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Original Contribution

Differentiation of SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress

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ABSTRACT

Cell differentiation is associated with changes in metabolism and function. Understanding these changes during differentiation is important in the context of stem cell research, cancer, and neurodegenerative diseases. An early event in neurodegenerative diseases is the alteration of mitochondrial function and increased oxidative stress. Studies using both undifferentiated and differentiated SH-SY5Y neuroblastoma cells have shown distinct responses to cellular stressors; however, the mechanisms remain unclear. We hypothesized that because the regulation of glycolysis and oxidative phosphorylation is modulated during cellular differentiation, this would change bioenergetic function and the response to oxidative stress. To test this, we used retinoic acid (RA) to induce differentiation of SH-SY5Y cells and assessed changes in cellular bioenergetics using extracellular flux analysis. After exposure to RA, the SH-SY5Y cells had an increased mitochondrial membrane potential, without changing mitochondrial number. Differentiated cells exhibited greater stimulation of mitochondrial respiration with uncoupling and an increased bioenergetic reserve capacity. The increased reserve capacity in the differentiated cells was suppressed by the inhibitor of glycolysis 2-deoxy-D-glucose. Furthermore, we found that differentiated cells were substantially more resistant to cytotoxicity and mitochondrial dysfunction induced by the reactive lipid species 4-hydroxynonenal or the reactive oxygen species generator 2,3-dimethoxy-1,4-naphthoquinone. We then analyzed the levels of selected mitochondrial proteins and found an increase in complex IV subunits, which we propose contributes to the increase in reserve capacity in the differentiated cells. Furthermore, we found an increase in MnSOD that could, at least in part, account for the increased resistance to oxidative stress. Our findings suggest that profound changes in mitochondrial metabolism and antioxidant defenses occur upon differentiation of neuroblastoma cells to a neuron-like phenotype.

Mitochondrial dysfunction and oxidative stress are early characteristics and key contributing factors to neurodegeneration in diseases, including Parkinson disease [1]. Postmitotic neurons are highly dependent on mitochondria to meet their bioenergetic demands, in contrast to rapidly dividing cells or tumor cells that largely depend upon glycolysis as a primary energy source [2]. Neuronal cells maintain a bioenergetic capacity sufficient to meet physiological energy demands, with a reserve or spare capacity that can be utilized by the cells under stress [2]. For example, during signal transmission across synapses, neurons have high energy demands that maintain and allow rapid recovery from depolarization [3]. Bioenergetic reserve capacity is utilized when excessive glutamatergic stimulation causes a cellular Ca^{2+} overload and increased energy demand in the cell [4]. The recruitment of the bioenergetic reserve capacity under these conditions is essential to prevent cell death [4]. Additionally, postmitotic neurons cannot divide to remove or dilute out damaged components and do not have high levels of antioxidants compared to other cells, such as the glia making their bioenergetic capacity a potentially important factor in protecting against oxidative stress [5].

In a recent series of studies, we and others have proposed that the reserve or spare bioenergetic capacity is critical to resist the toxicity associated with increased oxidative stress [6]. In the case of neurodegenerative diseases, such as Parkinson, in which mitochondrial respiratory chain

Abbreviations: DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; 2-DG, 2-deoxy-D-glucose; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; HNE, 4-hydroxynonenal; OCR, oxygen consumption rate; RA, retinoic acid; ROS, reactive oxygen species.

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proteins are damaged [7], reserve capacity is likely to be compromised, rendering the cells more susceptible to oxidative insults. It has been suggested by the Warburg hypothesis that rapidly dividing undifferentiated cells have a greater dependence on glycolysis for metabolic intermediates needed for cell division [8–10]. This also results in a down-regulation of mitochondrial function, which suggests that the mitochondria may be functioning at near-maximal rates resulting in loss of bioenergetic reserve capacity. This paradigm also suggests that as cells differentiate, the metabolic requirements change, resulting in a greater requirement for mitochondrial ATP production. In this study, we have used the well-established cell line SH-SY5Y because it can be maintained in an undifferentiated state and can be stimulated to differentiate into a neuron-like phenotype in cell culture [11–18]. SH-SY5Y human neuroblastoma cells are derived from a thrice-cloned cell line, SK-N-SH, originally from a neuroblastoma patient [19]. SH-SY5Y cells contain many characteristics of dopaminergic neurons [11] and have therefore been used extensively to study neuron-like behavior in response to neurotoxins in the context of Parkinson disease [11].

Neurodegenerative diseases are frequently associated with increased oxidative stress, including increased production of lipid peroxidation products [20,21]. An important secondary lipid peroxidation product that is present in Parkinson disease brain is the aldehyde 4-hydroxy-2-nonenal (HNE). HNE is electrophilic, which allows it to react with nucleophilic protein residues, thus modulating their functions [22–28]. Furthermore, accumulation of HNE can damage key proteins in the mitochondrial respiratory chain [29,30], inhibit NADH-linked respiration [31,32], and deplete cardiolipin [33]. In addition the ability of the mitochondria to resist the toxic effects of reactive lipid species has not been investigated in this neuronal cell model and was tested in this study. Using both undifferentiated and differentiated SH-SY5Y cells as a model system, we characterized the mitochondria and the bioenergetics of these cells under basal conditions and in response to oxidative stress induced by exposure to the oxidized lipid HNE and the generator of intracellular reactive oxygen species (ROS), 2,3-dimethoxy-1,4-napthoquinone (DMNQ) [34]. This is particularly relevant to Parkinson disease because hydrogen peroxide is produced by dopamine metabolism and is thought to be a major contributor to the early dopaminergic cell death [35]. In this study we compared susceptibility to DMNQ and HNE in differentiated and undifferentiated cells and propose that mitochondrial function plays a key role in the differential response to oxidative stress in neuronal cells.

Materials and methods

Materials

HNE was obtained from Calbiochem. DMNQ was obtained from Alexis Biochemicals. All materials for the extracellular flux assays were from Seahorse Biosciences. Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), oligomycin, and antimycin A were from Sigma. MitoTracker red was purchased from Molecular Probes. JC-1 was purchased from Invitrogen.

Cell cultures

We cultured and used early passage P11–17 human neuroblastoma SH-SY5Y cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin. Differentiation was induced by 10 μM all-*trans*-retinoic acid on day 1 after plating and continued for 5 days. Medium was replaced with fresh medium containing retinoic acid every 48 h in 1% DMEM supplemented with 1% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin.

Immunocytochemistry

Cells were grown in 24-well plates in which a glass coverslip was added to the well before plating. Cells were fixed with 4% paraformaldehydeparaformaldehyde for 1 h at room temperature, permeabilized by 0.1% Triton X-100, incubated in 5% bovine serum albumin blocking buffer for 1 h, followed by primary antibodies and then Cy3- or FITC-conjugated secondary antibodies, and counterstained with Hoechst. Glass coverplates were taken out and mounted to glass slides. Staining was visualized on a Leica TCS SP5 confocal microscope. MitoTracker images were taken of live cells grown on chamber slides using the same Leica TCS SP5 confocal microscope.

Measurement of mitochondrial membrane potential by JC-1 staining

Cells were washed with phenol red and serum-free DMEM and then incubated with 5 μg/ml JC-1 in phenol red and serum-free DMEM for 45 min. Cells were then washed three times with serum-free DMEM. The last wash was without (control) or with 10 μM FCCP. The cells were incubated in medium or medium + FCCP for 10 min and then were imaged on a PerkinElmer Life Sciences Wallace 1420 multilabel plate reader. Green was imaged with 485 nm excitation and 530 nm emission and red was imaged with 530 nm excitation and 590 nm emission. The ratio of red/green fluorescence (aggregated/nonaggregated JC-1) was calculated.

Assessment of cell viability

We measured cell viability using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay as described previously [29,36] and confirmed it by a calcein AM assay and cell counting by trypan blue exclusion. For the MTT assay, we seeded SH-SY5Y cells in the XF24 culture plates at 80,000 cells/well. Sixteen hours after HNE or DMNQ treatment, the cells were incubated with medium containing 0.4 mg/ml thiazoyl blue tetrazolium in a 37 °C incubator for 2 h. Then we removed the medium, solubilized the resulting formazan crystals in 250 μl dimethyl sulfoxide, and read the absorbance at 550 nm.

Citrate synthase assay

Citrate synthase activity is based on the reaction of Ellman's reagent, DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], with the thiol group of free CoA producing TNB (5-thio-2-nitrobenzoic acid) measured at 412 nm. Whole-cell lysates were incubated with 100 mM Tris/0.1% (v/v) Triton X-100, pH 8.0, containing 0.2 mM DTNB, 0.1 mM acetyl-CoA. Reactions were initiated by the addition of oxaloacetate (0.2 mM). Rates are reported as nmol TNB formed/min/mg protein.

Quantification of mitochondrial DNA (mtDNA)

DNA was extracted from SH-SY5Y cells at the indicated days of differentiation. Quantitative real-time PCR was performed by using a LightCycler DNA Master SYBR Green I kit (Roche) as described previously [37]. The primer sequences used for mtDNA were mtF (5′-CACCCAAGAACAGGGTTTGT-3′) and mtR (5′-TGGCCATGGGTATGTTGTTAA-3′). The primer sequences for the nuclear DNA were 18SF (5′-TAGAGGGACAAGTGGCGTTC-3′) and 18SR (5′-CGCTGAGCCAGTCAGTGT-3′). Cycling conditions were as follows: 95 °C for 150 s, followed by 40 cycles at 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 20 s.

Measurement of mitochondrial function

To measure mitochondrial function in differentiated and undifferentiated SH-SY5Y cells, a Seahorse Bioscience XF24 extracellular flux analyzer was used. The XF24 creates a transient, 7-μl chamber in specialized microplates that allows for the determination of oxygen and