from the Ministry of Science and Technology of China (2007CB947100), the National Natural Science Foundation of China (30470544, 30471452, and 30570580), the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-R-115), and Science and Technology Commission of Shanghai Municipality (Grant No. 07DJ14005). Dr Z.-J. Ke was also supported by the One Hundred Talents Program of the Chinese Academy of Sciences. Dr J. Luo was also supported by a grant from NIH/NIAAA (AA015407).

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