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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.

2.7. Confocal microscopy and immunocytochemistry

Cells transfected with vector or EGFP-synphilin-1 were washed after treatment, fixed with 4% paraformaldehyde in PBS, stained with DAPI and mounted under ProLong antifade medium (Molecular Probes). To stain for synphilin-1, cells were fixed as above, permeabilized with 0.1% (w/v) saponin in PBS, blocked with PBS plus 5% goat serum, 5% FBS and 0.1% TWEEN 20, followed by incubation with primary antibody (overnight at 4 °C) and secondary rhodamine-conjugated goat anti-mouse (1:10,000; KPL Biomedical), and DAPI staining. Fluorescence confocal images were captured utilizing LSM 700 confocal microscope and assisted with ZEN 2009 software (Zeiss, New York, NY).

3. Results

3.1. Differential nuclear staining assay to quantify cytotoxicity

Results from the cells differential nuclear staining assay (DNS) are shown in Fig. 1. To define and analyze survival and death of SH-SY5Y, dead cells were detected by using PI (Fig. 1B); and, entire, nucleated cells were stained with membrane permeable Hoechst dye (Fig. 1A). Co-localization (magenta color) of nuclear fluorescence signals as shown in Fig. 1C represents the dead cells. The DNS assay adapted to HTS revealed cytotoxicity of NaβHB itself and preventive effect of NaβHB against rotenone induced cytotoxicity in SH-SY5Y cell. Fig. 1D shows the dose response assay of NaβHB in SH-SY5Y cell line. Our data reveals no significant cytotoxicity up to 500 μM NaβHB.

The cytotoxicity by rotenone insult was measured in the presence and absence of NaβHB. Pre-incubation with 100 μM NaβHB lowered the level of 300 nM rotenone cytotoxicity (Fig. 1E). Our results illustrated in Fig. 1D showed no significant differences between control after DMSO treatment and 100 μM NaβHB treatment for 24 h. We found more than 70% toxicity at 300 nM rotenone on SH-SY5Y cells after 24 h of incubation (Fig. 1E). It is clear in Fig. 1E that pre-treatment with 100 μM NaβHB (6 h) followed by rotenone insult for 24 h resulted in reduction of cytotoxicity to a significant level ($\geqslant$25%). Based on these results it is obvious that, NaβHB, classified as a ketone body, can prevent the mitochondrial inhibitor rotenone mediated cell death.

3.2. Apoptosis/necrosis assay

Fig. 2 depicts annexin V-FITC and propidium iodide (PI) flow cytometric analysis to quantitatively estimate the apoptotic/necrotic profiling of SH-SY5Y cells upon different treatment. Each histogram in Fig. 2 is fragmented in four quadrants; left top quadrant – necrosis, cells permeable to PI that have lost their membrane integrity, without Annexin V-FITC signal, one color (red); right top quadrant – late apoptosis, cells with compromised plasma membrane, permeable to PI, but also with Annexin V-FITC signal, two colors (green and red); lower left quadrant – alive unstained cells, without PI or Annexin V-FITC fluorescent signal; lower right

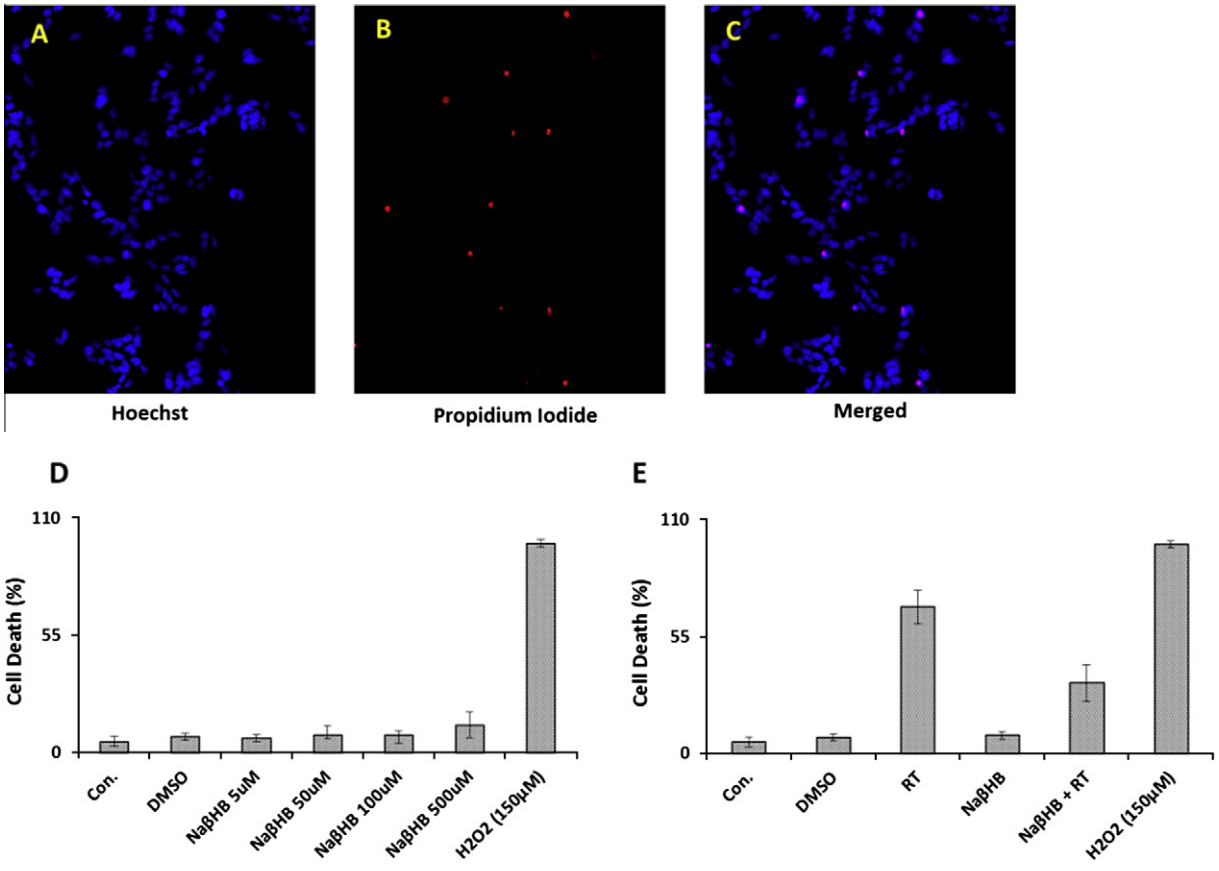

Fig. 1. The cytotoxicity and preventive effect of NaβHB was tested utilizing SH-SY5Y cell line, measured by using differential nuclear staining assay (DNS) adapted to high throughput screening (HTS). (A) Hoechst emission signal indicates the total number of nuclei (cells), shown in blue. (B) Propidium iodide emission signal indicates the number of death cell, shown in red. (C) Magenta color is an outcome of co-localization of red (PI) and blue (Hoechst) colors indicating the number of death cells in the image. (D) The cytotoxicity of NaβHB at different concentration after 24 h of treatment and (E) Preventive effect of NaβHB against rotenone toxicity in SH-SY5Y cells. Each bar represents average of triplicate values, and error bars their corresponding standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

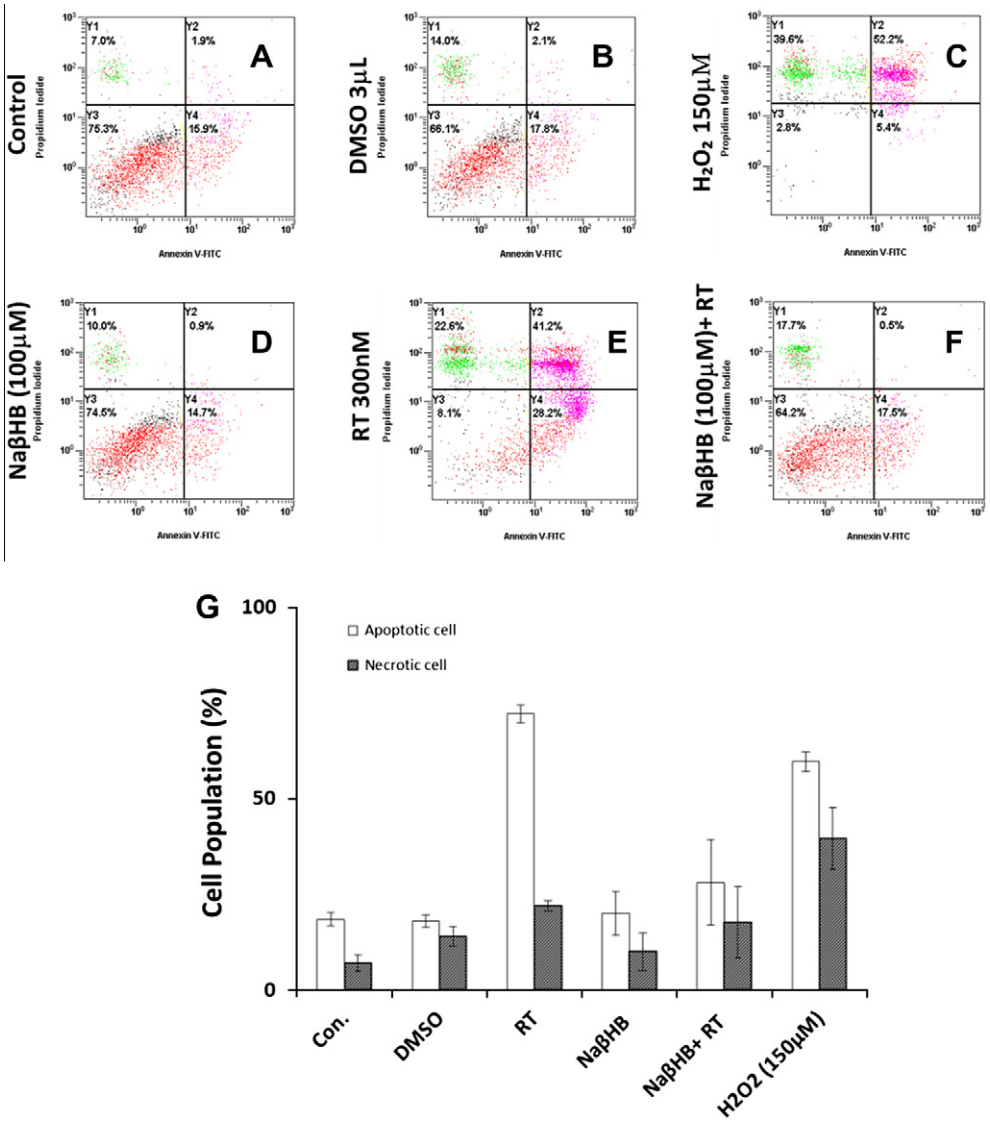

Fig. 2. Representative flow cytometric dot plots used to determine the percentages of apoptosis/necrosis effects on SH-SY5Y cells. (A) untreated cells (control), (B) vehicle control (dimethyl sulfoxide; DMSO), (C) positive control (150 µM H_2O_2), (D) cells treated with sodium beta hydroxy butyrate (NaβHB; 100 µM), (E) cells treated with rotenone (300 nM), (F) Cells were pretreated with NaβHB (100 µM) for 6 h and exposed to 300 nM of rotenone for another 24 h. (G) represent the percentage of apoptotic and necrotic cell population.

quadrant-early apoptosis, cells with only Annexin V-FITC signal, one color (green). Representative histograms of untreated cells (Fig. 2A); cells treated with DMSO (Fig. 2B) and 100 µM NaβHB (Fig. 2D), did not show much detrimental effect on the viability of SH-SY5Y cells. Approximately, 2% of cells survive the exposure of 150 µM H_2O_2 (positive control) as shown in Fig. 2C. Apoptotic cell death occurred of nearly 70% (early and late apoptosis combined) after 24 h exposure to 300 nM rotenone (RT) in SH-SY5Y cells (Fig. 2E). Pretreatment with 100 µM NaβHB for 6 h resulted in $\geqslant$40% and $\geqslant$10% protection against rotenone (300 nM) induced late and early apoptotic cell death respectively (Fig. 2F) and almost preserves the characteristic as of control.

Total percentage of apoptotic cells were expressed as the sum of both early and late stages of apoptosis (light colored bars; Fig. 2G). Cells permeable to propidium iodide without Annexin V-FITC signal were considered as necrotic cells (dark colored bars; Fig. 2G). Untreated cell (Con.), vehicle control (DMSO) as well as 100 µM NaβHB treated cells showing negligible apoptotic and necrotic cell death (Fig. 2G) as anticipated from previous data (Fig. 1D and E). Rotenone induced excessive generation of NOx resulted in cell death mostly *via* apoptotic pathway. Interestingly, rotenone even outcast 150 µM H_2O_2 induced apoptotic cell death by $\geqslant$10%. Necrotic cell death due to rotenone aggression was very low over control. Our data clearly suggest that rotenone, a known NOx producer, can activate the caspase pathway through mitochondrial membrane depolarization leading towards apoptotic cell death. Fig. 2 also revealed that cells pre-incubated with 100 µM NaβHB for 6 h can clearly prevent rotenone induced apoptosis by $\geqslant$40% in SH-SY5Y.

3.3. PARP assay

In Fig. 3, lane 1, 2, 3, 4 and 5 (Ln = 1–5) indicates untreated cells, vehicle control (DMSO), cells treated with sodium beta hydroxy butyrate (NaβHB; 100 µM), cells pretreated with NaβHB (100 µM) for 6 h and exposed to 300 nM of rotenone for another 24 h and cells treated with rotenone (300 nM) alone respectively. Our data show that stress with 300 nM rotenone on cells for 24 h increases cleavage of PARP at a very high level over control (Fig. 3). Excess generation of NOx, upon rotenone insult, facilitates

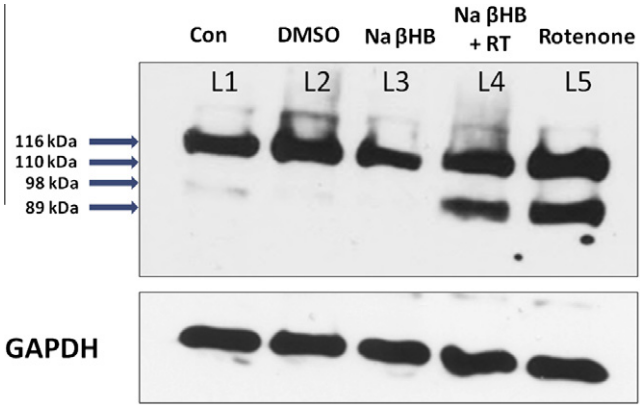

Fig. 3. Protective effect of NaβHB (100 μM) against rotenone (300 nM) induced poly(ADP-ribose) polymerase (PARP) cleavage, marker of apoptosis progression, in SH-SY5Y cells. PARP cleavage was analyzed *via* Western Blot analysis.

cleavage of the PARP carboxy-terminal catalytic domain (89 kDa) from amino-terminal DNA binding domain (24 kDa) (Fig. 3, Ln5), which activates the apoptotic pathway. In this experiment we show that 100 μM NaβHB can prevent the cleavage of PARP (Fig. 3, Ln4), suggesting the generation of low nitrosative stress and thus protecting the SH-SY5Y cells from going through apoptosis.

3.4. S-Nitrosylation of PDI mediates synphilin-1 aggregation in model cells of PD

Fig. 4 shows the protective effect of NaβHB on aggregation of GFP-tagged synphilin-1 by confocal microscopy in transiently transfected SH-SY5Y cells as a function of rotenone insult. The results clearly indicate the aggregation of synphilin-1 when exposed to 300 nM rotenone (Fig. 4D). Pre-treatment of cells with 100 μM NaβHB prior to 300 nM rotenone exposure shows markedly diminished level of synphilin-1 aggregation (as evidenced through GFP fluorescence; Fig. 4E). Fig. 4A revealed a relatively homogeneous cytosolic distribution of GFP in cells transfected with pEGFP C2 plasmid alone. In contrast, cells transfected with EGFP- synphilin-1 constructs show a punctuated (or speckled) cytosolic distribution of green fluorescent signal (Fig. 4B). This observation indicates that over expression of synphilin-1 fused to EGFP protein does not display homogenous cytosolic distribution, instead, accumulating subcellularly in the form of aggresomes. Cells, treated with the vehicle alone (DMSO; Fig. 4C) or 100 μM NaβHB (Fig. 4F) did not differ in the expression of EGFP-synphilin-1 as compared to untreated cells (Fig. 4B).

4. Discussion

In eukaryotes the mitochondrial respiratory chain (complexes I–V) is the major site of ATP production; additionally, it significantly impacts apoptosis. Inhibition of the mitochondrial respiratory chain by rotenone is linked to ROS production and serves to study the role of the mitochondrial respiratory chain in apoptosis [22]. NOx-stress, an outcome of ROS elevation, results in caspase-9 and caspase-3 activation, PARP cleavage, and DNA fragmentation eventually leading to apoptotic cell death.

The endoplasmic reticulum is a specialized compartment with a redox potential designed to facilitate the (oxidative) formation of disulfide bonds in secretory or membrane-bound proteins [3]. This is an essential process preceding their export from the ER and is catalyzed by protein-disulfide isomerase, the chief ER-resident oxidoreductase chaperone. The catalytic function of PDI, executed through two redox-active cysteine-containing active sites is essential to balance the flux between incoming nascent polypeptides and outgoing biologically viable folded proteins [3]. Compromise or failure in the catalytic efficiency of PDI can reduce the maturation processing of nascent substrates and lead to terminal misfolding, retrotranslocation along the endoplasmic reticulum associated degradation (ERAD) pathway and debris accumulation in the cytosol. This sequence of events is perhaps the rosetta-stone for the onset of apoptotic cell death related neuropathies [3].

Here, we hypothesized that the NaβHB can mitigate the incidence of apoptotic cell death propagated by nitrosative stress.

To test our hypothesis, we employed rotenone to initiate nitrosative stress in an SH-SY5Y cell line. We first determined the cytotoxicity of rotenone and prophylactic effect of NaβHB against rotenone through differential nuclear staining cytotoxicity assay. Although we were unable to detect the mechanism of cell death (apoptotic or necrotic) by this assay, out study demonstrated that rotenone induced cytotoxicity was reduced to a significant level in the presence of NaβHB.

Next, we determined the mechanism by which rotenone induces nitrosative-stress-related cell death (i.e. *via* the apoptotic and/or necrotic pathway). We used flow cytometric assay to determine the pathway of rotenone induced cytotoxicity in SH-SY5Y cell line; additionally, we examined the preventive effect of NaβHB against rotenone-induced nitrosative-stress-related apoptotic or necrotic cell death. Our data indicate that rotenone causes cytotoxicity in SH-SY5Y cell line primarily through the apoptotic pathway and NaβHB mitigates apoptotic cell death at a very significant level by attenuating nitrosative stress.

PARP analysis also confirmed that rotenone-induced nitrosative stress leads to cell death essentially through apoptosis. Excessive generation of NOx by mitochondrial complex-I inhibitor rotenone leads PARP cleavage *via* caspase activity and that activates apoptotic stimuli in eukaryotic cell. We determined that rotenone induced PARP cleavage is significantly reduced in the presence of NaβHB. This reinforces evidence for prophylactic ability of NaβHB against apoptotic cell death.

Collectively, our data reveal that rotenone-induced nitrosative stress activates programmed cell death stimuli and leads to apoptotic cell death *via* caspase-9 and caspase-3 activation → PARP cleavage → DNA fragmentation. NaβHB prophylactic effect against nitrosative-stress-related apoptotic cell death might arise from its inhibitory behavior towards one or more elements within this cascade.

Initially, we examined the cytosolic aggregation of synphilin-1 in the SH-SY5Y cell line under rotenone-induced nitrosative stress as previously demonstrated [12]. GFP-labeled synphilin aggregation was monitored using confocal microscopy. In comparison to control experiments, incubation of the cell line with rotenone clearly demonstrated cytosolic aggregation of synphilin-1 consistent with previous results suggesting that healthy PDI inhibits aggregation of synphilin-1. In other controls, we examined whether unstressed cells expressing PDI could prevent synphilin-1 Lewy-body-like inclusions in the cytosol after synphilin-1 overexpression. Our data revealed very limited diffused synphilin-1 localization in cytosol (Fig. 4B and C). In contrast, rotenone treated cells showed discrete inclusions of synphilin-1 in the cytosol. These results suggest that rotenone-dependent elevation of nitric oxide attenuated the protective effect of PDI on synphilin-1 inclusions (Fig. 4D).

Next, cells were preincubated with NaβHB prior to rotenone exposure to determine whether NaβHB can prevent rotenone induced aggregation formation of misfolded protein. Confocal microscopy data monitoring GFP-tagged synphilin-1 clearly indicate that unlike rotenone-induced nitrosative stressed cells (Fig. 4D), cells pre-treated with NaβHB followed by rotenone treat-

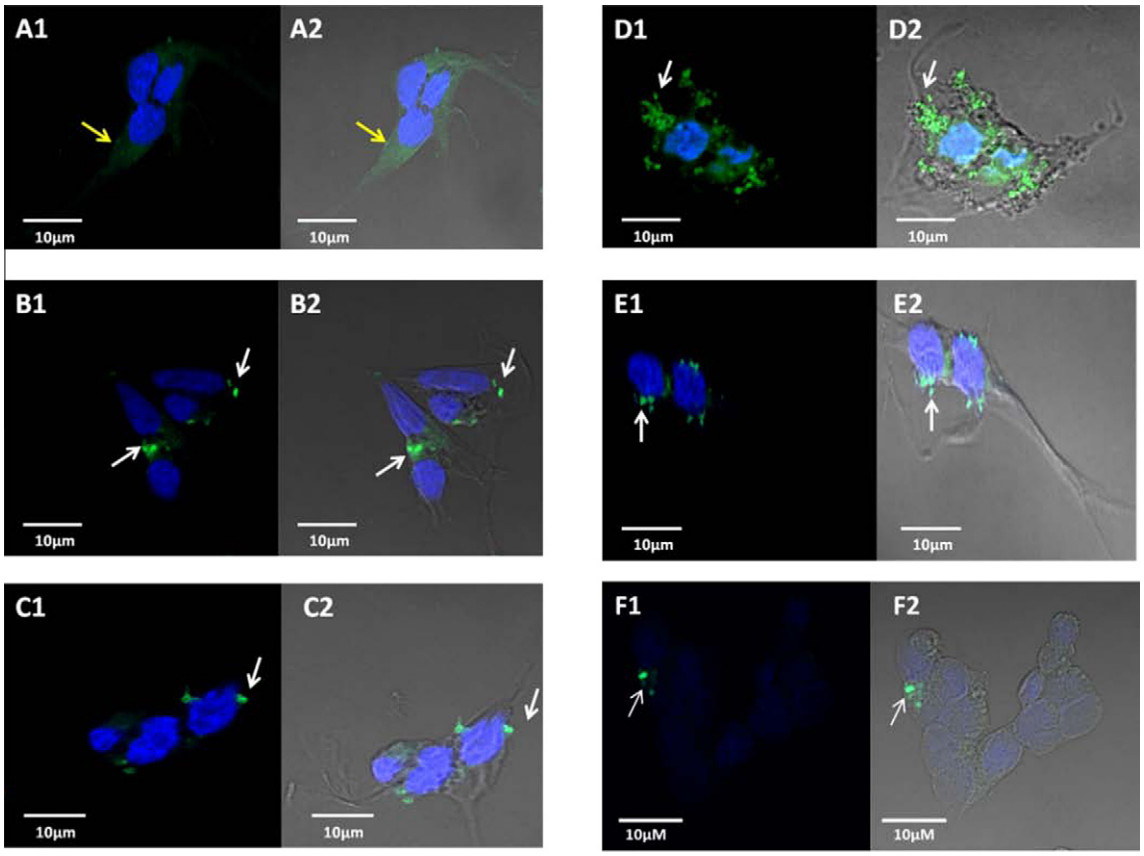

Fig. 4. Expression of synphilin-1 in SH-SY5Y cells and rotenone induced aggregation. (A) Cells transfected with pEGFP-C2 without synphilin-1 insert; (B) cells untreated; (C) cells treated with DMSO 0.2% v/v; (D) cells exposed to 300 nM rotenone for 12 h; (E) cells pretreated with 100 μM NaβHB for 6 h were exposed to 300 nM rotenone for 12 h; (F) cells exposed to 100 μM NaβHB. All the cells were counterstained with DAPI to delimitate the nucleus (blue color). White arrows indicate the presence of aggregates corresponding to the recombinant fusion protein and yellow arrow represents GFP expression. Second part of each figure represents the differential interference contrast (DIC) picture merged with fluorescence images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ment showed a drastic decrease in discrete Lewy-like inclusions in cytosol (Fig. 4E). These results suggest that NaβHB can intracellularly rescue *S*-nitroso modification of PDI as seen under elevated levels of nitrosative stress and prevent Lewy-neurite formation in our model system.

In conclusion, our data reveals that the innate metabolite NaβHB can serve as a potent prophylactic against nitrosative stress induced pathogenesis of PD. It remains to be investigate whether NaβHB can intervene in other reactive oxygen species initiated sporadic neuropathies such as Alzheimer's disease.

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