Inhibitory effect of nicardipine on rotenone-induced apoptosis in SH-SY5Y human neuroblastoma cells

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Abstract. Previous studies have demonstrated that calcium channel blockers have protective effects on damaged brains. In the present study, the protective effect of the calcium channel blocker nicardipine against rotenone-induced apoptosis in SH-SY5Y human neuroblastoma cells was investigated, focusing on mitogen-activated protein kinases (MAPKs) and caspase (CASP)-mediated apoptotic events. Nicardipine was found to decrease rotenone-induced apoptosis through 4,6-diamidino-2-phenylindole staining and the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay. In addition, nicardipine was identified to inhibit rotenone-induced elevation of intracellular Ca\(^{2+}\) concentration measured using the Fluo-4 AM fluorescent dye.

Introduction

Rotenone is a natural hydrophobic pesticide derived from the roots and barks of the Derris and Lonchorcarpus species. Rotenone has been used as a botanical insecticide for over 150 years to control crop pests (1). The pesticidal activity of rotenone is attributed to irreversible binding and inactivation of NADH ubiquinone reductase (complex I) in the mitochondrial electron transport chain (2). When incorporated in the brain, rotenone decreases intracellular ATP levels, increases production of reactive oxygen species (ROS) (3,4), releases glutamate from presynaptic terminals (5,6), elevates intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (7,8) and initiates neurodegenerative processes. In addition, rotenone is known to cause Parkinson's disease-associated motor dysfunction (9,10) and degeneration of dopaminergic neurons via apoptotic pathways by activation of mitogen-activated protein kinases (MAPKs) and caspases (CASPs) (11-13).

Previous studies have revealed that calcium channel blockers play a role as neuroprotectants in neurodegenerative disorders (14-16). Nicardipine is a hepatically metabolized dihydropyridine-type calcium channel blocker that causes vasodilation through blockade of L-type calcium channels in vascular smooth muscle cells. Nicardipine is widely employed for the treatment of specific cardiovascular and cerebrovascular disorders and esophageal cancer in animals (17,18). In the brain, nicardipine has been demonstrated to block voltage-gated calcium channels (VGCCs) in the neuronal terminus which modulate glutamate release (19). Indeed, in ischemia and reperfusion rats, nicardipine improved motor neurological outcomes and reduced infarction and edema volume (20). Nicardipine also decreased plasma levels of the neuron-specific enolase, a specific marker for the incidence of neuronal injury (20). Therefore, we hypothesized that nicardipine may exhibit protective effects against rotenone-induced apoptosis in neuronal cells.

In the current study, the protective effect of nicardipine in rotenone-treated SH-SY5Y neuroblastoma cells was investigated. We specifically focused on the c-Jun N-terminal protein kinase (JNK)/p38 MAPK and CASP pathways, which have been hypothesized to be important for rotenone-induced apoptosis in neuronal cells (11-13).

Materials and methods

Materials. Rotenone, nicardipine, Fluo-4 AM, the CASP3 assay kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were
obtained from Gibco-BRL (Grand Island, NY, USA). The in situ cell death detection terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit was obtained from Roche Diagnostics (Indianapolis, IN, USA). Anti-JNK, anti-phospho-JNK (Thr 183 and Tyr 185), anti-p38 MAPK, anti-phospho-p38 MAPK (Thr 180 and Tyr 182), anti-cleaved CASP9, anti-cleaved CASP3, anti-cleaved poly (ADP-ribose) polymerase-1 (PARP) and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-B-cell lymphoma protein 2 (Bcl2) and anti-Bcl2-associated X protein (BAX) antibodies and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatment. SH-SY5Y cells were obtained from American Type Culture Company (Rockville, MD, USA). Cells were grown in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air. Cell culture medium was changed every 2 days.

Rotenone was freshly prepared in dimethyl sulfoxide (DMSO) prior to each experiment. Nicardipine was freshly prepared in saline and was added 4 h prior to rotenone addition to the cultures.

Cell viability assay. Cell viability was determined by MTT assay. For detecting cytotoxicity of rotenone on SH-SY5Y cells, cells were seeded in triplicate at a concentration of 2x10⁴ cells/ml on a 96-well plate. Cells were exposed to rotenone (0.1, 1, 5, 10 and 20 µM) containing 0.1% (v/v) DMSO or vehicle for 24 h. To test the effect of nicardipine, cells were pretreated with nicardipine or saline for 4 h prior to rotenone treatment of concentrations of 0.1, 0.5, 1 and 5 µM. Following this, cells were exposed to rotenone (10 µM) containing 0.1% (v/v) DMSO or vehicle for 24 h. MTT (0.5 mg/ml) was added to each group and the cells were incubated for 4 h. Then, cells were incubated for an additional 1 h in the solution in which MTT was dissolved. Viability was read with an absorbance microplate reader (Molecular Devices, Toronto, ON, Canada) at a test wavelength of 595 nm with a reference wavelength of 690 nm. Optical density (OD) was calculated as the difference between the reference and test wavelength. Percent viability was calculated as \(\frac{OD_{\text{drug}} - OD_{\text{control}}}{OD_{\text{control}}} \times 100\).

DAPI staining. SH-SY5Y cells (1x10⁵ cells/ml) were cultured on four-chamber slides (Nalge Nunc International, Naperville, IL, USA). Following pretreatment with nicardipine (5 µM, 4 h) or saline, cells were incubated with rotenone (10 µM) or vehicle for 24 h. Then, cells were fixed in methanol and incubated in 1 µg/ml DAPI solution for 30 min in the dark. The stained cells were observed with a fluorescence microscope (Carl Zeiss, Oberköchen, Germany).

TUNEL assay. TUNEL assays were performed according to the manufacturer’s instructions. SH-SY5Y cells (1x10⁵ cells/ml) pretreated with 5 µM nicardipine (4 h) or saline were exposed to 10 µM rotenone or vehicle for 24 h and then fixed in acetic acid at -20°C. Fixed cells were incubated with the TUNEL reaction mixture (terminal deoxynucleotidyl transferase and nucleotide) for 1 h at 37°C, followed by the addition of peroxidase-conjugated detection antibody. DNA fragments were stained using 3,3’-diaminobenzidine as the substrate for the peroxidase.

Measurement of [Ca²⁺]. Cells were pretreated with nicardipine (5 µM) for 4 h, incubated with 3 µM Fluo-4 AM (dissolved in DMSO) in serum-free growth medium at 37°C for 45 min in a CO₂ incubator and then washed with calcium-free Hank's balanced salt solution (Gibco-BRL). Ca²⁺ fluorescence was assessed at intervals of 10 sec in an absorption spectrum (488 nm) and an emission wavelength (515 nm) using laser scanning confocal microscope (Leica Lasertechnik, Heidelberg GmbH, Wetzlar, Germany). Baseline [Ca²⁺], was observed for 100 sec and 10 µM rotenone was added and changes in [Ca²⁺] were measured. Results are expressed as relative fluorescence intensity (21).

Western blot analysis. Cells were lysed in RIPA buffer containing protease inhibitors. The protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (80 µg) were separated on sodium dodecyl sulfate-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Postfach, Germany). Following blocking with 5% skimmed milk, membranes were probed with rabbit anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38 MAPK, anti-cleaved CASP9, anti-cleaved CASP3, anti-cleaved PARP or mouse anti-β-actin antibodies overnight at 4°C. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG were used as the secondary antibodies. Band detection was performed using the Enhanced Chemiluminescence detection system (Amersham Biosciences, Uppsala, Sweden). Results of western blot analysis were quantified using ImageJ image analysis software (v1.4; National Institutes of Health, Bethesda, MD, USA).

CASP3 activity assay. CASP3 activity was measured using an assay kit according to the manufacturer’s instructions. SH-SY5Y cells (2x10⁴ cells/ml) were lysed following treatment with 5 µM nicardipine and 10 µM rotenone for 24 h. The CASP3 substrate (Ac-DVED-p-NA) was added to cell lysates and the mixtures were incubated overnight in a humidified environment at 37°C. Control lysates were preincubated with the CASP3 inhibitor Ac-DEVD-CHO to determine on-specific background substrate breakdown. The concentration of p-NA released from the CASP3 substrate was measured using an absorbance microplate reader (Molecular Devices) as absorbance values at 405 nm and calculated from a calibration curve of p-NA standards.

Statistical analysis. Data are presented as mean ± SEM from three independent experiments. All experiments were performed at least three times independently. Data were analyzed by one-way ANOVA, followed by Tukey’s HSD post hoc test, using the SPSS software (v13.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.
Results

Effect of nicardipine on rotenone-induced cell death in SH-SY5Y cells. SH-SY5Y cells were treated with rotenone of various concentrations (0.1-20 µM) for 24 h. Rotenone revealed significant cytotoxic effects in a dose-dependent manner in SH-SY5Y cells (Fig. 1A; P<0.05 vs. non-treated cells). The viability of SH-SY5Y cells exposed to rotenone at concentrations of 10 and 20 µM was 58.3±3.7 and 33.7±3.3% of the control value, respectively.

Effect of nicardipine pretreatment on rotenone-induced cell death was investigated. Cells were treated with rotenone (10 µM, 24 h) and nicardipine (5 µM, 4 h prior to rotenone treatment). As demonstrated in Fig. 1B, nicardipine pretreatment (4 h prior to rotenone treatment) increased cell viability in rotenone-treated cells in a dose-dependent manner. The viability of SH-SY5Y cells was 61.8±4.1, 69.9±4.5, 80.5±1.3 and 91.8±1.6% at concentrations of 0.1, 0.5, 1 and 5 µM nicardipine, respectively. Further experiments were performed using 5 µM nicardipine to determine the precise effects of nicardipine.

Effect of nicardipine on rotenone-induced apoptosis. Apoptosis of rotenone-treated cells was determined by DAPI staining and TUNEL assay. As revealed in Fig. 1C (upper panel), 5 µM nicardipine protected against shrinkage of SH-SY5Y cells treated with 10 µM rotenone for 24 h. DAPI staining demonstrated nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies upon treatment of rotenone, whereas nicardipine pretreatment inhibited these apoptotic features (Fig. 1C, middle panel). TUNEL assay also revealed DNA strand breaks, indicative of rotenone-induced apoptosis. By contrast, nicardipine prevented the induction of apoptosis by rotenone (Fig. 1C, lower panel).

Effect of nicardipine on rotenone-induced Ca^{2+} elevation. Increased [Ca^{2+}] has been postulated to be associated with rotenone-induced cell death in previous studies. As demonstrated in Fig. 2, [Ca^{2+}] was rapidly increased by treatment with 10 µM rotenone and increased [Ca^{2+}] was sustained to the end of the experiment. By contrast, nicardipine prevented rotenone-induced elevation of [Ca^{2+}]. Nicardipine did not affect [Ca^{2+}] in rotenone-untreated cells.

Effect of nicardipine on rotenone-induced increase of phosphorylation of JNK and p38 MAPK. The effect of nicardipine on the phosphorylation of JNK and p38 MAPK in SH-SY5Y cells treated with rotenone was investigated. Cells were treated with
10 µM rotenone for up to 120 min. Fig. 3A indicates that rotenone induced phosphorylation of JNK in a time-dependent manner. Phosphorylation of p38 MAPK was also transiently upregulated following exposure to rotenone, with peak expression observed at 10 min. The effect of nicardipine on phosphorylation of JNK and p38 MAPK was examined in cells treated with rotenone for 120 and 10 min, respectively. In nicardipine (5 µM)-pretreated cells, the phosphorylation of JNK and p38 MAPK triggered by rotenone was abrogated (Fig. 3B and C).

**Effect of nicardipine on the decrease of Bcl2 and increase of BAX by rotenone.** The effect of nicardipine on Bcl2 family proteins, including Bcl2 and BAX, was analyzed in SH-SY5Y cells exposed to rotenone through western blot analysis. Rotenone (10 µM, 6-24 h) decreased the expression of the anti-apoptotic protein Bcl2 and increased the expression of pro-apoptotic protein BAX in a time-dependent manner (Fig. 4A). As demonstrated in Fig. 4B and C, 5 µM nicardipine pretreatment prevented the reduction of Bcl2 and the elevation of BAX induced by rotenone (10 µM, 24 h).

**Effect of nicardipine on rotenone-induced activation of caspases.** The effect of nicardipine on CASP9 and 3 and PARP in SH-SY5Y cells treated with rotenone (10 µM) for up to 24 h and (B) the effect of nicardipine (5 µM, 4 h prior to rotenone treatment) on CASP3 and 9 and PARP expression in cells treated with 10 µM rotenone for 24 h. β-actin was detected as an internal control. (C) Histogram shows expression of target proteins divided by expression of β-actin. CASP, caspase; PARP, poly (ADP-ribose) polymerase-1.
in SH-SY5Y cells treated with 10 µM rotenone for 24 h. The activity was measured by hydrolysis of the peptide substrate, Ac-DEVD-p-NA. Nicardipine pretreatment significantly attenuated the cleavage of Ac-DEVD-p-NA elevated by rotenone (Fig. 6).

Discussion

In the present study, nicardipine reduced rotenone-induced cytotoxicity, apoptosis and elevation of [Ca\(^{2+}\)], in SH-SY5Y cells. Rotenone increased the phosphorylation of JNK and p38 MAPK, whereas nicardipine inhibited these increases. Nicardipine also inhibited the upregulation of Bcl2 expression and downregulation of BAX expression by rotenone. In addition, nicardipine protected rotenone-induced cleavage of CASP9 and 3 and PARP and increased CASP3 enzyme activity. These results indicate that nicardipine exhibits a protective effect against rotenone-induced apoptotic cell death in SH-SY5Y cells.

Ca\(^{2+}\) has been implicated in the induction of apoptosis and the regulation of the apoptotic signaling pathways. Negre-Salvayre and Salvayre (22) demonstrated the importance of Ca\(^{2+}\) for apoptosis as well as the protective effect of Ca\(^{2+}\) channel blockers and chelators. A number of studies have also reported that rotenone elevates [Ca\(^{2+}\)]\(_i\) by inducing Ca\(^{2+}\) influx into cells (7,8). Wang and Xu (8) identified that rotenone (10 µM) induced an elevation in [Ca\(^{2+}\)]\(_i\) through Ca\(^{2+}\) influx by opening VGCCs in SH-SY5Y cells, inducing apoptotic events, including ROS production, G\(_i/M\) cell cycle arrest and CASP activation. In addition, treatment with the intracellular Ca\(^{2+}\) chelator 1,2-bis-(2-aminophenoxy) ethane-N,N,N\(_{\prime},N\_{\prime}\)-tetracetic acid tetrakis/acetoxymethyl ester prevented rotenone-induced apoptosis (8). Therefore, it was hypothesized that the increase in Ca\(^{2+}\) influx via VGCCs was associated with rotenone-induced apoptotic events. Freestone et al (7) identified rotenone-induced elevation of [Ca\(^{2+}\)]\(_i\) in substantia nigra pars compacta neurons, hypothesizing that the increase in Ca\(^{2+}\) influx resulted from transient receptor potential M2 channels activated by increased ROS production. However, a low toxin concentration (5 nM) was used in the study. In the present study, rotenone increased [Ca\(^{2+}\)]\(_i\) and induced apoptosis, decreasing anti-apoptotic protein Bcl2 expression and increasing pro-apoptotic protein BAX expression and CASP activation. Nicardipine inhibited rotenone-induced elevation of [Ca\(^{2+}\)]\(_i\) and changes in expression of these apoptotic proteins. These results, together with previous studies, indicate that rotenone-induced apoptosis may be suppressed by blocking Ca\(^{2+}\) influx.

JNK and p38 MAPK play diverse roles in neuronal differentiation, survival and death and are activated in response to a variety of cellular stresses and toxicants (11). Prolonged activation of these signaling pathways has been implicated in several forms of neuronal apoptosis (12,23). Activation of JNK and p38 MAPK is responsible for inhibition of Bcl2 and induction of phosphorylation of c-Jun, a nuclear transcription factor and a known target of JNK, which further promotes the release of cytochrome c from the mitochondria to the cytoplasm and leads to activation of CASPs (11,24). In addition, rotenone-induced apoptosis has been found to require activation of JNK and p38 MAPK via phosphorylation and may mediate the regulation of Bcl2 family proteins and activation of CASPs (12,13). Previous studies have also reported that MAPKs are activated by Ca\(^{2+}\) influx through VGCCs (25,26). Thus, we hypothesized that calcium channel blockers may inhibit the activation of MAPKs by rotenone leading to Ca\(^{2+}\) influx via VGCCs. In our study, nicardipine reduced the rotenone-induced phosphorylation of JNK and p38 MAPK in SH-SY5Y cells. Results indicate that nicardipine protects rotenone-induced apoptosis through regulation of MAPKs as well as Bcl2 family proteins and CASPs.

In conclusion, the present study demonstrates that nicardipine has a potent protective effect on rotenone-induced apoptosis in SH-SY5Y cells, inhibiting phosphorylation of JNK and p38 MAPK and activation of CASPs with modulation of Bcl2 family proteins. These observations indicate that nicardipine may be useful for impairment of rotenone-induced neurotoxicity.

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References


