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Nitrosative stress mediated misfolded protein aggregation mitigated by Na-D-β-hydroxybutyrate intervention

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ABSTRACT

Mitochondrial dysfunction, leading to elevated levels of reactive oxygen species, is associated with the pathogenesis of neurodegenerative disorders. Rotenone, a mitochondrial stressor induces caspase-9 and caspase-3 activation leading proteolytic cleavage of substrate nuclear poly(ADP-ribose) polymerase (PARP). PARP cleavage is directly related to apoptotic cell death. In this study, we have monitored the aggregation of green-fluorescent protein (GFP)-tagged synphilin-1, as a rotenone-induced Parkinsonia-onset biomarker. We report that the innate ketone body, Na-D-β-hydroxybutyrate (NaβHB) reduces markedly the incidence of synphilin-1 aggregation. Furthermore, our data reveal that the metabolic byproduct also prevents rotenone-induced caspase-activated apoptotic cell death in dopaminergic SH-SY5Y cells. Together, these results suggest that NaβHB is neuroprotective; it attenuates effects originating from mitochondrial insult and can serve as a scaffold for the design and development of sporadic neuropathies.

1. Introduction

Pathologically, Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the substantia nigra and the formation of Lewy-body inclusions [1]. Although the molecular mechanisms are not clearly understood, mitochondrial dysfunction is known to be an important factor among other known intra- and extracellular etiological factors [1]. Studies showed that in a particular sporadic form of PD, mitochondrial complex I activities are compromised in the nigro-striatal pathway [2–4]. Rotenone, a plant derived pesticide, induces cell destruction by inhibiting complex I (NADH ubiquinone oxidoreductase) which mimic the biochemical lesions of PD, both *in vivo* and *in vitro* [5,6].

The mitochondrial respiratory chain is a key site of reactive oxygen species (ROS) production under physiological conditions which in turn, orchestrates apoptosis [4,7,8]. Rotenone is a model ROS generator *via* the induced production of NOx. Earlier studies have shown the mechanism of rotenone-induced apoptosis through mitochondrial ROS production [2]. Apoptotic stimuli instigate the release of cytochrome c from the mitochondria into the cytosol, where it triggers autocatalytic processing of procaspase-9. Caspase-3 gets activated along with other effector proteins by caspase-9, resulting in the proteolytic cleavage of substrate nuclear poly(ADP-ribose) polymerase (PARP) [9]. In human PARP, the cleavage occurs between Asp214 and GLY215, which separates the PARP amino-terminal DNA binding domain (24 kDa) from the carboxy-terminal catalytic domain (89 kDa) [5,10]. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [11].

A hallmark event characteristic of PD is the accumulation of aggregated proteins to often form Lewy-bodies in the cytosol of human neuronal cells, which results apoptotic cell death of dopaminergic neuronal cells [3]. A common feature observed in the neuronal cells of PD victims in this sporadic variant was the attachment of nitric oxide (NO) to the redox-active cysteines of protein-disulfide isomerase (PDI) to form *S*-nitroso-PDI because of high levels of nitrosative stress [3,12]. The formation of *S*-nitroso-PDI coupled with the pathogenesis of PD making the oxidoreductase a chief target for the prevention of PD in the nitrosative-stress-linked variant of the diseases [12].

Na-D-β-hydroxybutyrate (NaβHB; $C_4H_7NaO_3$) is a ketone body produced by hepatocytes and serve as an alternative source of energy in the brain during starvation [13,14]. Neuronal damage induced by glucose deprivation and mitochondrial poisoning is prevented by NaβHB [15,16]. Ketone bodies decrease the need for glycolysis [17], bypass the blockade of the pyruvate dehydrogenase multienzyme complex, and reduce the mitochondrial [NAD+]/[NADH] ratio [18,19].

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In this study, we investigated the neuroprotective effect of NaβHB against rotenone induced caspase-activated apoptosis by using SH-SY5Y dopaminergic neuroblastoma cells. Our results reveal that NaβHB attenuate the apoptotic stimuli by acting against rotenone toxicity. Furthermore, we have monitored the aggregation of overexpressed green-fluorescent protein tagged synphilin-1 in SH-SY5Y cells. Our results show that exposure of this cell line to rotenone leads to the aggregation of synphilin-1, as observed by fluorescence microscopy and consistent with previous reports that NO influences Lewy-body formation *via* PDI modification [12]. Importantly, cells that were pre-incubated with NaβHB prior to rotenone insult demonstrated a marked resilience to synphilin-1 aggregation. These results suggest that it may be possible to mitigate nitrosative-stress induced aggregates in cell lines using ketone body-analogs. Our work opens avenues for the design and development of more effective prophylactics against nitrosative-stress linked PD.

2. Materials and methods

2.1. Reagents, cell line and plasmid

Sodium beta hydroxy butyrate (NaβHB) and rotenone (RT) were purchased from Sigma–Aldrich (St. Louis, MO). Other reagents were commercially sourced: mouse monoclonal for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and PARP (Cell Signaling Technology, Danvers, MA); apoptosis/necrosis kit (Beckman Coulter, Miami, FL), horseradish peroxidase (HRP)-conjugated goat anti-mouse (KPL Biomedical); Hoechst 33342 (Invitrogen, Eugene, OR); propidium iodide (PI) (MP Biomedicals, Solon, OH); human neuroblastoma cell line SH-SY5Y (ATCC, Manassas, VA). Cells were transfected with the pEGFP-C2 or synphilin-1/pEGFP-C2 plasmid as previously described [3].

2.2. Cell culture and treatment

SH-SY5Y cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin. Cells were grown at 37 °C in humidified 5% carbon dioxide atmosphere. SH-SY5Y cells (1×10^6 cells/well) were seeded onto glass coverslips in 6-well plates and incubated for 12 h. Cell transfections were performed the following day with pEGFP-C2 control (without insert) or pEGFP-C2 carrying the fusion protein GFP-synphilin-1, as recommended by manufacturer using Effectene reagent (Qiagen, Valencia, CA). After transfection, the cells were incubated overnight to allow expression of proteins. Cells were treated with vehicle (DMSO) or with 100 μM NaβHB for 6 h followed by exposure to 300 nM of rotenone for 12 h. After incubation, cells were prepared for microscopy as described below.

2.3. Differential nuclear staining cytotoxicity assay

Cells were grown for 24 h to allow attachment to multi-well plates. Cells were treated with rotenone or with different concentrations (5–500 μM) of NaβHB alone, to determine its possible cytotoxic effect. As control for non-specific effects, DMSO vehicle control, as contained in the experimental samples, was included at final concentration of 0.2% v/v. Cells were pretreated with 100 μM of NaβHB for 6 h prior to rotenone exposure. Subsequently, cells were incubated by an extra 24 h and images were captured in live mode [20].

A mixture of PI and Hoechst 33342 at a final concentration of 1 μg/ml was added to each well 1 h prior to imaging [20]. Images were acquired in a live-cell mode utilizing a BD Pathway 855 Bioimager system (BD Biosciences Rockville, MD). Montages (3×3) from nine adjacent image fields were captured per well utilizing a 20× objective. Captured images and data analysis determining the percentage of death cells from each individual well was performed by using BD AttoVision™ v1.6.2 software (BD Biosciences Rockville, MD). Data were assessed in quintuplicate.

2.4. Apoptosis/necrosis assay

SH-SY5Y cells were seeded on 24-well micro plate at density of 20,000 cells/well and cultured as described. Cells were incubated overnight followed by 6 h pre-incubation in presence of 100 μM NaβHB and then added with 300 nM rotenone and incubated for additional 24 h. Cells from each individual well were collected, washed and processed essentially as described previously [21]. Briefly, cells were concurrently stained by resuspending them in a solution containing Annexin V-FITC and PI dissolved in 100 μl of binding buffer (Beckman Coulter, Miami, FL). After incubation for 15 min on ice in the dark, ice-cold binding buffer (400 μl) was added to the cell suspensions, gently homogenized, and immediately analyzed by flow cytometry. The percentage of total apoptotic cells per sample is annotated as the sum of both early and late stages of apoptosis (Annexin V-FITC positive), bottom right quadrant and top right quadrant, respectively. For each sample, approximately 10,000 individual events were acquired using flow cytometer (Cytomics FC 500; Beckman Coulter, Miami, FL) and data analyzed with CXP software (Beckman Coulter, Miami, FL). Every experimental point, as well as all controls, was assessed in quintuplicate.

2.5. Western Blotting

Total cell lysates were prepared by washing the cells with cold Tris-buffered saline, collected by centrifugation (3003*g*, 5 min at 4 °C, and extracted by sonication in buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM EDTA, 0.5% (v/v) SDS and protease inhibitors (Sigma). Total protein concentrations were measured using a bicinchonic acid kit (Pierce, Rockford, IL) and BSA as standard. Equal amounts of protein (approximately 10 μg per lane) were separated using SDS–polyacrylamide gel electrophoresis and then transferred to polyvinyldifluoride (PVDF) membranes. Blots were incubated in blocking buffer (5%, w/v, dried skimmed milk in Tris–buffered saline, pH 7.4, and 0.1% Tween 20) followed by incubation with anti-PARP rabbit polyclonal antibody (1:1000) or anti-GAPDH (1:1000 dilution) diluted in blocking buffer for 1 h followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit in 1% BSA/TBST for 30 min. Chemiluminescence (ECL-plus or SuperSignal West Pico Chemiluminescent Substrate) was used according to the manufacturer's instructions (Amersham or Pierce Biotechnology Inc.). GAPDH was used as housekeeping protein loading control.

2.6. Transfection and cell treatment

SH-SY5Y cells (1×10^6 cells/well) were seeded onto glass coverslips in 6-well plates and incubated at 37 °C in 5% CO2 for 12 h. Cell transfections were performed in the following day as recommended by manufacturer using Effectene reagent. Cells were then incubated with transfection complexes under normal growth condition for expression of pEGFP-C2 control or the fusion protein GFP-synphilin-1 gene.

Transiently transfected SH-SY5Y cells were incubated overnight to allow expression of proteins. Cells were treated with DMSO vehicle or with 100 μM NaβHB for 6 h followed by exposure to 300 nM of the toxicant rotenone for 12 h. After attachment, cells were prepared for microscopy as described below.