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Abstract

Cadmium, a toxic environmental contaminant, induces oxidative stress leading to various neurodegenerative disorders, where it interferes with homeostasis of intracellular free calcium ($[Ca^{2+}]_i$), leading to cellular damage and apoptosis. We investigated whether resveratrol, a plant-derived antioxidant could offer protection against cadmium-induced neuroapoptosis. Primary cortical neurons were exposed to cadmium (10 or 20 μ M) with/without prior exposure to resveratrol (5, 10 or 20 μ M) for 12 hours and unexposed cells served as control. Resveratrol caused marked reduction in cadmium-induced neuronal apoptosis and down-regulated caspase-3 expressions. Cadmium-induced marked elevations in reactive oxygen species, and $[Ca^{2+}]_i$ levels were potentially reduced by resveratrol. Resveratrol effectively regulated the alterations observed in the activation levels of mitogen-activated protein kinases (MAPKs) and proteins of mammalian target of rapamycin (mTOR) pathways. Thus, resveratrol effectively protected the cortical neurons exposed to cadmium by modulating the $[Ca^{2+}]_i$ levels and regulating the MAPK/mTOR pathways.

Introduction

Cadmium is an extremely toxic environmental pollutant commonly found in industrial workplaces, released from metal refining, smelting, and burning of fossil fuels and municipal wastes. Cadmium is toxic to many body organs including liver, kidney and brain (Lopez et al., 2006; Prabu et al., 2011; Oliveira et al., 2012; Napolitano et al., 2012; Coccini et al., 2013). Cadmium has high blood-brain barrier permeability and chronic exposure affects nervous system leading to memory deficits, olfactory dysfunction and hypernociception (Pihl and Parkes, 1977; Lukawski et al., 2005; Nishimura et al., 2006; Wright et al., 2006). Increasing evidences indicate cadmium in the etiology of many neurodegenerative diseases (Okuda et al., 1997; Panayi et al., 2002; Jiang et al., 2007; Chen et al., 2011c).

Cadmium induced oxidative stress interferes with proteins, lipids and DNA and alters their functions (Stohs and Bagchi, 1995; Figueiredo-Pereira et al., 1998; Green and Peers, 2002; Kim et al., 2005). Cadmium interacts with the functions of many Ca^{2+} dependent enzymes as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) (Lohmann and Beyersmann, 1993; Beyersmann and Hechtenberg, 1997; Misra et al., 2002). Intracellular calcium homeostasis is pivotal in cellular function, survival and death (Clapham, 2007). Cadmium induced disruption in intracellular free calcium ($[Ca^{2+}]_i$) homeostasis leads to apoptosis (Lemarie et al., 2004; Liu and Templeton, 2008; Yang et al., 2008; Son et al., 2010; Xie et al., 2010).

Ca^{2+} mediates numerous physiological responses of neurons (Cheng et al., 2003; Neher and Sakaba, 2008; Surmeier et al., 2010) and disruptions in Ca^{2+} levels



disturbs many signalling cascades (Liu and Templeton, 2008). Ca^{2+} levels are critical for activation of mammalian target of rapamycin (mTOR) (Gulati et al., 2008). mTOR is important for development and survival of neurons. Altered mTOR activity has been observed in neurodegenerative disorders and brain tumors (Ravikumar et al., 2004; Swiech et al., 2008). Chen et al. (2008; 2011b, c) demonstrated that MAPK and mTOR pathways are involved in cadmium induced neuronal apoptosis. Thus, targeting these pathways could be a potential strategy in therapy of neurodegenerative disorders and in cadmium-induced toxicities.

Resveratrol (3,5,4'-trihydroxystilbene), a polyphenolic phytoalexin is found in grapes, berries and also in many other plants (Sovak, 2001). Numerous pharmacological properties have been attributed to resveratrol including antioxidant, cardioprotective, and antiproliferative activities (Fremont, 2000; Fulda, 2010; Lin et al., 2013; Cullberg et al 2014; Lephart et al 2014). With this background, we investigated the effect of resveratrol in cadmium-induced neuroapoptosis.

Materials and Methods

Reagents and chemicals

Dulbecco's modified eagle's medium (DMEM)-F12 (1:1), 2-aminoethoxydiphenyl borate (2-APB), 2',7'-dichloro-dihydrofluorescein diacetate (H₂-DCF-DA), trypsin, penicillin/streptomycin, BAPTA (1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid) and Poly-D-lysine (PDL), resveratrol were obtained from Sigma-Aldrich (USA). Cadmium chloride (Sigma, USA) was dissolved in sterile double distilled water and the stock solutions (0-20 mM) were prepared. For western blot analysis, following antibodies were used: phospho-p38 (Thr180/Tyr182), phospho-Erk1/2 (Thr202/Tyr204), phospho-mTOR (Ser2448), mTOR, phospho-Akt (Ser473), phospho-S6K1 (Thr389), 4E-BP1, phospho-S6 (Ser235/236), S6, caspase-3, cleaved caspase-3 (Asp175), PTEN (Cell Signaling Technology, USA), JNK, phospho-JNK (Thr183/Tyr185), c-Jun, phospho-c-Jun (Ser63), Erk1/2, p38, Akt, S6K1 (Santa Cruz Biotechnology, USA), β -tubulin (Sigma-Aldrich, USA), goat anti-rabbit IgG horseradish peroxidase (HRP), goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP (Pierce, Rockford, IL, USA). NEUROBASAL™ medium, B27 supplement and fluo-4/AM were procured from Invitrogen (USA). The other chemicals that were used in this study were obtained from Sigma Aldrich (USA), unless otherwise mentioned.

Cell isolation and culture

Primary rat cerebral cortical neurons were cultured from fetal Sprague-Dawley rats (18-19 days of gestation) as described formerly by Yan et al. (2012). The isolated cells were seeded at a density of 2×10^4

cells/well in 96-well plates which were coated with 100 mg/L poly-L-lysine in NEUROBASAL™ medium supplemented with L-glutamine (1 mM), penicillin (100 U/mL), streptomycin (100 U/mL) and B27 supplement at 2% and were cultured in a humid incubator at 37°C in 5% CO₂. The cells were used for analysis after 7 days of culture. Fetal Sprague-Dawley rats were obtained from Laboratory Animal Center in Yangzhou University (Yangzhou, China). This research study was performed with strict adherence to the recommendations specified in the Guide for the Care and Use of Laboratory Animals of the National Research Council and was approved by the Institutional ethical committee.

Assessment of cell viability and morphological changes

DAPI staining for morphology analysis

The neuronal cells were seeded at a density of 5×10^5 cells/well in a 6-well plate comprising a PDL coated glass coverslip per well and cells were cultured for 24 hours, following which cells were then treated with various concentrations of resveratrol (5, 10 and 20 μ M) for 12 hours. After treatment with resveratrol, cells were incubated with/without cadmium (10 and 20 μ M) for 12 hours and observed for morphological changes (fragmented and condensed nuclei) using DAPI staining as described by Chen et al. (2008) and cells were observed with fluorescence microscope (Nikon 80i, Japan)

Cell viability assay

Primary neurons exposed to resveratrol and/or cadmium was washed twice with phosphate buffered saline and were analysed for cell viability. Apoptosis was determined by LIVE/DEAD viability (LIVE/DEAD cell viability kit, Invitrogen) assay. The assay determines plasma membrane integrity to assess the viability of cells. Treated or untreated cells were stained with 5 μ mol/L ethidium homodimer and 5 μ mol/L calcein-AM and incubated at 37°C for 30 min following which the cells were analyzed for viability under a Nikon labophot-2 fluorescence microscope. The assay relies on the intracellular esterase activity within living cells, through which the calcein-AM, a cell permeable fluorogenic esterase substrate, hydrolyzes to the green fluorescent product calcein. Live cells will retain calcein-AM, and produce a green fluorescence. Further, ethidium homodimer enters damaged membranes of the dead cells and binds to nucleic acids thereby producing a red fluorescence.

Analysis of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

For $[Ca^{2+}]_i$ detection, cortical neurons were cultured in 6-well plates and incubated with BAPTA-AM (10 mM), or 2-APB (50 mM) for 30 min and cells were subjected to various concentrations of resveratrol and/or Cd for another 12 hours. The harvested cells were incubated with Fluo-4/AM (5 mmol/L final concentration) for 30

min at 37°C in dark and were washed with PBS, analyzed for fluorescence using fluo-4/AM as an intracellular free Ca²⁺ fluorescent probe to analyse [Ca²⁺]_i in cadmium exposed cerebral cortical neurons. [Ca²⁺]_i levels signified by intensity of fluorescence was recorded (494 nm and 516 nm) and data was analyzed by Cell Quest program (Becton Dickinson).

Assessment of ROS generation

Generation of intracellular ROS was measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining by flow cytometry where DCFH-DA is a non-fluorescent compound which gets converted to DCF enzymatically in presence of ROS. After exposure of BAPTA-AM (10 mM) for 30 min, the cells were treated with resveratrol followed by cadmium (10 and 20 μM) for 12 hours. Cells were further incubated with DCFH-DA (10 μM) for 30 min at 37°C in dark and were washed twice with PBS and intensity of fluorescence was measured (Lu et al., 2004).

Western blot analysis

After treatment with cadmium and/or resveratrol, the cells were treated and washed with cold PBS and prior to cadmium or resveratrol exposure, they were incubated with/without BAPTA-AM as stated before. The cells were lysed in RIPA buffer (50 mM Tris, pH 7.2; 1 mM Na₃VO₄; 1% sodium deoxycholate; 0.1% SDS; 1% Triton-X 100; 150 mM NaCl; 10 mM NaF; 1:1000 protease inhibitor cocktail) and the lysate was further sonicated for 10 sec; centrifuged at 14,000 rpm for 10 min at 4°C. Using bovine serum albumin as standard, the protein concentration was determined where equivalent amounts of proteins were separated on SDS-polyacrylamide gel (7.5–12%) and blotted to polyvinylidene difluoride membranes (Millipore, USA). Membranes were incubated with blocking solution (phosphate buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk) to block non-specific binding and were incubated with primary antibodies followed by equivalent horseradish peroxidase conjugated secondary antibodies. Positive bands were visualized using enhanced chemiluminescence solution (GE Healthcare). The band signals of other proteins were normalized to those of β-actin using anti-β-actin (1:2000 dilution) (Cell Signaling Technology, USA).

Statistical analysis

The results were represented as mean ± SD. Values at p<0.05 are considered significant as determined by ANOVA (one way analysis of variance) and analyses were performed using SPSS statistical package (version 17.0).

Results

In the control group, majority of cerebral cortical

neurons had homogeneously stained nuclei, and the chromatin of normal nuclei were unaltered and spread equally throughout the entire nucleus (Figure 1A). However, in cells that were exposed to cadmium at 10 and 20 μmol/L for 12 hours, prominent nuclear morphological changes archetypal to apoptosis were observed. The cells presented condensed nuclear chromatin and fragmented nuclei. Nevertheless, in cells that were pre-treated with resveratrol, the intensity of morphological changes were considerably less.

Following cadmium exposure for 12 hours, neurotoxicity was observed as a striking decline in cell viability percentage with 20 μM cadmium presenting neurotoxic effects greater than 10 μM (Figure 1B). Resveratrol at various concentrations (5, 10 and 20 μM) resulted in a significant (p<0.05) improvement in cell viability percentage. The viable neuron counts increased with increasing concentrations of resveratrol. The 20 μM concentration caused a marked raise in viability when compared to lower doses (5 and 10 μM).

Expression of activated caspase-3 was comparatively less following exposure to 10 μM cadmium. Incubation with resveratrol for 24 hours markedly reduced the expression of cleaved caspase-3 in a dose-dependent manner that was in line with the results of the cell viability as in LIVE/DEAD assay and DAPI staining. Resveratrol at 20 μM was more effective in down-regulating the expression of activated caspase-3.

Studies have shown that cadmium disrupts [Ca²⁺]_i homeostasis leading to apoptosis and to determine [Ca²⁺]_i levels following the treatment of cerebral cortical neurons with resveratrol and/or cadmium, the cells were stained with Fluo-4 AM, a calcium indicator dye. We found that the exposure to cadmium (10 and 20 mM) resulted in a concentration-dependent increase of [Ca²⁺]_i in cerebral cortical neurons (Figure 2). However, resveratrol caused a significant reduction in [Ca²⁺]_i levels.

Additionally, the combined effect of resveratrol and BAPTA-AM on cadmium-induced perturbation of [Ca²⁺]_i homeostasis was evaluated where the cells were pre-loaded with 10 mM BAPTA-AM for 30 min. BAPTA-AM, an effective membrane permeable intracellular Ca²⁺ chelators gets trapped in the cells after cytoplasmic hydrolysis. As exemplified in Figure 2, chelating intracellular Ca²⁺ with BAPTA-AM prevented the elevation of [Ca²⁺]_i. Further on incubation of the neurons with 2-APB, a ER calcium channel (inositol-1,4,5-trisphosphate receptor; IP₃R) blocker, the elevated levels of [Ca²⁺]_i induced by cadmium was found to be suppressed. However exposure to resveratrol without BAPTA-AM caused minor decrease in levels of [Ca²⁺]_i. The combined exposure to BAPTA-AM and resveratrol was found to be more effective in suppressing the levels of raised intracellular calcium levels. Resveratrol at 20 μM was more effective in reducing the levels of [Ca²⁺]_i.

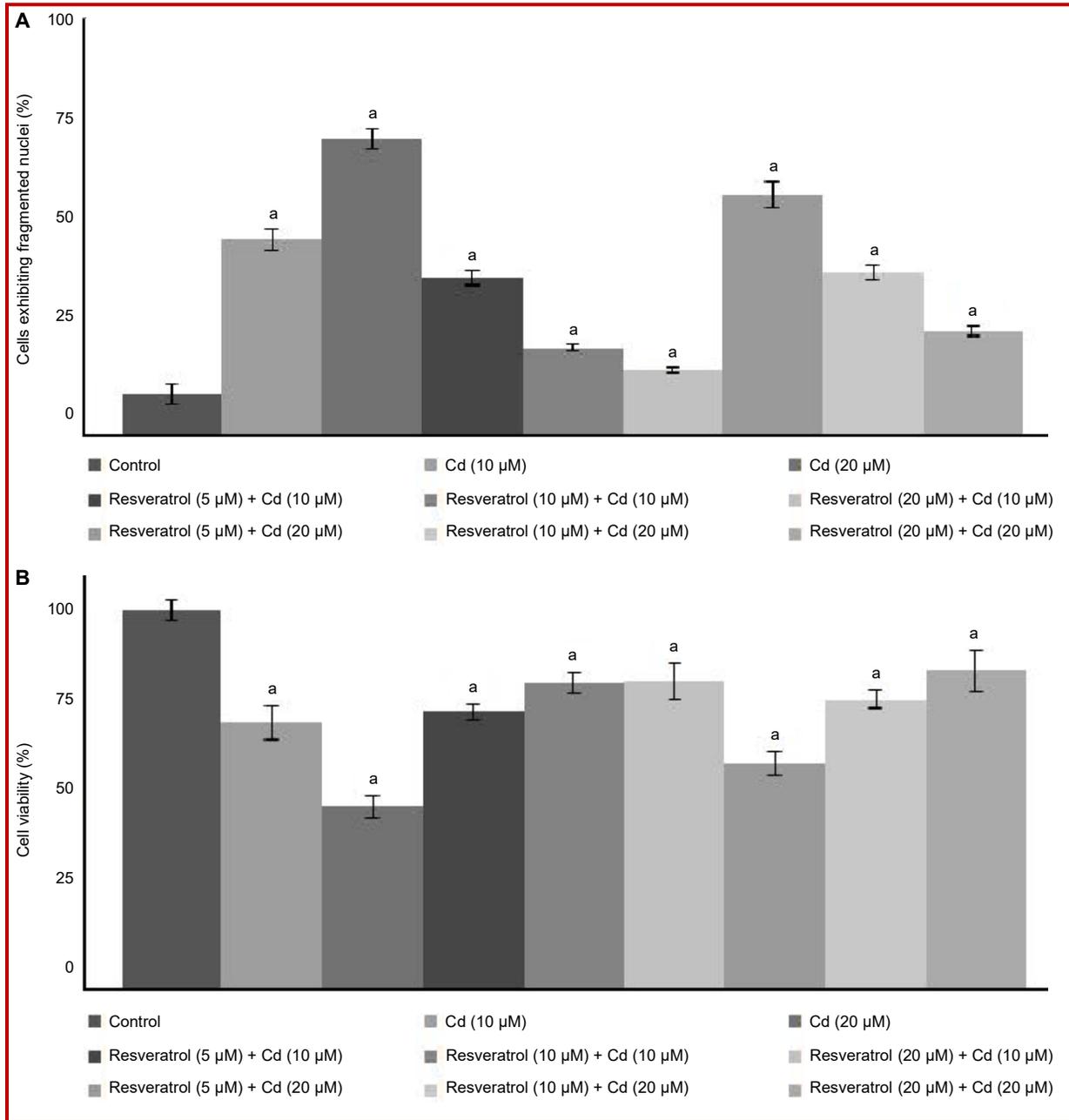


Figure 1: Resveratrol inhibits cadmium induced neuroapoptosis

Pre-treatment with resveratrol markedly reduction in morphological changes as seen by DAPI staining (A) and improved the viable cell percentage (B); Values are represented as mean \pm SD; n=6; ^arepresents statistical significance at $p < 0.05$ compared against control as determined by one-way ANOVA

(Figure 2). While APB reduced the discharge of calcium from ER, resveratrol also caused alterations against the cadmium-induced raise in $[Ca^{2+}]_i$ along with APB. These observations suggest that resveratrol affects cadmium-induced raised levels of intracellular calcium.

The cells that were treated with cadmium at 20 μ M exhibited marked increase in the levels of ROS as compared to lower dose of 10 μ M (Figure 3). Incubation with BAPTA-AM and/or resveratrol resulted in a multi

-fold decline in ROS level. Further resveratrol and cadmium exposure in the absence of BAPTA-AM also caused a significant decrease in ROS levels, with the higher dose (20 μ M) of resveratrol exhibiting more potent effects than lower doses. Nevertheless, exposure to both BAPTA-AM and resveratrol presented sharp declines in ROS generation irrespective of concentration of resveratrol.

Suppression of ROS in the combined treatment was in

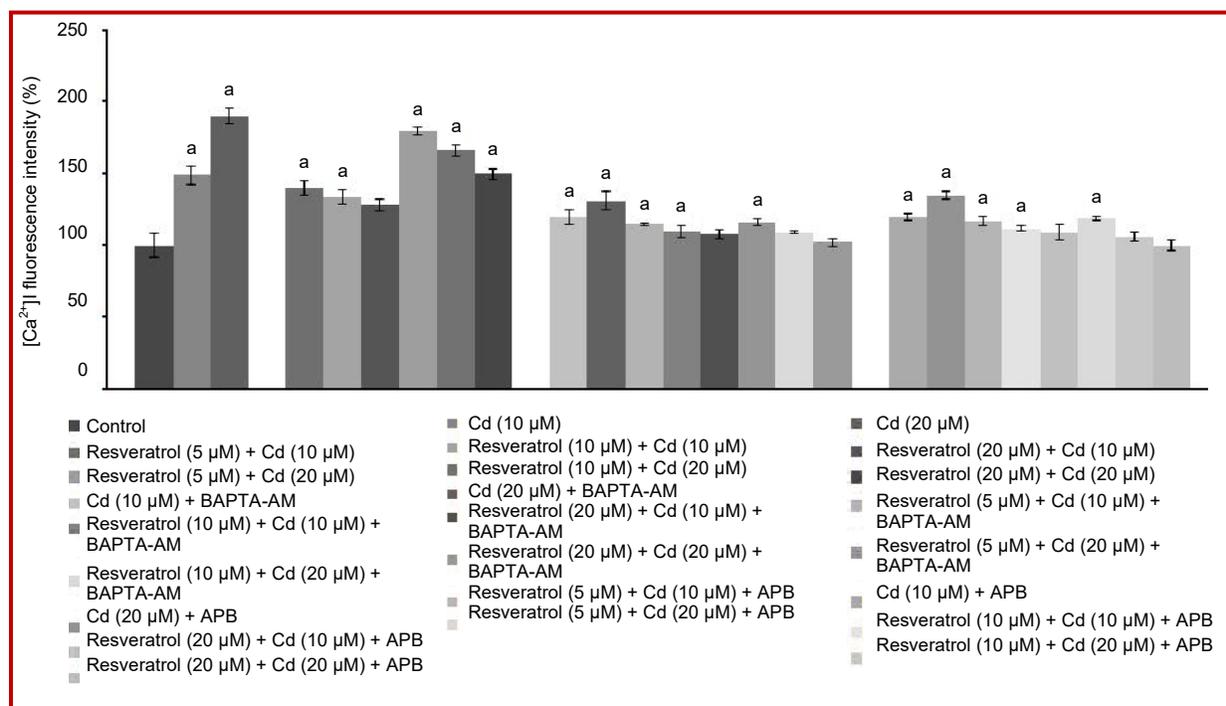


Figure 2: Resveratrol inhibits Cd-induced alterations in intracellular free calcium levels

Resveratrol with/without BAPTA-AM or APB treatment caused significant reduction in $[Ca^{2+}]_i$ levels; Values are represented as mean \pm SD; n=6; *represents statistical significance at $p < 0.05$ compared against control as determined by one-way ANOVA

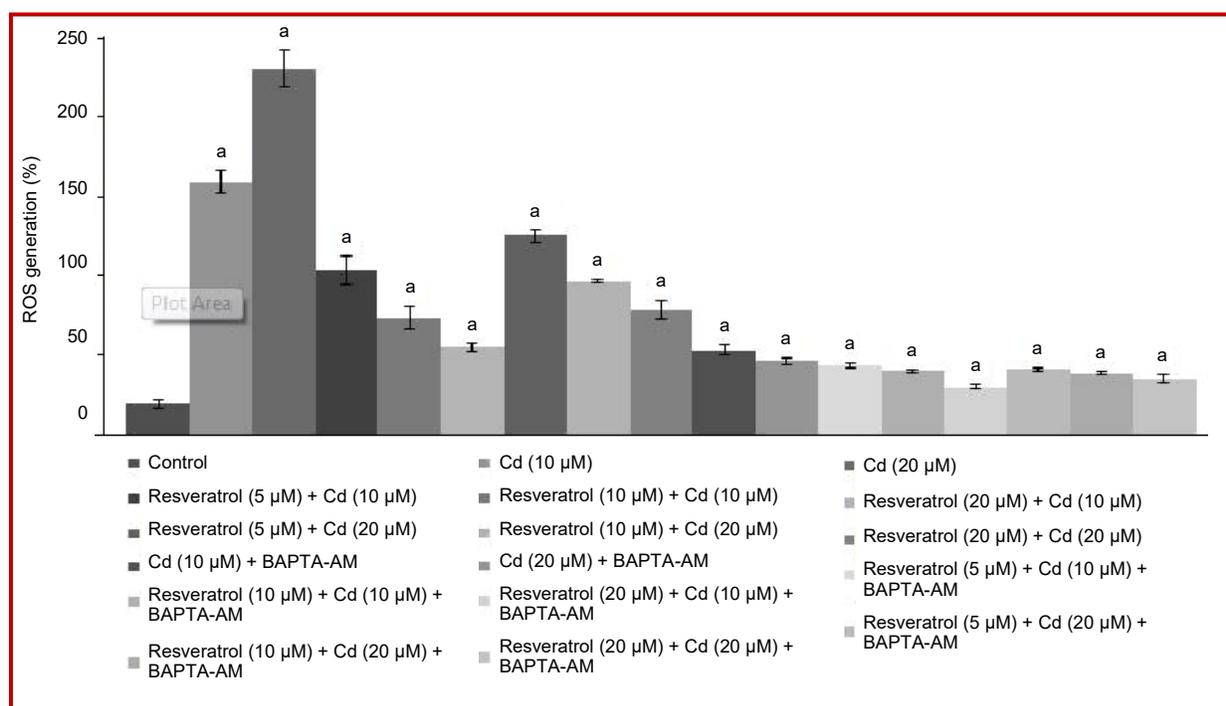


Figure 3: Resveratrol inhibits Cd-induced ROS generation

Values are represented as mean \pm SD; n=6; *represents statistical significance at $p < 0.05$ compared against control as determined by one-way ANOVA

the order: BAPTA-AM and resveratrol (20 μ M) > BAPTA-AM and resveratrol (10 μ M) > BAPTA-AM and resveratrol (5 μ M). The results suggest that effective

decrease in ROS levels by resveratrol could be attributed to its antioxidant potency. BAPTA-AM also caused reduction in ROS level suggesting BAPTA-AM

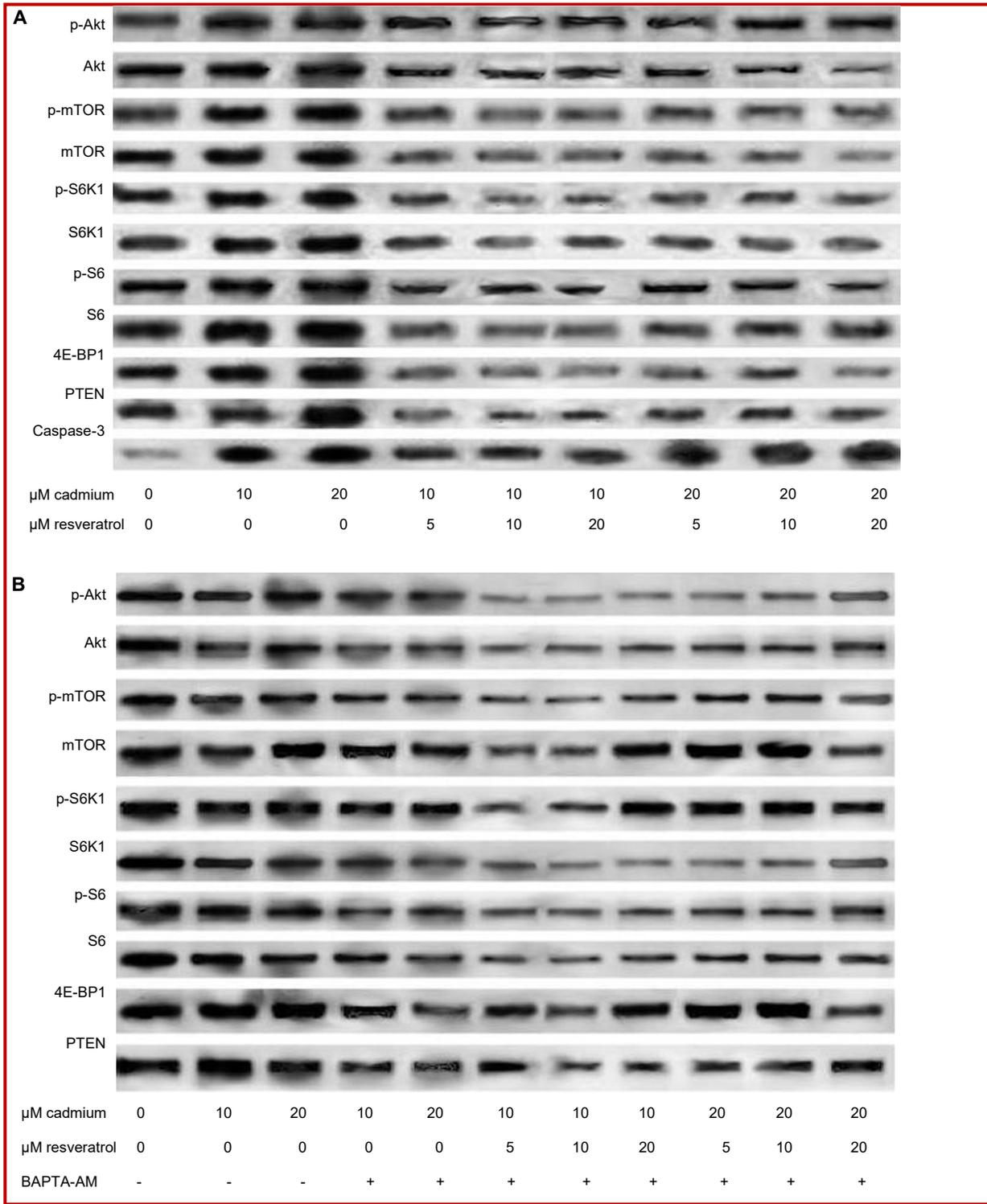


Figure 4: Resveratrol prevents cadmium induced neuronal cell death by modulating PTEN expression and Akt/ mTOR network

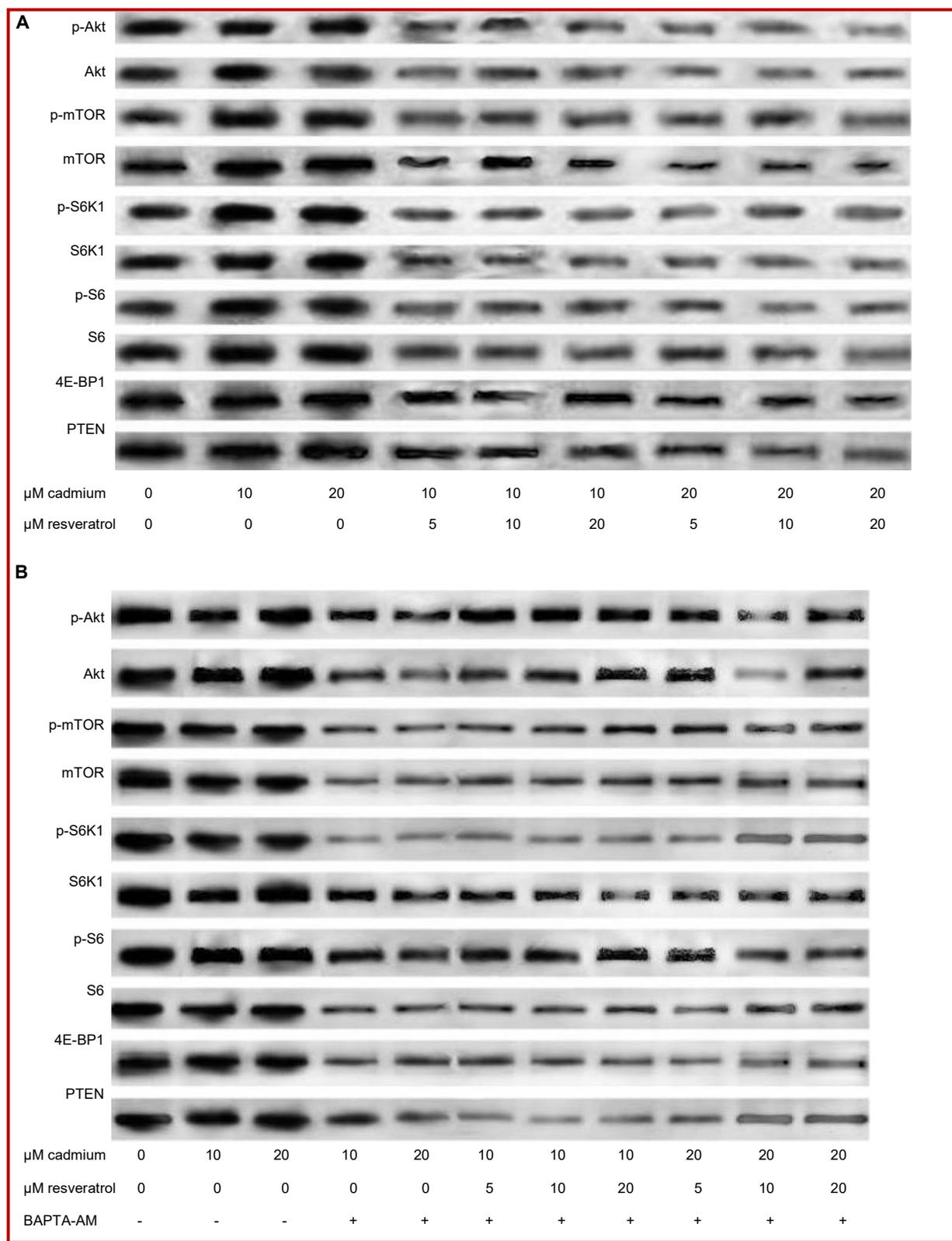


Figure 5: Resveratrol regulates the expression of MAPK pathway proteins

blocked calcium levels that could have reduced the production of ROS.

Effect of resveratrol on cadmium induced phosphorylation of Akt, S6K and 4E-BP1 was determined. Pre-treatment with varying concentrations of resveratrol markedly inhibited cadmium induced phosphorylation of Akt, S6K and 4E-BP1 in the primary cortical neurons (Figure 4A, B). At 20 μ M, resveratrol entirely blocked the phosphorylation. Furthermore, significant down-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10) was observed following induction of cadmium. Resveratrol caused dose-dependent up-regulation of PTEN. The results suggest that resveratrol was able to effectively inactivate Akt/mTOR proteins and increase PTEN expression.

Cadmium-induced relative expressions of PTEN and Akt/mTOR pathway proteins under the influence of resveratrol without (a)/with BAPTA-AM (b). Resveratrol effectively modulates the expressions of the proteins.

The effects of resveratrol on the expressions of the three main members of the MAPK family: c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 MAPK were determined. Resveratrol was found to potentially block the phosphorylation of JNK, ERK1/2, and p38 in primary neurons (Figure 5). However, no observable change in the basal expression of JNK, Erk1/2 and p38 was found. Resveratrol inhibited cadmium-induced phosphorylation of JNK and phosphorylation of c-Jun as well; c-Jun is a specific substrate of JNK (Figure 5). The effects were however dose-dependent, with 5 μ M of resveratrol exhibiting no obvious alterations in the expression patterns of c-Jun, JNK, ERK1/2 and p38. Maximum effects were exhibited by 20 μ M resveratrol. The data thus suggest that resveratrol was able to potentially inhibit cadmium induced expressions of MAPK family proteins.

Cadmium-induced alterations in the expression of MAPK pathway proteins were effectively modulated by resveratrol. The relative expressions of the proteins under the influence of resveratrol alone and with BAPTA-AM are shown in a and b respectively.

Discussion

Use of chelators for effective therapy of cadmium poisoning may not hold effective for long-term exposure and also due to the side-effects associated with chelating agents (Sinicropi et al., 2010). Recent studies have shown efficacy of plant-derived compounds in cadmium poisoning. Celastrol and Epigallocatechin 3-gallate have been reported to possess potential neuroprotective effects against cadmium induced neurotoxicity (Abib et al., 2011; Chen et al., 2014).

The exact mechanism through which cadmium induces neuronal apoptosis is yet to be resolved completely, while recent reports indicate the sustained activation of mitogen-activated protein kinases signalling pathways (Kim and Sharma, 2004; Kim et al., 2005) and mTOR networks (Chen et al., 2008; 2011) in cadmium induced neuroapoptosis. The present study was undertaken to evaluate whether resveratrol was able to effectively modulate cadmium induced alterations of MAPK and mTOR pathways.

Resveratrol treatment caused effective reduction in the morphological alterations in cadmium induced primary cortical neurons. The intensity of fragmented nuclei significantly reduced in resveratrol pre-treatment. Studies have shown that cadmium may trigger cell death by caspase-dependent and/or independent apoptotic mechanisms, depending on cell types (Coutant et al., 2006; Mao et al., 2007). Resveratrol was able to potentially improve the cell viability and markedly down-regulate cadmium induced robust up-regulation of caspase-3.

Cadmium disrupts $[Ca^{2+}]_i$ homeostasis, causing apoptosis in a variety of cells. In line with the previous reports (Biagioli et al., 2008; Wang et al., 2009; Xu et al., 2011), in the present study cadmium exposure caused a striking increase in the $[Ca^{2+}]_i$ levels. However, resveratrol caused a marked decrease in $[Ca^{2+}]_i$. The results were similar to the effects exerted by BAPTA-AM, that was used as a standard to chelate intracellular calcium in order to demonstrate cadmium induced elevation in $[Ca^{2+}]_i$. Xie et al. (2010) has reported cadmium induced apoptosis as mediated by the release of Ca^{2+} from intracellular Ca^{2+} stores. Pre-treatment with 2-APB, an membrane-permeable inhibitor of IP_3R (Ruiz et al., 2009), evidently reduced cadmium-induced elevation of $[Ca^{2+}]_i$ suggesting that this elevation possibly involves intracellular release from Ca^{2+} stores. Resveratrol exposure has also caused an obvious decrease in levels of intracellular release of Ca^{2+} , suggesting its effect against cadmium induced release of Ca^{2+} . As shown by Misra et al. (2003) an increase in IP_3 , an ligand of IP_3R following exposure to cadmium suggests that cadmium could activate IP_3R through increase of intracellular IP_3 which then causes release of calcium from ER which is an major calcium storage compartment of the cell. Pre-treatment with an inhibitor of the calcium channel of ER (IP_3R) suggests that the release of calcium from ER in stimulating apoptosis (Deniaud et al., 2008). The effect of resveratrol in reducing the levels of Ca^{2+} suggests that it contributes in further inhibiting the process of apoptosis by maintaining the levels of Ca^{2+} . Decrease in the Ca^{2+} contributed by resveratrol exposure is found to be in line with observations of the cell viability assay and DAPI staining.

Cadmium induced neuronal toxicity is also due to

induction of ROS leading to oxidative stress (Lopez et al., 2006; Chen et al., 2008, 2011; Thevenod et al., 2009). Xu et al. (2011) has demonstrated that cadmium elevated $[Ca^{2+}]_i$ induces the ROS and obtained results of this studies also showed cadmium induced $[Ca^{2+}]_i$ elevation resulting in the induction of ROS in cerebral cortical neurons after treatment with cadmium. Resveratrol and/or BAPTA-AM markedly attenuated cadmium induced ROS, suggesting calcium-related ROS production. Elevated $[Ca^{2+}]_i$ and ROS could lead to apoptosis. By causing a marked decrease in the levels of $[Ca^{2+}]_i$ and ROS generation, resveratrol significantly reduces neuronal apoptosis and offers protection against cadmium induced neurotoxicity.

Mitogen-activated protein kinases (MAPKs) are important signal transduction enzymes that are involved in many cellular processes including development, differentiation, proliferation and apoptosis (Hamanoue et al., 2007; Vacotto et al., 2008). Studies have demonstrated that ERK, JNK and p38 MAPK, play a critical role in cadmium induced apoptosis of many cell types including neuronal cells (Xu et al., 2011; Nemmiche et al., 2012). In the present study, cadmium exposure caused an increase in the phosphorylation levels of JNK, ERK1/2, c-Jun and p38. Further following cadmium exposure the increase in p-c-Jun levels correlated with c-Jun levels. Sustained activation of JNK, ERK1/2 and/or p38 is responsible for cadmium induced apoptosis in various cells, including neuronal cells (Chuang et al., 2000; Kim et al., 2005). Resveratrol significantly down-regulated the expression levels of the phosphorylated JNK, ERK1/2, C-Jun and p38. This down-regulation in the phosphorylation status of MAPK proteins is suggestive of the reversal of MAPK pathway to normal.

mTOR has been widely recognized as a central controller of cell proliferation, growth and survival (Bjornsti and Houghton, 2004). Cadmium-induced inhibition of cell proliferation and cell viability has been reported (Lopez et al., 2003; Kim et al., 2005). Akt positively regulates mTOR, leading to increased phosphorylation of ribosomal p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), the downstream effector molecules of mTOR (Bjornsti and Houghton, 2004). In the present study, cadmium exposure caused raised phosphorylation of mTOR and Akt. As reported by Bjornsti and Houghton (2004), cadmium-induced activation of mTOR caused consequent activation of S6K1 and 4E-BP1. Resveratrol induced significant down-regulation in the phosphorylation levels of Akt and mTOR, lead to subsequent decreases in p-4E-BP1 and pS6K1. Chen et al. (2011b) reported cadmium inactivation of PTEN resulting in activation of Akt/mTOR signalling and apoptosis of neuronal cells thus implying loss of PTEN function may promote development of neurodegenerative disorders. We also witnessed cadmium exposure resulting in

considerable inactivation of PTEN and the same being significantly attenuated by resveratrol. Resveratrol exposure effectively improved PTEN expression and also regulated the Akt/mTOR pathway.

The overall observations of the study indicates that cadmium-induced raised $[Ca^{2+}]_i$ in neuronal cells subsequently induced ROS generation and activated MAPK/mTOR pathways that were potentially regulated by resveratrol. The results suggest the efficacy of resveratrol in neuroprotection. These protective effects of resveratrol may possibly be attributed to its potent antioxidant capacity.

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