Resveratrol Ameliorates NMDA-induced Mitochondrial Injury by Enhanced Expression of Heme Oxygenase-1 in HT-22 Neuronal Cells

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N-methyl-D-aspartate (NMDA) receptors have received considerable attention regarding their involvement in glutamate-induced neuronal excitotoxicity. Resveratrol has been shown to exhibit neuroprotective effects against this kind of overactivation, but the underlying cellular mechanisms are not yet clearly understood. In this study, HT-22 neuronal cells were treated with NMDA in Mg^{2+}-free buffer and subsequently used as an experimental model of glutamate excitotoxicity to elucidate the mechanisms of resveratrol-induced neuroprotection. We found that NMDA treatment causes a drop in MTT reduction ability, disrupts inside-negative transmembrane potential of mitochondria, depletes cellular ATP levels, and stimulates intracellular ROS production. Double fluorescence imaging studies demonstrated an increased formation of mitochondrial permeability transition (MPT) pores accompanied by apoptotic cell death, while cobalt protoporphyrin and bilirubin showed protective effects against NMDA-induced mitochondrial injury. On the other hand, zinc protoporphyrin IX significantly attenuated the protective effects of resveratrol which was itself shown to enhance heme oxygenase-1 (HO-1) mRNA and protein expression levels. In cells transfected with HO-1 small interfering RNA, resveratrol failed to suppress the NMDA-induced effects on MTT reduction ability and MPT pore formation. The present study suggests that resveratrol may prevent mitochondrial injury in NMDA- treated HT-22 cells and that enhanced expression of HO-1 is involved in the underlying cellular mechanism.

Key words : Heme oxygenase-1, mitochondrial injury, NMDA excitotoxicity, neuroprotection, resveratrol

Introduction

Different classes of ionotropic and metabotropic receptors are involved in glutamate-mediated synaptic excitation. Among these receptors, N-methyl-D-aspartate (NMDA) receptors have received considerable attention regarding their involvement in neuronal excitotoxicity in addition to their roles in ordinary neurotransmission and synaptic plasticity [26]. Excessive or persistent activation of NMDA receptors above physiological levels may cause cellular Ca^{2+} overload and stimulate a cellular cascade of events, which in turn leads to neuronal dysfunction, damage or even death [36]. This process, called NMDA receptor-mediated excitotoxicity, is hypothesized to serve an important role in a wide range of neuronal diseases, such as infraction [11, 34], hemorrhage [11, 34], epilepsy [32], trauma [8], Alzheimer's disease [33] and Parkinson's disease [7].

Resveratrol (3,4′,5-trihydroxy-trans-stilbene, $C_{14}H_{12}O_3$) is one of the most widely studied natural polyphenols, and it's beneficial effects in various diseases have been extensively studied. Resveratrol is found in a wide variety of dietary ingredients including peanuts, grapes and wine, and can be extracted using various techniques, such as organic solvent extraction, ultrasound-assisted extraction and high-pressure processes [31]. Resveratrol is known to exhibit a variety of beneficial biological effects, such as anti-oxidative, anti-inflammatory and anti-carcinogenic activities [16, 23-25, 35]. Resveratrol has also been shown to pass through the blood-brain barrier [1] where it exhibits neuroprotective effects in experimental models of neurodegenerative diseases, such as stroke, Alzheimer's and Parkinson's diseases [1, 5, 10, 29]. In addition, several studies have elucidated the neuroprotective role of resveratrol against glutamate-induced excitotoxity [6, 12]. However, the cellular mechanism underlying resveratrol-induced neuroprotection is not clearly understood.

Mitochondria are the primary site responsible for the initiation of mitochondria-dependent apoptosis signaling and production of ATP and reactive oxygen species (ROS) [27].

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Accumulating evidence has shown that the deterioration of the functional and/or structural integrity of the mitochondria is implicated in pathological events associated with several neurodegenerative diseases. Impairment of mitochondrial integrity leads to the depletion of cellular ATP, opening of mitochondrial permeability transition (MPT) pores, release of cytochrome c and finally cell death [13]. Hence, over the last few decades, mitochondria and the mitochondria-dependent cellular pathways have been a focus of increasing attention as targets of pharmacological interventions to alleviate neuronal damage and restore neuronal viability and their physiological functioning in a variety of neurodegenerative diseases.

The aim of the present study was to examine the potential role of mitochondria as an important target of NMDA receptor-mediated neuronal cytotoxicity and the resveratrol-induced protective mechanisms. In addition, the potential role of heme oxygenase-1 (HO-1), which can act as a crucial cellular mechanism underlying resveratrol-induced neuroprotection, was investigated.

Materials and Methods

Chemicals and reagents

Medium and reagents for cell culture were purchased from Gibco (Thermo Fisher Scientific, Inc.). Tetramethylrhodamine methyl ester (TMRM), calcein acetoxymethyl ester (calcein/AM), $DiOC_6(3)$ and 2′,7′- Dichlorofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR, USA.) Anti-HO-1 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). NMDA, MK-801, cobalt protoporphyrin (CoPP), bilirubin and zinc protoporphyrin IX (ZnPP IX) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Resveratrol with a purity of ≥99% (purified using high-performance liquid chromatography) was also purchased from Sigma- Aldrich (St. Louis, MO, USA). Resveratrol stock solution (10 mM) was prepared by dissolving in DMSO before use.

Cell culture and induction of NMDA excitotoxicity

HT-22 cells (Salk Institute, San Diego, USA) were routinely grown in 75 cm^2 culture flasks with DMEM supplemented with 10% FBS, 150 IU/ml penicillin G and 50 μg/ml streptomycin. When cells were confluent, they were detached using 0.05% trypsin/0.53 mM EDTA solution and split (1:5). For biochemical or microscopic studies, cells were grown on the appropriate culture plates, 24 or 96-well plates, or on collagen-coated glass cover slips in six-well plates dependent on the experiment.

For cytotoxicity studies, growth medium was removed by washing three times with prewarmed Locke's buffer containing NaCl, 154 mM; KCl, 5.6 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2.3 mM; $NaHCO_3$, 3.6 mM; glucose, 5.6 mM; and HEPES, 5.0 mM (pH7.4). NMDA-induced excitotoxicity was achieved by incubation of cells with NMDA in Mg^{2+}-free Locke's buffer. The concentration and time for NMDA treatment were 20 mM and 3 hr, unless otherwise indicated.

MTT assay

Mitochondria reduce MTT to formazan [21]. Therefore, the ability of cells to produce formazan as a result of MTT reduction provides a suitable indicator of cell viability and intact mitochondrial function. After exposure to experimental protocols, cells were incubated with 50 ml of 5 mg/ml MTT at 37℃ for 4 hr. The rate of formazan production was determined using an automatic multi-well spectrophotometer at 540 nm.

Measurement of mitochondrial membrane potential

Mitochondrial transmembrane potential was measured using $DiOC_6(3)$. $DiOC_6$ accumulates into the mitochondria dependent on the mitochondrial membrane potential; accumulating when negative. Therefore, a decrease in $DiOC_6(3)$ staining indicates a lower mitochondrial transmembrane potential. Cells were loaded with $DiOC_6(3)$ at a final concentration of 50 nM for 20 min at 37℃ in the dark. Cells were washed and resuspended in PBS. The fluorescence intensity was analyzed using a FACsort (Becton, Dickinson and Company) flow cytometer.

Measurement of ATP content

Cellular ATP content was measured using a luciferin-luciferase assay [17]. Cells were solubilized with 500 ml 0.5% Triton X-100 and acidified using 100 μl 0.6 M perchloric acid and placed on ice. The cellular extract was then diluted with a buffer containing potassium 10 mM glutamate and 4 mM $MgSO_4$ (pH 7.4). Per 10 ml diluted sample, 100 ml luciferin-luciferase (20 mg/ml) was added and light emission was measured using a luminometer (MicroLumat LB96P; Berthold Detection Systems GmbH)

Analysis of ROS production

Intracellular production of ROS was determined using DCFH-DA, which is highly permeable to the cell membrane and readily penetrates the cell. DCFH-DA is then hydrolyzed to DCFH by the esterases which are abundantly present in the cytoplasm. In the presence of cellular peroxidase and ROS, DCFH is rapidly converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Therefore, the change in DCF fluorescence can be used as an indicator of cellular ROS levels. Cells were preloaded with DCFH-DA by incubation for 1 hr at 37℃ with 10 mM DCFH-DA. After exposure to experimental procedures, DCF fluorescence was analyzed by flow cytometry.

Detection of mitochondrial permeability transition (MPT)

To examine the formation of MPT pores, a double staining method with fluorescent dyes, calcein/AM and TMRM, was used as described by Lemasters *et al* [13]. These dyes penetrate into cells and are hydrolyzed to tetramethylrhodamine (TMR) and calcein by the cellular esterases. Intact mitochondria maintain a negative membrane potential which results in accumulation of the lipophilic cationic dye TMR, and thus fluoresces bright red. Due to the impermeability of the inner mitochondrial membrane, calcein cannot enter the mitochondria. However, mitochondrial dysfunction caused by a mechanism referred to as MPT makes the inner mitochondrial membrane permeable to solutes. Therefore, damaged mitochondria do not accumulate TMR and become permeable to calcein. After exposure to experimental procedures, cells grown on glass cover slips were incubated at 37℃ in HBSS containing 3 mM TMRM for 30 min followed by a further incubation for 30 min in the presence of 0.5 mM calcein/AM. After washing four times with PBS, glass cover slips were mounted on glass slides using PBS:Glycerol (1:1 w/w) solution and observed using confocal microscopy (Carl Zeiss AG) at room temperature. Excitation wavelengths for calcein and TMR were 488 and 568 nm, respectively.

Analysis of apoptosis

For detection of apoptotic cells, a TUNEL assay was performed using an ApoTag peroxidase *in situ* apoptosis detection kit (Sigma-Aldrich, Merck KGaA) according to the manufacturer's protocol. Briefly, cells were cultured on collagen-coated cover slips and exposed to the various experimental conditions. The cells were then fixed using 1% paraformaldehyde for 10 min and incubated with digoxigenin-conjugated dUTP in a TdT-catalyzed reaction for 60 min at 37℃. After incubation, the cells were immersed in a stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-peroxidase-conjugated antibody for 30 min. The DNA fragments were stained using DAB as a substrate and analyzed using a microscope.

Reverse transcription-quantitative (RT-q)PCR

RT-qPCR was used to measure the HO-1 mRNA expression levels. Total RNA was prepared using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed using SYBR Green MasterMix (Invitrogen; Thermo Fisher Scientific, Inc.) with 15 pmol primers of both forward and reverse primers for HO-1 and β-actin. Reactions were performed in triplicate and specificity was monitored using melting curve analysis after amplification. The sequences of the primers used to detect were: HO-1 forward 5′-GGTGAT GGCTTCCTTGTACC-3′ and reverse 5′-AGTGAGGCCCAT ACCAGAAG-3′; and β-actin forward 5′-AGAGGGAAATCG TGCGTGAC-3′ and reverse 5′-CAATAGTGATGACCTGGC CGT-3′.

Western blotting

Western blot analysis was used to determine the HO-1 protein expression levels. Total intracellular protein was isolated by freeze-thaw lysis repeated five times in a buffer containing 600 mM KCl, 20 mM Tris-Cl, 20% glycerol, 0.4 mg/ml Pefabloc, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 5 μg/ml aprotinin (pH 7.8). Protein concentration was measured using a Bradford assay (Bio-Rad Laboratories, Inc.). Protein samples (40 mg) were loaded on 10% SDS-gels, resolved using SDS-PAGE and transferred to immobile PVDF membranes (EMD Millipore). The membranes were then blocked with 5% skimmed milk in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 hr at room temperature. Subsequently, the membranes were incubated with primary antibodies against HO-1 (1:1,000) or b-actin (1:1,000). The membranes were washed three times with TBST, and incubated with goat anti-mouse IgG-horseradish peroxidase with a 1:5,000 dilution in TBST for 2 hr at room temperature. The membranes were washed three times for 10 min with TBST again. Signals were visualized using enhanced chemiluminescent horseradish peroxidase substrate detection method.

Transfection with HO-1 siRNA

A pool of three target-specific small interfering RNAs (siRNAs) were used to transiently knockdown the expression of the HO-1 gene. The sequences of the siRNAs were as follows: Sequence 1 forward, 5′-GCUUCCUUGUACCAU AUCUtt-3′ and reverse, 5′-AGAUAUGGUACAAGGAAGC tt-3′; sequence 2 forward, 5′-CCUUCCUGCUCAACAUUGA tt-3′ and reverse, 5′-UCAAUGUUGAGCAGGAAGGtt-3′; and sequence 3 forward, 5′-CUCUAACUUCUGUGUGAAAtt-3′ and reverse, 5′-UUUCACACAGAAGUUAGAGtt-3′. Transient transfection with siRNA or scrambled siRNAs was performed using Superfect® transfection reagent (Qiagen, GmbH). Briefly, aliquots of 2×10^5 cells/well were plated in 60 mm dishes on the day before transfection and grown to ~80% confluence. On the day of transfection, 5 mg siRNA or scrambled siRNA dissolved in TE buffer (pH 7.0) was diluted with 150 ml growth medium containing no serum,

proteins or antibiotics. To the RNA mixtures, 30 ml Super Fect® transfection reagent was added and vortexed. The mixture was then incubated for 5-10 min at room temperature to allow formation of the transfection complex. Cell growth medium (1 ml, containing serum and antibiotics) was added to the reaction tube containing the transfection complexes. After mixing by pipetting up and down twice, the total volume was immediately transferred to the cells in the 60 mm dishes. The cells were then incubated with the transfection complexes for 2-3 hr. When incubation was completed, medium was removed by gentle aspiration and cells were washed once with PBS. The cells were then assayed for expression of the HO-1 gene.

Statistical analysis

Data are presented as the mean ± standard error of the mean. Comparisons between two groups were performed
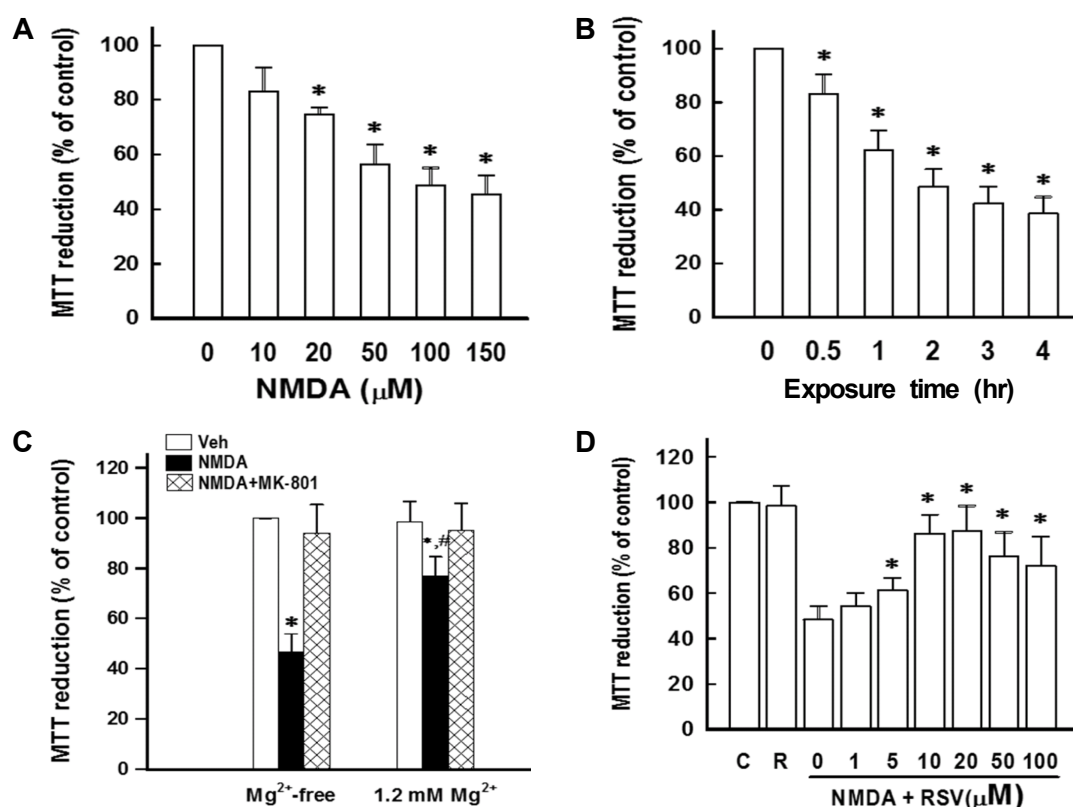

Fig. 1. Effects of NMDA and RSV on MTT reduction ability. (A) Dose-dependent effect of NMDA. Cells were treated with the indicated concentrations of NMDA for 3 hr in Mg^{2+}-free Locke's buffer. (B) Time-dependent effect of NMDA. Cells were treated with 100 mM NMDA for the indicated time periods in Mg^{2+}-free Locke's buffer. (C) Effects of Mg^{2+} and MK-801. Cells were treated with 100 mM NMDA for 3 hr in Mg^{2+}-free or buffer supplemented with 1.2 mM Locke's buffer in the presence and absence of 20 mM MK-801. (D) Protective effect of RSV on the NMDA-induced impairment of MTT reduction ability. Cells were treated with 100 mM NMDA for 3 hr in the presence of the indicated concentrations of RSV. Data are represented as the mean ± the standard error of the mean of 5 repeats. $^*p<0.01$ vs. respective control. $^#p<0.01$ vs. Mg^{2+}-free. C, control; R, 20 μM resveratrol alone; Veh, vehicle; RSV, resveratrol.

using an unpaired Student's t-test. Differences between multiple groups were compared using a one-way ANOVA followed by a post-hoc Tukey's test. $p<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of NMDA and resveratrol on MTT reduction ability

To determine the effects of NMDA and resveratrol on cell viability and mitochondrial functional integrity, MTT reduction ability was examined. There was a dose- and time-dependent decrease in MTT reduction ability in the NMDA-treated cells (Fig. 1A, Fig. 1B). In subsequent experiments,

unless otherwise indicated, a concentration of 20 mM and a treatment time of 3 hr were used to examine the effect of NMDA. In the presence of Mg^{2+} (1.2 mM as $MgCl_2$) or MK-801, the effect of NMDA to impair MTT reduction ability was remarkably reduced, suggesting that the NMDA-induced impairment of MTT reduction ability resulted from interaction with NMDA receptor channels (Fig. 1C). In the presence of resveratrol, the NMDA-induced impairment of MTT reduction ability was significantly ameliorated, and the effect was concentration-dependent up to a concentration of 20 mM (Fig. 1D).

Changes in mitochondrial membrane potential, ATP production and ROS generation
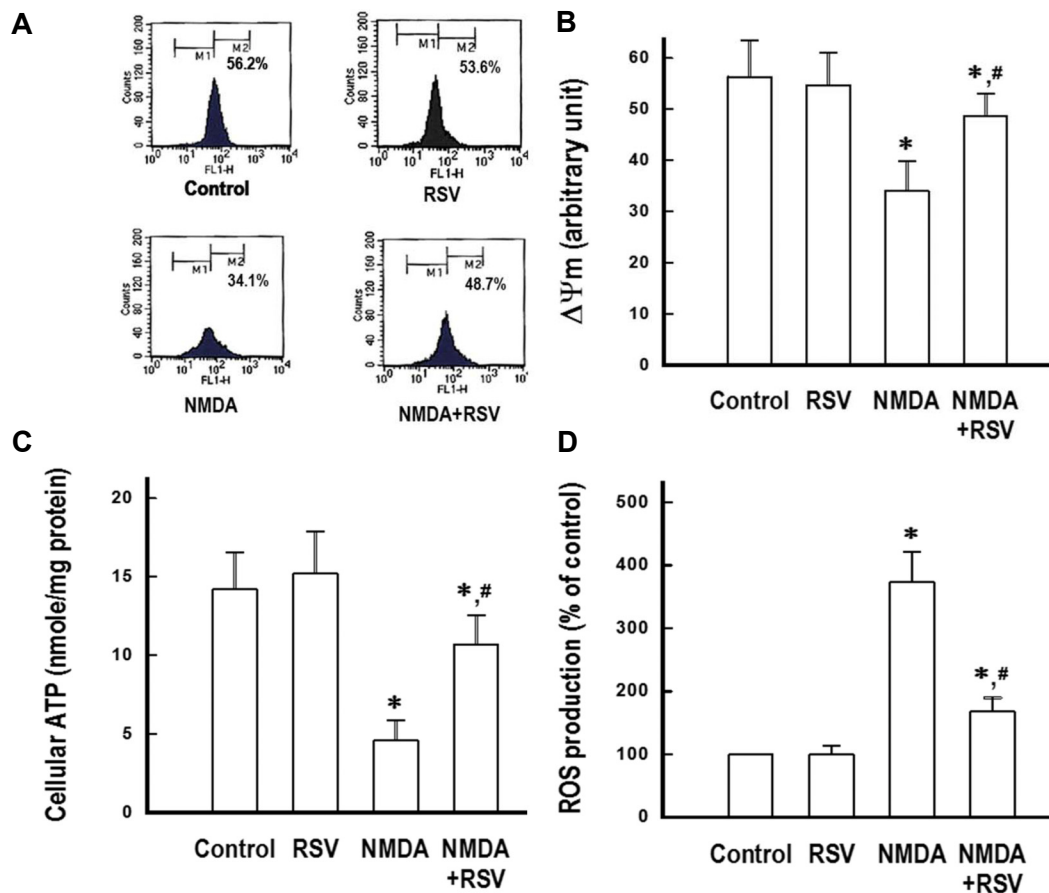

Fig. 2. Protective effect of RSV on the NMDA-induced changes in mitochondrial membrane potential, cellular ATP content and ROS production. (A) Representative flow cytometry analyses of mitochondrial membrane potential. Cells were treated with 100 mM NMDA for 3 hr in the presence and absence of 20 μM RSV. Cells were then loaded with $DiOC_6(3)$ for flow cytometry analysis of mitochondrial membrane potential. (B) $\Delta\Psi m$ estimated from flow cytometry analysis was expressed in arbitrary units. (C) ATP content was measured using a luciferin-luciferase assay in cells treated with NMDA for 3 hr in the presence and absence of RSV. (D) ROS production was measured by flow cytometry analysis of DCF fluorescence in cells treated with NMDA for 3 hr in the presence and absence of RSV. Data were represented as the mean ± standard error of the mean of 5 repeats. $^*p<0.01$ vs. respective control; $^\#p<0.01$ vs. NMDA alone. ROS, reactive oxygen species; RSV, resveratrol; $\Delta\Psi m$, mitochondrial membrane potential; DCF, 2',7'-dichlorofluorescein.

To further elucidate the effects of NMDA and resveratrol on mitochondrial functional integrity, we examined mitochondrial membrane potential, cellular ATP levels and ROS production as indicators of mitochondrial functional integrity. Fig. 2A shows representative flow cytometry analysis of DiOC$_6$(3)-stained cells. In these graphs, the left shift of events from strong (designated as M2) to weak (designated as M1) DiOC$_6$(3) fluorescence region indicates the disruption or depolarization of mitochondrial membrane potential. NMDA-treated cells exhibited marked depolarization of mitochondrial membrane potential. Resveratrol significantly suppressed the NMDA-induced depolarization of mitochondrial membrane potential (Fig. 2A, Fig. 2B).

ATP is produced primarily by mitochondria through oxidative phosphorylation, and therefore the intracellular ATP levels are a reliable indicator of mitochondrial functional integrity. There was a significant reduction in intracellular ATP content in NMDA-treated cells. Resveratrol significantly reversed the NMDA-induced reduction of intracellular ATP content (Fig. 2C).

Oxidative stress is known to be implicated in NMDA-induced neurotoxicity. Thus to examine whether the protective effects of resveratrol against NMDA-induced mitochondrial dysfunction was related to its effect on oxidative stress, the changes in ROS production in NMDA-treated cells in the presence and absence of resveratrol were assessed. In cells treated with NMDA, there was a significant increase in ROS production, and this was significantly suppressed in the presence of resveratrol (Fig. 2D).

NMDA-induced MPT and the protective effects of resveratrol

As shown in the representative confocal micrographs of cells that were double stained with calcein/AM and TMRM in Fig 3A, control cells exhibited intact mitochondria that could be discriminated by punctuated bright red spots as a consequence of TMR accumulation due to their negative membrane potential. In cells where formation of MPT pores was progressing, the mitochondria eventually lost TMR and became permeable to calcein, and as a consequence, lost their visible contours. In NMDA-treated cell preparations, there was a significant increase in the number of cells showing MPT, and this was significantly suppressed when treated with resveratrol (Fig. 3B).

NMDA-induced apoptosis and the protective effects of resveratrol
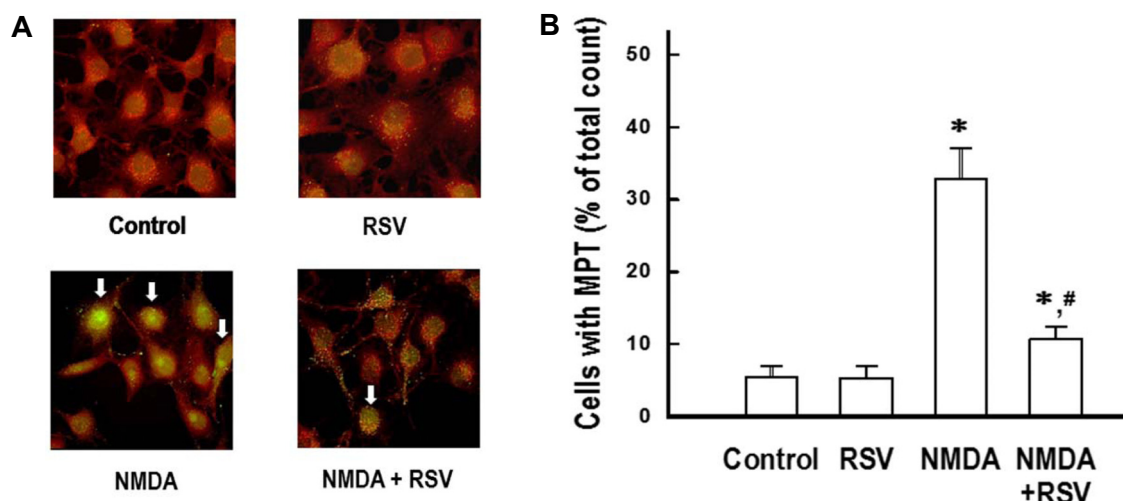
As shown in the representative micrographs of TUNEL-

Fig. 3. NMDA-induced MPT and the protective effects of RSV. After treatment of cells with 100 mM NMDA for 3 hr in the presence or absence of 20 μM RSV, a double staining method with fluorescent dyes, calcein/AM and TMRM was used to analyze the formation of MPT pores. (A) Representative confocal microscopic images showing cells with intact mitochondria and cells with MPT in progress. The control cells exhibited intact mitochondria that could be discerned by the punctuated bright red spots, indicative of TMR accumulation. Conversely, in the cells with MPT in progress, as indicated by arrows, mitochondria came to lose TMR and became permeable to calcein, and as a consequence, lost their visible contours. (B) Cells with MPT in progress were counted and expressed as a percentage of total cell count. Data are presented as the mean ± standard error of the mean of 4 repeats. $^*p<0.01$ vs. control; $^\#p<0.01$ vs. NMDA alone. MPT, mitochondrial permeability transition; RSV, resveratrol; TMRM, tetramethylrhodamine methyl ester; TMR, tetramethylrhodamine.

stained cells (Fig. 4A) and the quantification of the staining (Fig. 4B), there was marked increase in the number of apoptotic cells (4.8%±1.2% to 41.3%±6.4% of the total cell population) in the NMDA-treated cells, and this was significantly suppressed by resveratrol (12.1%±1.7%).

Resveratrol-stimulates expression of HO-1

The effect of resveratrol on HO-1 expression was next assessed. RT-qPCR and Western blot analyses showed that resveratrol significantly increased the mRNA (Fig. 5A) and protein expression levels (Fig. 5B, Fig. 5C). The effect of re-
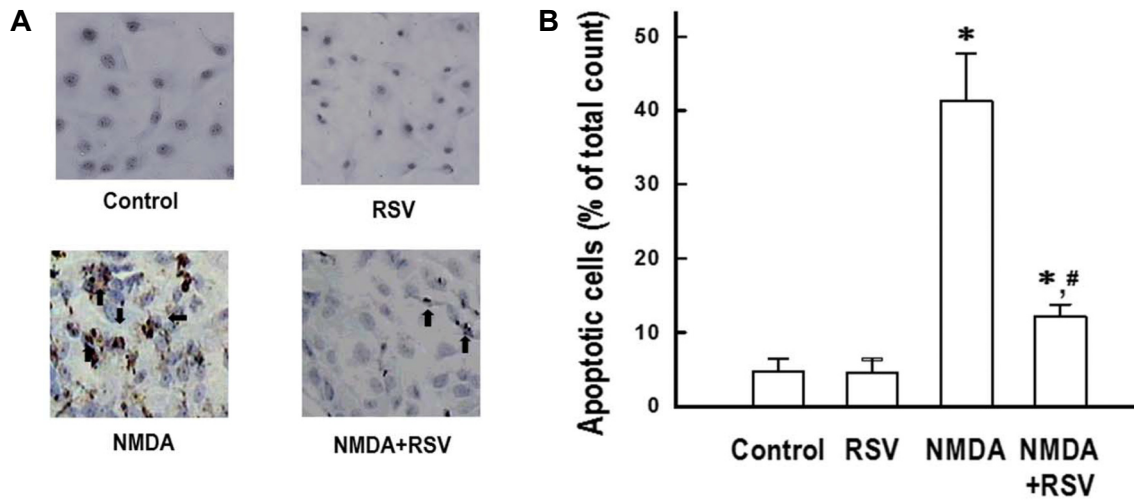

Fig. 4. NMDA-induced apoptosis and the protective effects of RSV. After treatment of cells with 100 mM NMDA for 3 hr in the presence or absence of 20 μM RSV, a TUNEL assay was performed to detect apoptotic cells. (A) Representative micrographs of TUNEL-stained cell preparations. Arrows indicate TUNEL-positive cells. (B) Quantitative analysis of TUNEL-positive cells. Data are presented as the mean ± the standard error of the mean of 5 experiments. $^*p<0.01$ vs. control; $^\#p<0.01$ vs. NMDA alone. RSV, resveratrol.
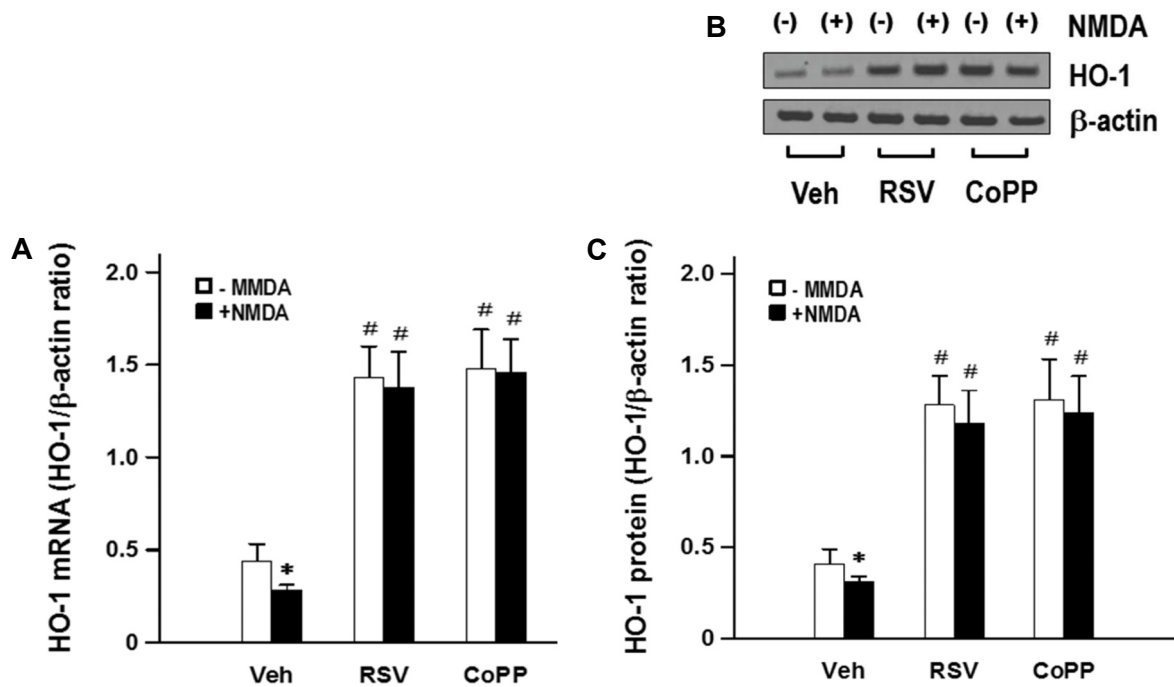

Fig. 5. RSV increases HO-1 expression. After treatment of cells with 20 μM RSV or 20 μM CoPP for 3 hr in the presence or absence of 100 mM NMDA, the expression of HO-1 was determined. (A) mRNA and (B and C) protein expression levels of HO-1 were determined. Data are presented as the mean ± the standard error of the mean of 5 experiments. $^*p<0.01$ vs. without NMDA; $^\#p<0.01$ vs. Veh. HO-1, heme-oxygenase-1; CoPP, cobalt protoporphyrin; RSV, resveratrol.

sveratrol in increasing HO-1 expression was comparable to that of the HO-1 inducer CoPP. NMDA alone exhibited some inhibitory effect on HO-1 expression, but the effect did not sufficiently alter the effects of resveratrol-induced stimulation (Fig. 5A - Fig. 5C).

Effects of HO-1 related molecules

To elucidate whether the protective effects of resveratrol resulted from the increased expression of HO-1, the effects of HO-1-related molecules on the NMDA-induced changes in MTT reduction and MPT were examined. A well-known inducer of HO-1, CoPP [18] and the metabolic product of HO-1, bilirubin [18] restored NMDA-induced impairment in MTT reduction ability. Conversely, a HO-1 inhibitor ZnPP IX [18] significantly suppressed the effect of the resveratrol (Fig. 6A). Similar results were obtained from experiments to examine the effects of HO-1-related molecules on NMDA-induced MPT (Fig. 6B). These results strongly suggest that the resveratrol-induced protective effects are closely associated with the increased expression of HO-1.

Effect of knockdown of HO-1

In order to further confirm the role of HO-1 in the protective effects of resveratrol against NMDA-induced neurotoxicity, cells were transfected with siRNA targeting HO-1. In cells transfected with siRNA, resveratrol did not significantly alter HO-1 expression at the mRNA (Fig. 7A) or protein level (Fig. 7B, Fig. 7C). Similarly, in the siRNA-transfected cells, resveratrol failed to restore the NMDA-induced changes in MTT reduction ability (Fig. 8A) and MPT formation (Fig. 8B). These results further confirmed that the increased expression of HO-1 was a crucial event underlying the protective effects of resveratrol.

Discussion

Over-activation of glutamate receptors leads to neuronal cell injury, commonly referred to as glutamate excitotoxicity [26]. An increasing number of studies have suggested that a large of Ca^{2+} influx through the NMDA receptor channel serves a crucial role in initiating glutamate excitotoxicity [36]. In the present study, HT-22 cells treated with NMDA in Mg^{2+}-free Locke's buffer were used as the experimental model of glutamate excitotoxicity. In these cells, NMDA induced cell injury in a dose- and time-dependent manner as evidenced by using an MTT assay. In the presence of Mg^{2+} or the NMDA receptor blocker MK-801 [30] NMDA-induced cell injury was significantly attenuated or abolished, suggesting that NMDA targets cells via the opening of the NMDA receptor channels.

A variety of neurodegenerative diseases have been shown to be associated with altered mitochondrial structure and function [3]. The disruption of inner mitochondrial membrane potential and formation of MPT pores is known to
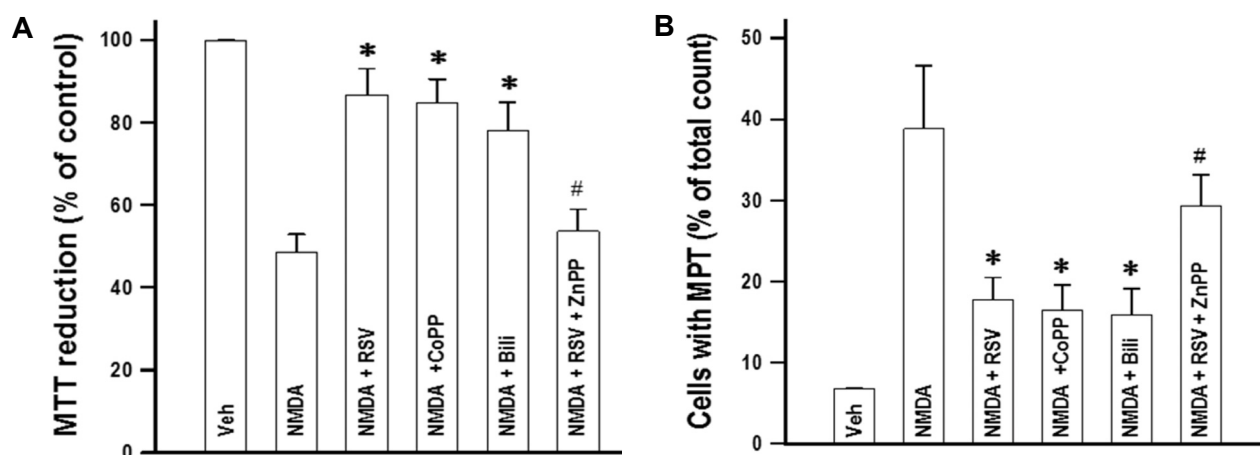

Fig. 6. Effects of HO-1 related molecules on the NMDA-induced changes in MTT reduction ability and MPT pore formation. Cells were treated with 100 mM NMDA for 3 hr in the presence of 20 μM RSV, CoPP, Bili or ZnPP. (A) MTT reduction ability was determined by colorimetric analysis of formazan formation. (B) A double staining method with fluorescent dyes, calcein/AM and TMRM was used to analyze the formation of MPT pores. Data are presented as the mean ± the standard error of the mean of 5 experiments. $^*p<0.01$ vs. NMDA alone; $^\#p<0.01$ vs. NMDA+RSV. HO-1 heme-oxygenase-1; RSV, resveratrol; CoPP, cobalt protoporphyrin; Bili, bilirubin; ZnPP, zinc protoporphyrin IX; MPT, mitochondrial permeability transition; TMRM, tetramethylrhodamine methyl ester.
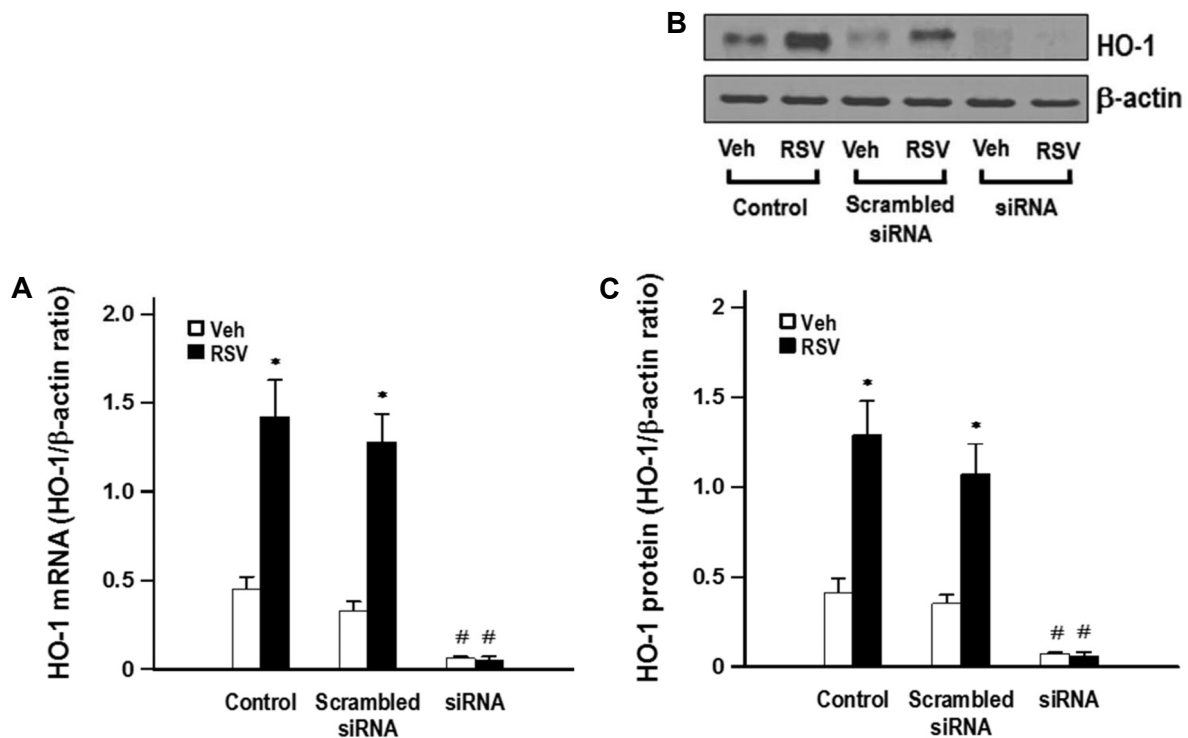
Fig. 7. Effect of siRNA transfection on HO-1 expression. Cells were first transfected with 30 pmol HO-1 siRNA or scrambled siRNA, and then treated with 20 μM RSV or CoPP. (A) mRNA and (B and C) protein expression levels of HO-1 were determined. Data are presented as the mean ± the standard error of the mean of 5 experiments. $^*p<0.01$ vs. Veh $^#p<0.01$ vs. scrambled siRNA. siRNA, small interfering RNA; HO-1 heme-oxygenase-1; CoPP, cobalt protoporphyrin; RSV, resveratrol; Veh, vehicle.
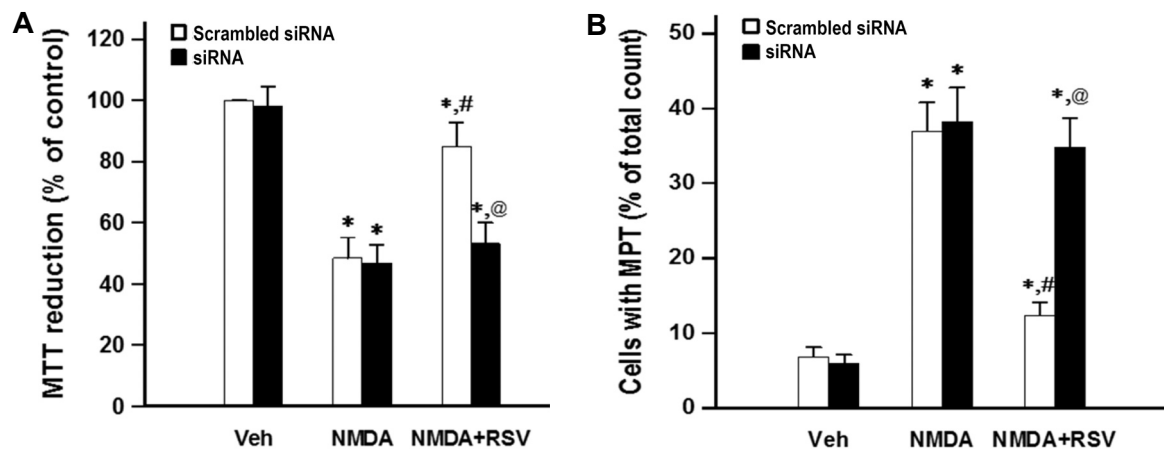

Fig. 8. Suppression of the RSV-induced protective effects in the si-HO-1 transfected cells. Cells were first transfected with 30 pmol HO-1 siRNA or scrambled siRNA and then treated with 100 mM NMDA for 3 hr in the presence and absence of 20 μM RSV. (A) MTT reduction ability was determined using colorimetric analysis of formazan formation. (B) A double staining method with fluorescent dyes, calcein/AM and TMRM was used to analyze the formation of MPT pores. Data are presented as the mean ± the standard error of the mean of 5 experiments. $^*p<0.01$ vs. Veh; $^#p<0.01$ vs. NMDA alone; $^@p<0.01$ vs. scrambled siRNA control. MPT, mitochondrial permeability transition; RSV, resveratrol; Veh, vehicle; HO-1 heme-oxygenase-1; TMRM, tetramethylrhodamine methyl ester; siRNA, small interfering RNA.

be a major event preceding mitochondria-dependent apoptotic signaling. The involvement of mitochondrial dysfunction in the mechanism of cell damage has also been suggested in glutamate excitotoxicity [2, 20]. The present study provided strong evidence that alterations in structural and functional mitochondrial integrity were closely associated

with cell injury in NMDA-induced excitotoxicity. NMDA treatment resulted in deterioration of mitochondrial membrane potential, suppressed cellular ATP production and stimulated intracellular ROS generation. Double fluorescence imaging studies with TMRM and calcein/AM demonstrated increased formation of MPT pores. These findings, taken together, strongly suggest that mitochondrial dysfunction may be a crucial event underlying NMDA-induced neuronal injury in HT-22 cells.

Resveratrol is one of the most extensively studied natural polyphenols due to its potential beneficial properties. However, the current body of literature from clinical and *in vitro* studies concerning the protective effects of resveratrol against diseases are still contested, and the reasoning behind the conflicting results remain to be elucidated. However, differences in the characteristics of the cells tested or enrolled patients, and the doses and duration of the resveratrol treatment have been proposed as possible explanations [28]. Several investigators have reported that resveratrol at high doses (100 mM) can be cytotoxic for cancer cells [14, 15, 30]. In our previous study, it was shown that resveratrol exhibited a cytotoxic effect on ovarian cancer cells [9]. Conversely, at lower concentrations, resveratrol may exhibit provide a protective effects. Several studies have shown that resveratrol has a neuroprotective effect against oxidative injuries [6, 12]. In the present study, resveratrol was shown to exhibit a protective effect against NMDA-induced injury. Resveratrol facilitated the reductive abilities of mitochondria of MTT and to produce ATP (Fig. 2C) in NMDA-treated cells. In addition, resveratrol prevented the disruption of mitochondrial membrane potential and suppressed intracellular ROS generation in NMDA-treated cells. It also suppressed NMDA-induced formation of MPT pores, which was accompanied by apoptotic cell death. These results strongly suggest that resveratrol provides a beneficial effect to protect mitochondria against NMDA-induced injuries.

Enhanced HO-1 expression has been shown to be a potential tool to protect neurons against oxidative injuries [4]. HO-1 produces carbon monoxide and bilirubin as products of heme metabolism, and bilirubin acts as a strong antioxidant [22]. Since the generation of ROS is known to serve an important role in a variety of neurodegenerative diseases, increased bilirubin production may prove helpful to suppress oxidative injuries. In the present study, it was suggested that HO-1 was significantly involved in resveratrol-induced protection against NMDA-induced mitochon-

drial injuries. CoPP, a HO-1 inducer [19] and bilirubin, the metabolic product of HO-1 [19] showed a protective effect against NMDA-induced mitochondrial injury. On the other hand, in cells treated with the HO-1 inhibitor ZnPP IX [19], the protective effects of resveratrol were significantly attenuated. In RT-qPCR and Western blot analyses, resveratrol was shown to increase HO-1 mRNA and protein expression levels. Knockdown of HO-1 provided further support of its involvement in the underlying mechanism of resveratrol; following knockdown of HO-1, resveratrol failed to suppress the NMDA-induced effects on MTT reduction ability and MPT pore formation. These results further confirmed that enhanced expression of HO-1 is crucially related to the protective effects of resveratrol.

In summary, the present study showed that resveratrol may exhibit beneficial effects to prevent NMDA-induced mitochondrial injuries in HT-22 neuronal cells, and the enhanced expression of HO-1 is involved in the underlying cellular mechanism.

Acknowledgement

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : NMDA를 처리한 HT-22 신경세포에서 미토콘드리아 손상을 완화하는 레스베라트롤의 보호 효과와 헴 산화효소-1의 역할

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뇌 등 신경 조직에서 흥분성 신경전달물질의 일종인 글루탐산(glutamate)에 의해 유도되는 신경세포 독성에 N-methyl-D-aspartate (NMDA) 수용체가 중요하게 관여함은 잘 알려져 있다. 레스베라트롤(resveratrol)은 자연 식품에서 얻어지는 가장 잘 알려진 폴리페놀(polyphenol)의 일종으로 글루탐산에 의해 유도되는 신경세포 독성을 완화하는 효과가 있는 것으로 보고되었으나 그 기전은 명확히 밝혀져 있지 않다. 본 연구에서는 NMDA를 처리한 HT-22 신경세포를 신경세포 독성 모델로 이용하여 미토콘드리아 손상에 미치는 레스베라트롤의 보호 효과와 그 기전을 연구하고자 하였다. NMDA를 처리한 HT-22 신경세포에서 MTT 환원능의 감소와 미토콘드리아 막전위의 소실, 세포 내 ATP 농도의 감소, 활성산소종 생성의 증가, 미토콘드리아 막 투과성의 증가(mitochondrial permeability transition) 등 미토콘드리아의 기능적, 형태학적 손상을 시사하는 지표 변화들이 관찰되었다. 또한 미토콘드리아 손상의 결과로 세포사멸(apoptosis)이 증가함도 확인하였다. 레스베라트롤은 NMDA에 의한 미토콘드리아 손상과 세포사멸을 현저히 방지하는 보호 효과를 보였다. 헴 산화효소-1(heme oxygenase-1) 활성 억제제인 아연 프로토포르피린-9(zinc protoporphyrin IX)을 전처리한 세포에서는 레스베라트롤의 보호 효과가 현저히 약화되었으며, 반면에 heme oxygenase-1 활성 촉진제인 코발트 프로토포르피린(cobalt protoporphyrin)과 빌리루빈(bilirubin)은 레스베라트롤과 유사한 보호 효과를 나타내었다. 실시간 정량중합효소연쇄반응(RT-qPCR) 검사와, 웨스턴 블롯(Western blot) 검사로 확인한 결과 레스베라트롤은 헴 산화효소-1의 mRNA와 단백 발현을 증가시킴을 확인할 수 있었다. 짧은 간섭 RNA (small interfering RNA)를 형질주입(transfection)하여 헴 산화효소-1의 발현을 일시적으로 차단(knock down)한 세포에서는 레스베라트롤의 보호 효과가 관찰되지 않았다. 이상의 결과를 종합하면 레스베라트롤은 NMDA를 처리한 HT-22 신경세포에서 미토콘드리아의 기능적, 형태학적 손상을 완화하여 신경세포 독성에 대한 보호 효과를 나타내며 그 기전에는 헴 산화효소-1의 발현 증가가 중요하게 작용함을 시사한다.