

Cytochrome b5 reductase, a plasma membrane redox enzyme, protects neuronal cells against metabolic and oxidative stress through maintaining redox state and bioenergetics

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Abstract The plasma membrane redox system (PMRS) containing NADH-dependent reductases is known to be involved in the maintenance of redox state and bioenergetics. Neuronal cells are very vulnerable to oxidative stress and altered energy metabolism linked to mitochondrial dysfunction. However, the role of the PMRS in these pathways is far from clear. In this study, in order to investigate how cytochrome b5 reductase (b5R), one of the PM redox enzymes, regulates cellular response under stressed conditions, human neuroblastoma cells transfected with b5R were used for viability and mitochondrial functional assays. Cells transfected with b5R exhibited significantly higher levels of the NAD^+/NADH ratio, consistent with increased levels of b5R activity. Overexpression of b5R made cells more resistant to H_2O_2 (oxidative stress), 2-deoxyglucose (metabolic stress), rotenone and antimycin A (energetic stress), and lactacystin (proteotoxic stress), but did not protect cells against H_2O_2 and serum withdrawal. Overexpression of b5R induced higher mitochondrial functions such as ATP production rate, oxygen consumption rate, and activities of complexes I and II, without formation of further reactive oxygen species, consistent with lower levels of oxidative/nitrative

damage and resistance to apoptotic cell death. In conclusion, higher NAD^+/NADH ratio and consequent more efficient mitochondrial functions are induced by the PMRS, enabling them to maintain redox state and energy metabolism under conditions of some energetic stresses. This suggests that b5R can be a target for therapeutic intervention for aging and neurodegenerative diseases.

Keywords Aging · Cytochrome b5 reductase · PMRS · NAD^+/NADH · Neuroprotection

Abbreviations

AD	Alzheimer's disease
ANOVA	Analysis of variance
b5R	Cytochrome b5 reductase
CoQ	Coenzyme Q
DCIP	Dichloroindolphenol
DMEM	Dulbecco's modified Eagle's medium
NAD	Nicotinamide adenine dinucleotide
NQO1	NADH-quinone oxidoreductase 1
PD	Parkinson's disease
PMRS	Plasma membrane redox system
ROS	Reactive oxygen species
UPS	Ubiquitin-proteasome system

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Introduction

Aging is a complex and irreversible degeneration process and associated with a wide range of age-related

diseases including neurodegenerative disorders, cardiovascular diseases, and cancer (Blasco 2005). Aging causes changes in antioxidant capacity followed by impairment of the mitochondrial function and oxidative damage. In fact, oxidative stress and mitochondrial dysfunction are known to be two of the causes of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Beal 2004, 2005; Jenner 2003).

The mitochondria are a main microorganelle, which produces ATP. However, a major portion of free radicals is produced inevitably during oxidative phosphorylation (Mattson et al. 2008; Murphy 2009). Mitochondrial dysfunction and shortage of ATP supply are commonly identified as early events in normal aging process and in many age-related diseases (Kim et al. 2008; Luft and Landau 1995). The brain requires a large amount of glucose and oxygen to produce ATP. Neuronal cells are very vulnerable to oxidative and energetic stresses (e.g., glucose limitation, drastic energy demand, etc.) because the brain has relatively low antioxidant capacity compared with other tissues such as the liver (Mattson et al. 2008; Sims 1992). A variety of neurodegenerative diseases such as Alzheimer's disease (AD) (Mattson 2004; Mosconi et al. 2008), Parkinson's disease (PD) (Ferrer 2009), and Huntington's diseases (Browne 2008) are known to occur by metabolic alteration and oxidative stress during the aging process. The mechanisms by which neurons survive under conditions of metabolic and oxidative stress can be a therapeutic target for aging intervention and prevention of neurodegenerative diseases.

When ATP supply by mitochondrial respiration is not sufficient, cells can survive through stimulation of alternative pathways such as glycolysis, fermentation, and plasma membrane redox system (PMRS) if they are supplemented with pyruvate and uridine (Miller et al. 1996). In fact, the plasma membrane has many cellular functions such as maintenance of cell homeostasis, cell signaling, and protection of cells against oxidative stress. The PM exhibits a compensatory mechanism in response to mitochondrial dysfunction via the PMRS, which is also involved in electron transport and energy metabolism (Hyun et al. 2006b; Villalba and Navas 2000). Previously, we reported that ρ^0 cells without functional mitochondria can survive and are more resistant to oxidative stress via enhanced activities of PMRS redox enzymes (Hyun et al. 2007). The PMRS contains NAD(P)H-dependent redox enzymes including cytochrome b5 reductase (b5R) and NAD(P)H-quinone

oxidoreductase 1 (NQO1), electron shuttles such as coenzyme Q (CoQ) and cytochrome c, and electron donors (e.g., NAD(P)H) (Hyun et al. 2006b). PM redox enzymes convert an oxidized form of CoQ to a reduced state by transferring electrons from NAD(P)H.

b5R is a monomeric flavoprotein of 32 kDa NADH-dependent reductase, which is noncovalently bound to FAD and located on the inner surface of the PM (Bewley et al. 2001; Rao et al. 1992; Sparla et al. 1997). A reduced form of CoQ is a lipid-soluble antioxidant molecule, which protects membrane lipids from oxidative damage by scavenging superoxide or other radicals alone or in association with α -tocopherol (Crane 2001; Turunen et al. 2004). The PMRS can maintain metabolic balance through modulating the NAD⁺/NADH ratio in response to metabolic fluctuation. In fact, the PMRS plays a key role in the regulation of pyrimidine nucleotide balance, making cells survive, grow, and differentiate under various physiological conditions (Buron et al. 1993; De Luca et al. 2005; Medina et al. 1997). For example, NADH-coenzyme Q reductase 1 (NQR1) in yeast, a homologue of NQO1, is coupled to the transition from fermentation to respiration, resulting in the extension of life span (Jimenez-Hidalgo et al. 2009). b5R can play a dual role in the regulation of oxidative and metabolic stresses through interaction between forkhead box class O 3a (FOXO3a) and nuclear factor (erythroid-derived 2)-like2 (Nrf2) (Siendones et al. 2014).

b5R is suggested to use plant polyphenols (e.g., quercetin, catechin, resveratrol) as well as NADH as an electron donor (Kesharwani et al. 2012). Although b5R can be a more potent therapeutic target for validating bioactive phytochemicals, the mechanisms of b5R responsible for aging intervention and neuroprotection are yet to be investigated. Other studies showed that PM-bound b5R is a component of protein complexes tightly associated with lipid raft microdomains of neuronal PM (Marques-da-Silva et al. 2010; Samhan-Arias et al. 2009). b5R is stimulated and clustered in the lipid microdomain enriched with caveolin in cerebellar granule neurons at an early stage of apoptosis induced by oxidative stress (Samhan-Arias et al. 2012). Interestingly, in aged mice, b5R activated by calorie restriction (CR) and aerobic exercise can attenuate oxidative damage (Rodriguez-Bies et al. 2015). These findings suggest a potentially essential role of b5R in cellular adaptation in response to metabolic alteration and oxidative stress known to occur in neurodegenerative diseases.

In this study, we used human neuroblastoma cells transfected with b5R to investigate how b5R protects neuronal cells. We found that elevated levels of b5R make cells more resistant to metabolic and oxidative stress. Cells overexpressing b5R maintained a higher NAD⁺/NADH ratio and enhanced mitochondrial functions without further production of reactive oxygen species (ROS), suggesting that this PMRS enzyme improves cellular bioenergetics.

Methods

Cell culture, transfection, and differentiation

SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (Invitrogen, Carlsbad, CA), 100 IU/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen) in a humidified 5 % CO₂/95 % air atmosphere. The cells were transfected with pCB6 vector containing the full-length b5R, as described previously (Hyun et al. 2010). Six different clones were selected using G-418. In order to induce cell differentiation, cells were treated with 10 µM retinoic acid (RA) and cultured in the DMEM containing 3 % FBS for 3 days.

Characterization of the transfected cells

Cells were lysed and levels of cytochrome b5 reductase proteins were determined by immunoblot analysis using a b5R monoclonal antibody, as described previously (Hyun et al. 2010; Seow et al. 2005). Levels of b5R proteins in the PM of the transfected cells were measured following isolation of the PMs by a two-phase partition, as described previously (De Cabo et al. 2004; Hyun et al. 2010).

Isolation of the plasma membranes

The PM isolation procedure was carried out using the two-phase partition, as described previously (De Cabo et al. 2004; Navas et al. 1989). Cells were homogenized in the ice-cold homogenization buffer with 37 mM Tris-maleate, 10 mg/ml dextran T-500, 50 mM magnesium chloride, 0.5 M sucrose, 28 mM sodium hydroxide, 1 mM ethylene diamine trichloroacetic acid (EDTA), 1 mM phenylmethyl sulfonyl fluoride (PMSF), and

5 mM dithiothreitol (DTT) (pH 6.4) using a Dounce homogenizer (Golgi grind). This homogenate was centrifuged at 5000g for 15 min. Then, one half of the pellet produced was resuspended in 1 mM sodium bicarbonate, homogenized in a Teflon glass tissue homogenizer, and centrifuged at 5000g for 15 min. The light brown, top portion of the pellet was combined with a mixture of 6.6 % (w/w) dextran and 6.6 % (w/w) polyethylene glycol in 0.1 M sucrose and 5 mM potassium phosphate (pH 7.2). The mixture was inverted vigorously 40 times at 4 °C and the two phases were separated by centrifugation at 550g for 3 min. The upper phase was then diluted with 1 mM sodium bicarbonate (pH 7.2) and centrifuged at 20,000g for 30 min. The PM fractions (pellets) were resuspended in the suspension buffer with 25 mM Tris, 10 % glycerol, 0.1 mM DTT, 1 mM EDTA, and 1 mM PMSF (pH 7.6).

Protein concentration in the PM fraction was determined by the Bradford assay (Bradford 1976). Immunoblotting assay using enzyme markers for PM and mitochondria was performed to measure the purity of the isolated fractions as described previously (De Cabo et al. 2004). For immunoblotting, anti-Na⁺/K⁺-ATPase α -subunit monoclonal antibody (1:1000 dilution, Affinity BioReagents, Golden, CO) and anticytochrome c oxidase subunit I monoclonal antibody (1:1000 dilution, Molecular Probe, Eugene, OR) were used.

Activity of b5R

The activity of NADH-ascorbate free radical (NADH-AFR) reductase (EC 1.6.5.4), which was mainly due to b5R, was determined using a spectrophotometer, as described previously (De Cabo et al. 2004). A decrease in absorbance at 340 nm was read following the addition of 66×10^{-3} units of ascorbate oxidase to the assay buffer (50 mM Tris-HCl buffer, 0.2 mM NADH, and 0.4 mM fresh ascorbate, pH 7.6) and 50 µg of PM. The extinction coefficient used to calculate specific activity was $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

Levels of metabolites

NAD⁺ and NADH levels were determined separately using a NAD/NADH Quantitation Kit (BioVision, Mountain View, CA). Briefly, in order to measure the total levels of NAD and NADH, lysates were transferred into a 96-well plate, and 100 µl NAD cycling buffer and

2 μ l NAD cycling enzyme mix were added. The mixtures were incubated at room temperature for 5 min to convert NAD to NADH, and NADH developer was then added to each well. The plate was incubated for 2 h and absorbance was read at 450 nm. In order to measure NADH, NAD was decomposed by heating the lysates at 60 °C for 30 min, followed by the same procedures described above. A standard curve was generated by serial dilution of standard NADH solution, and the NAD^+/NADH ratio was calculated as $[(\text{NAD}_{\text{total}} - \text{NADH})/\text{NADH}]$.

Cell viability assay

Cells were exposed to a normal culture medium containing 2-deoxyglucose (5, 10, 15, 20 mM) (Sigma, St. Louis, MO), rotenone (50, 100, 150, 200 μ M) (Sigma), antimycin A (50, 100, 150, 200 μ M) (Sigma), and lactacystin (10, 20, 30, 40 mM) (Sigma), or to a serum-free medium, for 24 h. In the case of H_2O_2 treatment, the cells were exposed to hydrogen peroxide (50, 100, 150, 200 μ M) in the absence of serum for 1 h and then incubated with normal culture medium for 24 h. Cell viability was measured using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), and intensity was read at 450 nm and by the trypan blue exclusion.

Isolation of the mitochondria and confirmation of the purity of the mitochondrial fractions

Mitochondrial fractions were isolated from the cells by centrifugation and kept at 4 °C, as described previously with minor modifications (Shim et al. 2011). Briefly, cells were washed with ice-cold PBS and homogenized in 10 mM Tris buffer (pH 7.6) with a protease inhibitor cocktail (1.5 mM) (Sigma). The homogenates were centrifuged at 600g for 10 min at 4 °C, and then the supernatants were centrifuged again at 14,000g for 10 min at 4 °C. The resulting pellets were carefully removed and resuspended in the assay buffer.

Immunoblot assays using enzyme markers for the cytosol and mitochondria were performed to establish the relative purity of the isolated mitochondrial fractions using antibodies against peroxiredoxin II (cytosol marker, 1:2000, AbFrontier, South Korea), peroxiredoxin III (mitochondrial marker, 1:2000, AbFrontier), and complex I (1:2000, Invitrogen). Briefly, the isolated mitochondrial fractions were lysed in PBS (pH 7.4)

containing 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin A, and 0.1 % Triton X-100 and placed on ice for 5 min. The lysates were centrifuged at 12,000g for 10 min, and the supernatants were transferred into new Eppendorf tubes. Protein levels were measured using the Bradford reagent (Bradford 1976), and 10 μ g of protein was separated by SDS-PAGE, as described previously (Hyun et al. 2006a, 2007). The separated proteins were transferred electrophoretically to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany), which was then incubated with the primary antibodies. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (1:5000, AbFrontier).

ATP production rate

Mitochondria were isolated from the cells and the ATP production rate (APR) was determined by the luciferase assay, as previously described, with minor modifications (Vives-Bauza et al. 2007). Isolated mitochondria (10 μ g) were suspended in 200 μ l of reaction buffer A (0.1 % BSA, 150 mM KCl, 0.1 mM MgCl_2 , 25 mM Tris-HCl, 10 mM potassium phosphate, pH 7.4) containing 160 μ M diadenosine pentaphosphate, 1 mM pyruvate, 100 μ M ADP, and either 5 mM NADH or 5 mM glutamate/malate or 5 mM succinate. Then, 10 μ l of buffer B (500 mM Tris-acetate, pH 7.75) containing 20 μ g/ml luciferase (Invitrogen) and 0.8 mM D-luciferin (Invitrogen) was added to the reaction mixture. The light emission was monitored using a luminometer (20/20ⁿ, Turner Biosystems, Sunnyvale, CA) for 5 min at 10 s intervals. A standard curve for luminescence was made by using solutions containing increasing concentrations of ATP (Sigma).

Oxygen consumption rate

Oxygen consumption rate (OCR) of living cells was assessed using an Instech Two Channel Fiber Optic Oxygen Monitor System (Instech Laboratories, Inc., Plymouth Meeting, PA, USA), as described previously (Hyun et al. 2007). Briefly, cell chambers were maintained at 37 °C in a circulating water bath. Cells were trypsinized and centrifuged at 1500g for 2 min at 4 °C. Cells were then resuspended in DMEM. Respiration rates were determined in the presence of 5 mM NADH, 5 mM glutamate/malate, or 5 mM succinate,

and the results were calculated as nanomoles of oxygen consumed per minute per milligram of protein. Activated OCR was measured following the addition of ADP. All measurements were assessed with Fiber Optic Oxygen Monitor Operating Software and Slope Calculator Software (Instech Laboratories). Resting respiration rate was determined as the OCR under conditions where no inhibitors or uncouplers were administered to the mitochondria. Nonmitochondrial oxygen consumption was determined as the respiration rate following incubation of cells with 100 mM potassium cyanide (KCN) (Fluka BioChemika, Bruchs, Germany).

Activities of complexes I and II

Activities of mitochondrial complexes I and II were assessed using decylubiquinone and dichloroindolphenol (DCIP), as described earlier (Janssen et al. 2007). Briefly, 10 µg of the isolated mitochondrial fractions was preincubated in the reaction buffer for complex I (70 µM decylubiquinone, 60 µM DCIP, 1 µM antimycin A, 0.35 % BSA, 25 mM potassium phosphate, pH 7.4) or complex II (70 µM decylubiquinone, 60 µM DCIP, 1 µM antimycin A, 50 µM rotenone, 500 µM EDTA, 200 µM ATP, 0.1 % BSA, 80 mM potassium phosphate, pH 7.4) at 37 °C. The reaction was initiated by the addition of substrates (complex I: 5 mM NADH or 5 mM glutamate/malate and complex II: 5 mM succinate and 300 µM potassium cyanide) to the mixture, and absorbance was read at 600 nm for 5 min with 20 s intervals.

Production of ROS

Mitochondrial fractions isolated freshly from isolated freshly were analyzed. Hydrogen peroxide (H₂O₂) released from the mitochondria was measured using the fluorescent dye Amplex Red (Molecular Probes, Eugene, OR) which reacts with H₂O₂ with a 1:1 stoichiometry in the presence of horseradish peroxidase and thereby produces the highly fluorescent chemical resorufin (Li and May 2002; Zhou et al. 1997). Briefly, a reaction buffer (50 mM Tris, pH 7.4) was supplemented with 5 µM Amplex Red, 0.5 U/ml HRP, and 20 U/ml Cu,Zn-superoxide dismutase to prevent the auto-oxidation of Amplex Red and interference with quantitative assessment of low rates of H₂O₂ production (Votyakova and Reynolds 2004). The

supplemented buffers were preincubated at 37 °C and mitochondrial fractions and electron donors (5 mM succinate or 5 mM glycerol 3-phosphate) were added to the reaction mixture. The detection of H₂O₂ in mitochondrial suspensions (0.1 mg protein/ml) was recorded as an increase in Amplex Red fluorescence using excitation and emission wavelengths of 560 and 590 nm, respectively. The response of Amplex Red to H₂O₂ was calibrated by incubation with known amounts of H₂O₂ solution from 100 to 1000 pmol. The concentration of commercial 30 % H₂O₂ solution (Sigma) was calculated from light absorbance at 240 nm using $E_{240\text{mM}}=43.6\text{ cm}^{-1}$, and the stock solution was diluted to 100 µM with water and used for calibration immediately.

Markers of oxidative/nitrative damage

Lipid peroxidation levels were assessed using the 8-Isoprostane Assay Kit (OxisResearch, Portland, OR). Briefly, following exposure to each of the mitochondrial toxins, cells were lysed and cell extracts (100 µl) were added to a 96-well plate and incubated with 100 µl horseradish peroxidase-conjugated antibody at room temperature for 1 h; a 200-µl substrate was added to the plate and it was incubated for 30 min. Absorbance was read at 450 nm after stopping the reaction by adding 50 µl 3 M sulfuric acid. Protein carbonyl content was determined as described previously (Lyras et al. 1997), except that the final cell extracts were dissolved in 1 ml of 6 M guanidinium hydrochloride (Sigma). Carbonyl content was calculated as nanomoles per milligram of protein (Reznick and Packer 1994). Measurement of protein-bound nitrotyrosine content of isolated plasma membranes was performed using the Nitrotyrosine Assay Kit (OxisResearch).

Apoptotic features

Following treatment with rotenone or antimycin A for 24 h, cell membrane damage and chromatin condensation were determined using propidium iodide or Hoechst 33258, as described previously (Kelner et al. 2000; Lee et al. 2001).

Statistical analysis

Statistical differences were analyzed by one- or two-way ANOVA test. Multiple comparisons were followed with post hoc Bonferroni *t* test where necessary.

Results

b5R is stably overexpressed in human neuroblastoma cells

Six different clones of SH-SY5Y cell lines stably overexpressing b5R were established, and the levels of b5R expression and enzymatic activity in these clones were examined. Among these clones, two expressed b5R at levels three- to fourfold greater than in vector-transfected control cell clones (Fig. 1). A clone with the highest levels of b5R expression and biochemical activity was used for further evaluation of the subcellular localization and functional activity of b5R in this study. Overexpressed b5R was present in both PM and cytosols, as confirmed by immunoblot analysis using a b5R antibody. Measurements of b5R enzyme activity also showed that b5R transfectants exhibited an approximately threefold

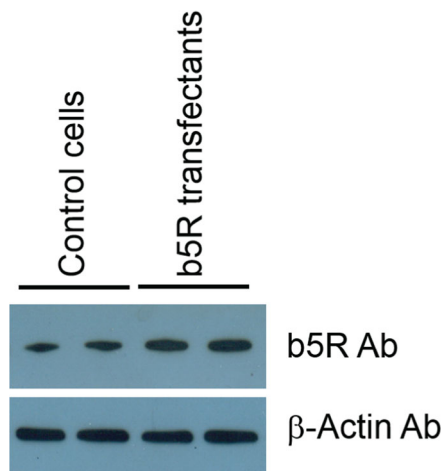


Fig. 1 Characterization of human neuroblastoma cells transfected with b5R. Control SH-SY5Y cells and the indicated clones of cells overexpressing b5R were cultured under normal culture conditions and lysed. Immunoblot analysis was performed using a b5R monoclonal antibody.

greater NQO1 activity compared with control cells, consistent with its expression level (Fig. 2a).

Overexpressed b5R increased the NAD⁺/NADH ratio

In order to assess the impact of b5R on cellular metabolic balance, the NAD⁺/NADH ratio was measured in neuroblastoma cells expressing basal or increased levels of b5R. The NAD⁺/NADH ratio was significantly elevated, by more than 800 %, in cells transfected with b5R compared with control cells (*p*<0.01) (Fig. 2b). However, the NAD⁺/NADH ratio in the mitochondria of b5R transfectants was decreased by about 50 %, compared with control cells.

Cells transfected with b5R protected cells against toxic insults

In order to investigate whether overexpressed b5R could make cell more resistant under stressed conditions, viability of the control and b5R transfectants was determined following exposure to six different toxic insults for 24 h: H₂O₂ (an oxidative stress inducer), 2-deoxyglucose (a glycolysis inhibitor), rotenone (a mitochondrial complex I inhibitor), antimycin A (a mitochondrial complex III inhibitor), lactacystin (a proteasome inhibitor), and serum-free medium (an apoptosis trigger).

In neuroblastoma cells with basal levels of b5R, cell viability was decreased following exposure to increasing concentrations of H₂O₂ (50–200 μM) (Fig. 3a). Overexpressed b5R tended to protect the cells against H₂O₂, but it was not significant. In control cells, cell viability was greatly reduced when treated with 2-deoxyglucose (10–20 mM), compared with normal culture conditions (Fig. 3b). However, overexpressed b5R made cells more resistant to being killed by 2-deoxyglucose. Cell viability was declined by the addition of rotenone in a dose-dependent manner with significant differences between control and cells overexpressing b5R (Fig. 3c). Antimycin A caused a decrease in cell viability in a dose-dependent manner, and cells transfected with b5R were less vulnerable to rotenone, compared with control cells (Fig. 3d). Treatment of control cells with lactacystin also induced cell viability loss, but it was attenuated by overexpression of b5R (Fig. 3e).

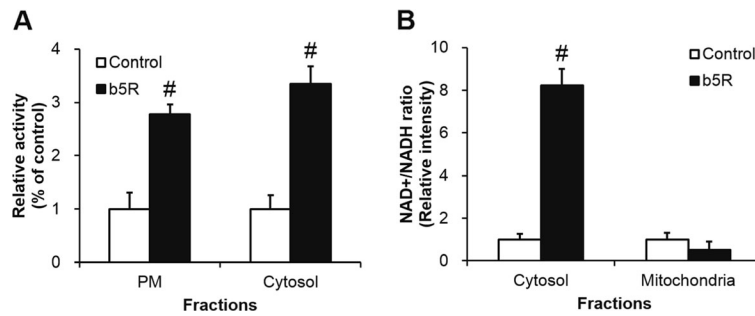


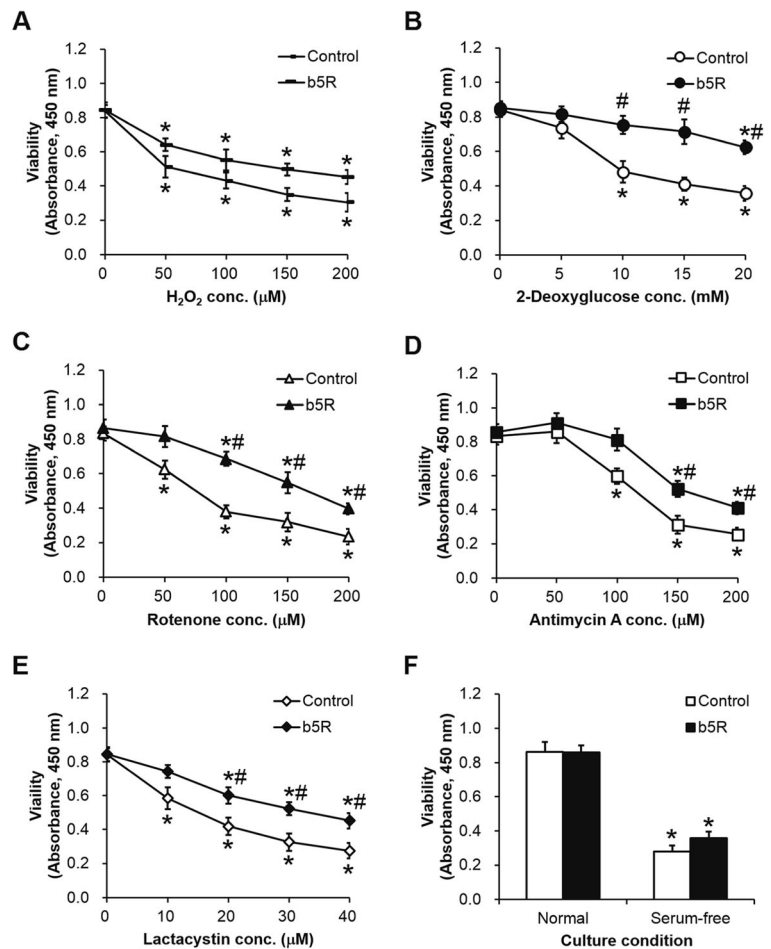
Fig. 2 Levels of b5R activity in the PMs and cytosols and cytoplasmic NAD⁺/NADH ratio are enhanced in cells overexpressing b5R. Plasma membranes (PMs) were isolated by a two-phase partition. **a** b5R activity was determined using menadione and cytochrome c as substrates. **b** Cells were lysed and the NAD⁺/

NADH ratio was measured using a NAD/NADH Quantitation Kit. Values are the mean and SEM ($n=6$). [#] $p < 0.01$ compared with the value between untransfected control cells and b5R transfectants in the same fractions

Finally, the vulnerability of SH-SY5Y cells to serum withdrawal, a way of inducing apoptosis (Pedersen et al. 2002), was not significantly different between

control cells and cells transfected with b5R (Fig. 3f). Following the addition of H₂O₂, rotenone, and antimycin A, cell viability assessed by the trypan blue

Fig. 3 Neuroblastoma cells overexpressing b5R exhibit reduced vulnerability to metabolic, oxidative, and proteotoxic insults, but are not protected against trophic factor withdrawal. Cells were exposed to H₂O₂ (**a**), 2-deoxyglucose (**b**), rotenone (**c**), antimycin A (**d**), lactacystin (**e**), or serum-free medium (**f**) for 24 h, and cell viability was measured using the WST-8 assay (see “Methods” for details). Values are the mean and SEM ($n=6$). * $p < 0.01$ compared with the value for untransfected control cells under normal culture conditions. [#] $p < 0.01$ compared with the value between untransfected control cells and b5R transfectants under the same culture conditions



exclusion (Supplementary Fig. 2A–C) also showed similarities to that assessed by the MTT assay.

Cells overexpressing b5R exhibited enhanced mitochondrial electron transport without further production of ROS

Previously, we published that mitochondrial activity is enhanced by NQO1, a PM redox enzyme (Kim et al. 2013), suggesting that mitochondrial function could be modulated by another PM enzyme, b5R. In order to verify the protective effects of b5R on cell viability and to examine the effects of b5R on bioenergetics, mitochondrial functions and ROS production were assessed in both control and b5R transfectants. Mitochondrial APR was determined by a luciferase assay. Mitochondrial APR was significantly increased by overexpression of b5R in the presence of NADH or glutamate/malate ($p < 0.01$) (Fig. 4a, b). APR tended to be elevated in cells transfected with b5R treated with succinate, but it was not significant (Fig. 4c). OCR was dramatically elevated in cells transfected with b5R when supplemented with NADH or glutamate/malate (Fig. 4d, e). The addition of succinate also showed increased OCR, but it was not significant (Fig. 4f).

In order to further elucidate the mechanism by which b5R influences mitochondrial electron transport in SH-SY5Y cells, activities of mitochondrial complexes I and II and ROS production were assessed. Mitochondrial complex I activity in cells overexpressing b5R was significantly higher than control cells ($p < 0.01$) (Fig. 5a, b). Cell differentiation induced a higher activity of the complex I activity (Supplementary Fig. 3B and C). Complex I activity was much higher in b5R transfectants. Complex II activity was also enhanced in b5R transfectants, compared with control cells ($p < 0.01$) (Fig. 5c). Although there was an increase in electron transport, ROS production (in the presence of NADH, glutamate/malate, or succinate) was slightly lower in b5R transfectants, compared with control cells (Fig. 5d–f).

Overexpressed b5R attenuated oxidative/nitrative damage

In order to investigate whether overexpressed b5R could protect cells against mitochondrial toxins, the levels of oxidative/nitrative damage were measured following

exposure to rotenone or antimycin A. The levels of protein carbonyls, a biomarker of protein oxidation, were slightly lower in cells transfected with b5R, compared with control cells, under normal culture conditions. The levels of protein carbonyls in control cells were greatly elevated following treatment with rotenone (Fig. 6a). These levels were significantly attenuated in cells overexpressing b5R ($p < 0.01$). Similarly, the levels of 3-nitrotyrosine, a marker of protein nitration, were dramatically increased in control cells after exposure to rotenone (Fig. 6b). This effect of rotenone was attenuated in cell transfected with b5R ($p < 0.01$). Antimycin A also caused elevated levels of both protein carbonyls (Fig. 6c) and 3-nitrotyrosine (Fig. 6d). These levels of both damages induced by antimycin A in b5R transfectants were greatly lower than those of control cells.

Overexpressed b5R protected cells against apoptotic cell death

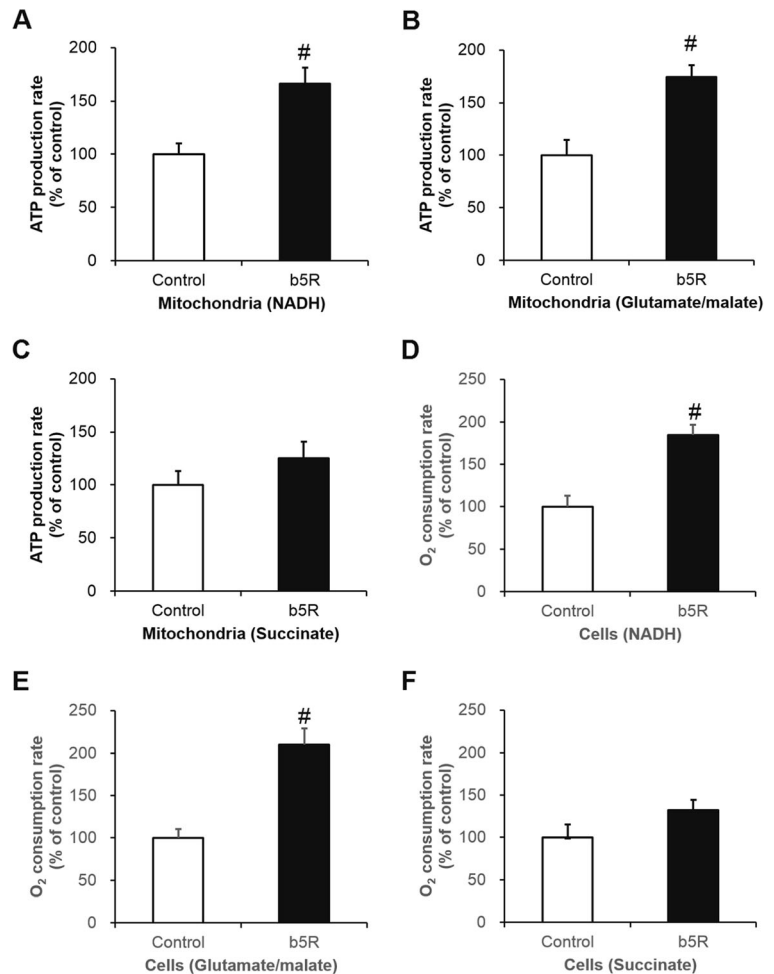
In order to investigate whether overexpressed b5R renders cells more resistant to apoptotic cell death induced by mitochondrial toxins, apoptotic features were examined. Following exposure to rotenone, the levels of propidium iodide-positive cells (Fig. 7a) and chromatin condensation (Fig. 7c) were decreased in cells transfected with b5R, compared with control cells. Similar patterns of resistance to the apoptotic features were also shown in cells overexpressing b5R after treatment with antimycin A (Fig. 7b, d).

Discussion

Previously, we showed that overexpressed NQO1, another PM redox enzyme, can protect cells against metabolic and proteotoxic stress through sustaining bioenergetics by increased mitochondrial function (Hyun et al. 2012; Kim et al. 2013). b5R is also one of the PM redox enzymes in the PMRS and may be involved in cell survival under stressed conditions. However, its protective mechanism is yet to be investigated.

This study shows that b5R can play an important role in the survival of human neuroblastoma cells under metabolic and oxidative stresses (Fig. 3). As expected, the NAD^+/NADH ratio was dramatically increased by overexpressed b5R (Fig. 2). Neural tumor cells (Nahimana et al. 2009) and normal neurons (Alano

Fig. 4 ATP production and O₂ consumption rate are increased by overexpression of b5R. Intact mitochondria were prepared, and ATP production and O₂ consumption rate were assessed in the presence of appropriate electron donors. ATP production rates in the presence of NADH (**a** and **d**), glutamate/malate (**b** and **e**), and succinate (**c** and **f**). Values are the mean±SEM (*n*=6). **p*<0.01 compared with the value for untransfected control cells under normal culture conditions. #*p*<0.01 compared with the value between untransfected control cells and b5R transfectants under the same culture conditions



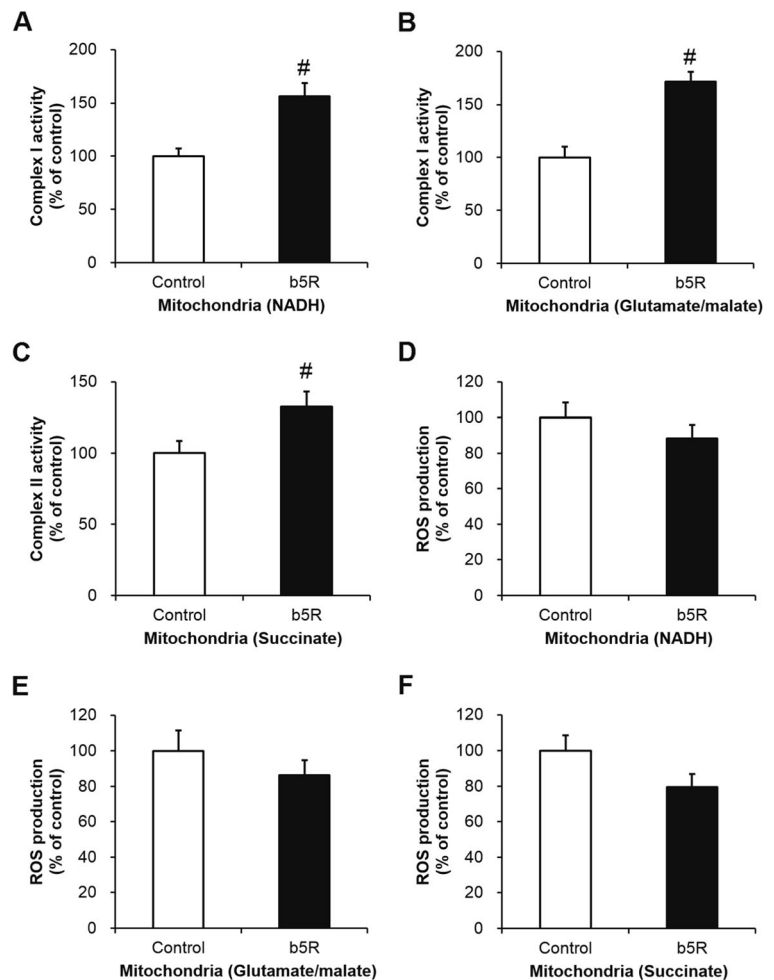
et al. 2010; Kruman et al. 2000; Liu et al. 2009) are very sensitive to a decline in the NAD⁺/NADH ratio because of their high energy demand. Impairment of energy metabolism and formation of protein aggregates are known to be responsible for a variety of neurodegenerative diseases. This study suggests a potential protective role for b5R in neurodegenerative diseases, consistent with a previous result (Incerpi et al. 2007). Failure of energy metabolism and altered abnormal protein handling are closely associated with an age-related decrease in PM redox enzymes (Hyun et al. 2006a).

Previous studies showed that NQO1 overexpression via a keap1-Nrf2 pathway using NQO1 inducers (e.g., sulforaphane, curcumin) renders neuroblastoma cells and primary neurons more resistant to metabolic and oxidative stress (Jia et al. 2008; Soane et al. 2010; Son et al. 2010). We also published that cells overexpressing

NQO1 are more resistant to metabolic and proteotoxic insults (Hyun et al. 2012). In this study, overexpression of b5R made neuroblastoma cells more resistant to several toxic insults such as 2-deoxyglucose, rotenone, antimycin A, and lactacystin (Fig. 3). We identified that b5R transfectants were more resistant to oxidative/nitrative stress and apoptosis following exposure to mitochondrial toxins.

Higher resistance of b5R transfectants to mitochondrial inhibitors (Fig. 3) suggests that b5R may also be involved in bioenergetics, because similar results have been published recently using another PM redox enzyme, NQO1 (Kim et al. 2013). NADH (a direct source of electron donor for complex I), glutamate/malate (an electron donor mediated by malate-aspartate shuttle for complex I), and succinate (an electron donor supplied in the TCA cycle for complex II) were used as the best

Fig. 5 Activities of mitochondrial complexes I and II are elevated, and production of ROS is reduced in human neuroblastoma cells overexpressing b5R. Cells were cultured and then mitochondrial fractions were isolated by centrifugal fractionation. Mitochondrial complex activities were measured using appropriate electron donors. Complex I activity in the presence of NADH (a) and glutamate/malate (b). Complex II activity in the presence of succinate (c). ROS production in the presence of NADH (d), glutamate/malate (e), and succinate (f). Values are the mean±SEM ($n=6$). * $p < 0.01$ compared with the value for untransfected control cells under normal culture conditions. # $p < 0.01$ compared with the value between untransfected control cells and b5R transfectants under the same culture conditions

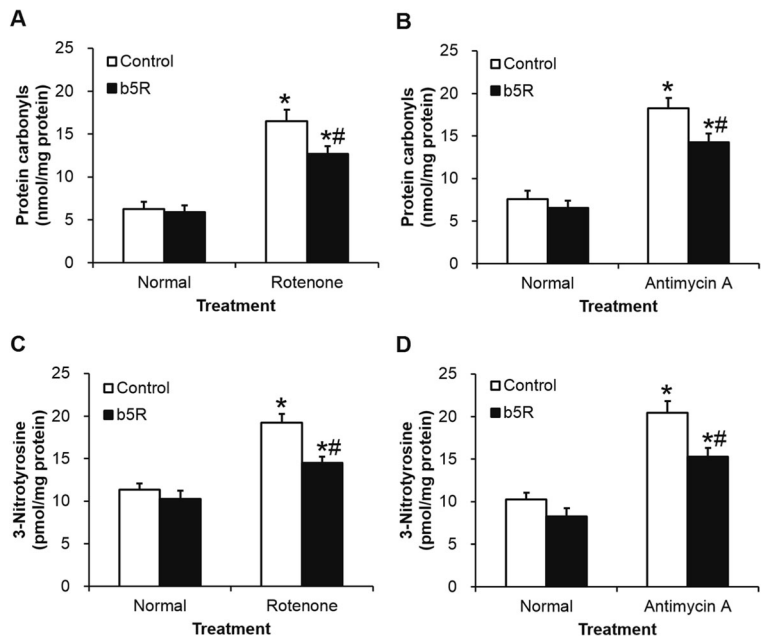


electron donors, as reported earlier (Panov et al. 2004). These phenomena were consistent with the data obtained from SH-SY5Y cells differentiated by RA (Supplementary Fig. 2). The complex I activity in b5R transfectants was further increased by RA. These data are consistent with a previous report that SH-SY5Y cells differentiated by RA exhibit increased OCR and higher mitochondrial respiratory capacity (Xun et al. 2012). In fact, RA can stimulate oxidative phosphorylation and mitochondrial biogenesis (Tourniaire et al. 2015).

Complexes I and III are a main site of mitochondrial ROS generation (Barja 1999; James et al. 2004). Levels of mitochondrial ROS are enhanced by treatment with mitochondrial inhibitors (e.g., rotenone, antimycin A) (Fig. 5) and in pathological conditions (e.g., hypoxia) (Kushnareva et al. 2002; Muller et al. 2004), consistent with the elevated levels of oxidative/nitrative damage

(Fig. 6) and resistance to apoptotic cell death (Fig. 7). Usually, the mitochondria can produce ROS as much as they consume oxygen and produce ATP (Murphy 2009). We found that neuroblastoma cells transfected with b5R did not produce further ROS, whereas they generated more ATP through enhanced mitochondrial electron transport under normal culture conditions, compared with control cells (Figs. 4 and 5). These phenomena can be explained by some possibilities. Firstly, as stated earlier, ROS (e.g., $O_2^{\cdot -}$) generated during oxidative phosphorylation can be scavenged directly by b5R. In fact, b5R can convert an oxidized form of CoQ to a reduced state by transferring one electron one by one. This might account for no significant decrease in ROS production in b5R transfectants, compared with cells overexpressing NQO1, which induces attenuated levels of ROS while higher ATP production and activity of

Fig. 6 Levels of oxidative damage to lipids and proteins are attenuated in cells with elevated b5R levels compared with control cells. Cell extracts were used to measure levels of protein carbonyls (**a, b**) and nitrotyrosine (**c, d**) following exposure to 50 μM rotenone (**a, c**) or 100 μM antimycin A (**b, d**) for 24 h. Values are the mean \pm SEM ($n=6$). * $p < 0.01$ compared with the value for untransfected control cells under normal culture conditions. # $p < 0.01$ compared with the value between untransfected control cells and b5R transfectants under the same culture conditions

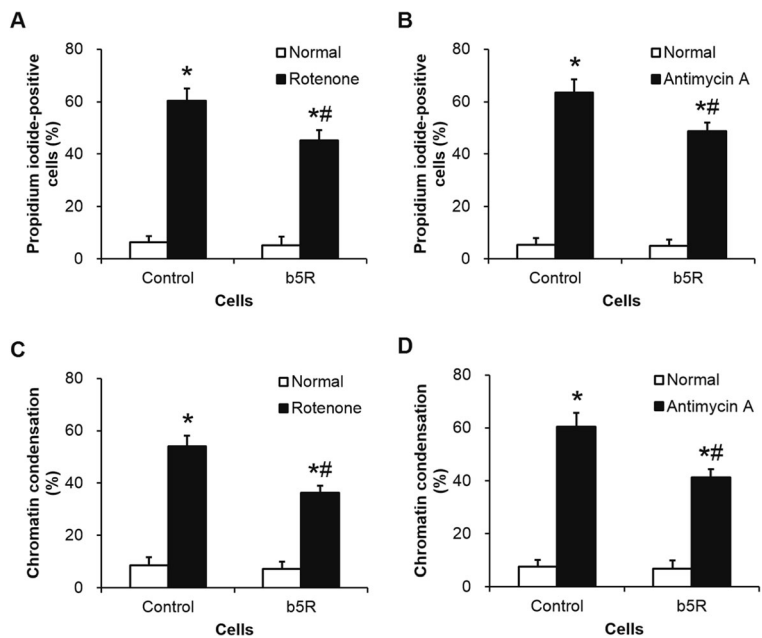


mitochondrial complexes I and II (Kim et al. 2013). Secondly, there could be causal relationship between higher QH₂/Q⁻ and NAD⁺/NADH ratio. Thirdly, b5R would induce electron transport from complex I or II to complex III without leakage of electrons, resulting in a more efficient production of ATP. This explanation is supported by increased NAD⁺/NADH ratio (Fig. 2) and

attenuated ROS production (Fig. 4) in cells overexpressing b5R.

CoQ is identified at significant levels in the PM and existed as three different states (Lopez-Lluch et al. 2010). Semi-ubiquinone is a state between oxidized and reduced forms of CoQ and transitionally formed by one electron transfer (Andreeva and Crompton 1994;

Fig. 7 Apoptotic cell death induced by mitochondria toxins is attenuated by overexpressed b5R. Levels of cell membrane permeabilization (propidium iodide-positive cells) (**a, b**) and chromatin condensation (**c, d**) were also measured after the addition of 50 μM rotenone (**a, c**) or 100 μM antimycin A (**b, d**) for 24 h. Values are the mean \pm SEM ($n=6$). * $p < 0.01$ compared with the value for untransfected control cells under normal culture conditions. # $p < 0.01$ compared with the value between untransfected control cells and b5R transfectants under the same culture conditions



James et al. 2004). Therefore, some studies showed that semi-ubiquinone can be a source of superoxide ($O_2^{\cdot-}$) production using purified b5R fractions (Samhan-Arias and Gutierrez-Merino 2014) and $O_2^{\cdot-}$ in the PM can be locally increased by b5R along with the formation of lipid rafts with caveolins (Samhan-Arias et al. 2012). However, another study reported that b5R is responsible for maintaining basal levels of the reduced form of CoQ (Lopez-Lluch et al. 2010). Although semi-ubiquinone is formed transiently by one electron transfer from NADH, it can immediately be converted to a fully reduced form of CoQ by consequent one electron transfer (Crane and Bottger 2001). This is supported by our finding that b5R can attenuate the levels of ROS following exposure to toxins (Fig. 5). These suggest that the PM b5R can neutralize overproduced $O_2^{\cdot-}$ by transferring one electron to oxidized CoQ. b5R can regulate CoQ levels and prevent oxidative damage in aged mice muscle through being stimulated by CR and aerobic exercise (Rodriguez-Bies et al. 2015). Also, phase I (e.g., b5R) and II (NQO1) enzymes can be activated by Aegle marmelos and protect against damage mediated by free radicals in mice (Singh et al. 2000). Consistent with our findings, these studies suggest a possible protective effect of b5R against oxidative stress.

Many studies demonstrated that impairment of the ubiquitin-proteasome system (UPS) is closely associated with many types of cell death identified in neurodegenerative diseases and cancer (Cecarini et al. 2007; Hoeller and Dikic 2009; Huang and Figueiredo-Pereira 2010; Sohns et al. 2010). Therefore, the UPS has been a hot spot in research to find therapeutic targets. This study found that b5R protects SH-SY5Y cells against cell death by lactacystin, a potent proteasome inhibitor (Fig. 3f), suggesting a new model of proteotoxicity, even though its protective mechanisms are still not clear. Some reports showed that heat shock proteins (e.g., HSP70) can be upregulated in models of proteotoxicity-mediated ATP depletion (Barja 1999) and cancer (Kushnareva et al. 2002). Previously, we also presented that overexpressed NQO1 is also involved in the protection of neuronal cells against proteotoxicity (Hyun et al. 2012). Because many neurodegenerative diseases are related to the accumulation of abnormal proteins such as amyloid β , α -synuclein, prions, and other damaged proteins, it suggests that upregulated PM redox enzymes (e.g., b5R, NQO1) can play a key role in removing abnormal proteins by increasing chaperon proteins during pathogenic processes in AD, PD, and prion disease.

Taken together, this study found that b5R can be a therapeutic target for neurodegenerative diseases focused on the regulation of mitochondrial function. Further work is required to improve mitochondrial energy metabolism using other cell culture and animal models.

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