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Mild mitochondrial metabolic deficits by α-ketoglutarate dehydrogenase inhibition cause prominent changes in intracellular autophagic signaling: Potential role in the pathobiology of Alzheimer's disease

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ABSTRACT

Brain activities of the mitochondrial enzyme α-ketoglutarate dehydrogenase complex (KGDHC) are reduced in Alzheimer's disease and other age-related neurodegenerative disorders. The goal of the present study was to test the consequences of mild impairment of KGDHC on the structure, protein signaling and dynamics (mitophagy, fusion, fission, biogenesis) of the mitochondria. Inhibition of KGDHC reduced its *in situ* activity by 23–53% in human neuroblastoma SH-SY5Y cells, but neither altered the mitochondrial membrane potential nor the ATP levels at any tested time-points. The attenuated KGDHC activity increased translocation of dynamin-related protein-1 (Drp1) and microtubule-associated protein 1A/1B-light chain 3 (LC3) from the cytosol to the mitochondria, and promoted mitochondrial cytochrome c release. Inhibition of KGDHC also increased the negative surface charges (anionic phospholipids as assessed by Annexin V binding) on the mitochondria. Morphological assessments of the mitochondria revealed increased fission and mitophagy. Taken together, our results suggest the existence of the regulation of the mitochondrial dynamism including fission and fusion by the mitochondrial KGDHC activity via the involvement of the cytosolic and mitochondrial protein signaling molecules. A better understanding of the link among mild impairment of metabolism, induction of mitophagy/autophagy and altered protein signaling will help to identify new mechanisms of neurodegeneration and reveal potential new therapeutic approaches.

1. Introduction

Decreased brain utilization of glucose always accompanies Alzheimer's disease (AD) and precedes its clinical manifestations by years (Furst et al., 2012). Reductions in the mitochondrial tricarboxylic acid (TCA) cycle enzymes may underlie the decline in glucose metabolism seen in neurodegeneration (Bubber et al., 2005). Diminished activity of the α-ketoglutarate dehydrogenase complex (KGDHC), critical and often rate-limiting step of the TCA cycle, has been extensively documented in studies of post-mortem brains from patients with AD (Bubber et al., 2005; Gibson et al., 2005). This reduction is highly correlated to the decline in the clinical dementia rating scale in patients with AD (Bubber et al., 2005). Animal models demonstrate that reducing the activity of

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KGDHC by one-half diminishes neurogenesis and memory, and stimulates the formation of AD-like pathology, including plaques and tangles (Karuppagounder et al., 2009; Yang et al., 2009). KGDHC is a thiamine-dependent enzyme and increasing thiamine largely prevents the AD pathology in a mouse model of AD (Pan et al., 2010).

In addition to their traditional and important role of providing energy, mitochondria are the central regulators in cellular protein signaling pathways. Protein signaling modulates mitochondrial fusion and fission, which are necessary for mitochondrial health (Losón et al., 2013; Youle and van der Bliek, 2012). Metabolically compromised mitochondria split to form smaller, spherical mitochondria by fission. Some of these spherical organelles fuse with healthy mitochondria by the process of fusion. Translocation of the cytosolic GTPase, dynamin-related protein-1 (Drp1), to the mitochondria is the critical step to initiate the mitochondrial fission (Chan, 2006). This translocation occurs when Drp1 is modified and activated by phosphorylation, SUMOylation, ubiquitination, S-nitrosylation or O-linked-N-acetyl glucosamine glycosylation. Activated Drp1 translocates to the mitochondria and assembles into helical structures which subsequently induce fragmentation or fission of the organelles (Cereghetti et al., 2008). Even mild oxidative stress can induce a Drp1-dependent increase in fission which promotes mitophagy (Frank et al., 2012). Drp1 is also involved in mitochondrial cytochrome c release (Estaquier and Arnoult, 2007; Kageyama et al., 2014).

Specific mitochondrial protein signaling also modulates autophagy which continuously repairs cells by removing damaged cytosolic components and organelles. Migration of some specific cytosolic proteins is a central part of autophagy that is particularly targeted towards the mitochondria (mitophagy), which in turn influences and promotes the selective survival of the existing healthy mitochondria within the cells (Banerjee et al., 2015; Lemasters, 2005). For example: (a) parkin migration to the mitochondria is a common early step in depolarization-induced mitophagy (Vives-Bauza et al., 2010); (b) microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein which is distributed ubiquitously in mammalian tissues and cultured cells, and upon autophagic activation the cytoplasmic LC3 (LC3I) protein is conjugated to phosphatidylethanolamine to form the LC3-phosphatidylethanolamine conjugate (LC3II) which, in turn, is recruited to autophagosomal membranes (Harris-White et al., 2015); (c) the movement of cardiolipin (CL) from the inner mitochondrial membrane (IMM) to the outer mitochondrial membrane (OMM) recruits LC3 and induces mitophagy (Chu et al., 2013). Depolarization and increased fission of the mitochondria make the organelles more fragmented and favor their selection for mitophagy. The fragmented and small mitochondria can be easily engulfed by autophagosome/lysosome (Gomes and Scorrano, 2013). Damaged mitochondria also release cytochrome c and apoptosis inducing factor (AIF) to the cytosol from the mitochondrial inter membrane space. Cytochrome c activates caspase-dependent apoptosis while, simultaneously, AIF translocates to the nucleus, inducing chromatin condensation and DNA fragmentation in a caspase-independent manner, ultimately culminating in cell death (Yu et al., 2002). Cytochrome c released from compromised and stressed mitochondria can induce caspase-dependent apoptosis, though the release of cytochrome c also occurs in the absence of apoptosis (Ghibelli et al., 1999). Release of cytochrome c can induce non-specific autophagy within the cell. Several different mechanisms can trigger the release of cytochrome c: CL peroxidation, leakage through the mitochondrial permeability transition (MPT) pore complex, Bax and Bak interactions with voltage-dependent anion channel (VDAC), and high K^+ level in extra-mitochondrial matrix (Gogvadze et al., 2006; Liu et al., 2013; Tan et al., 2005).

Since very little is known about the underlying mechanism(s) causing alterations of the cellular functions and the neurodegenerative processes induced by changes in KGDHC activity, a better understanding of these phenomena at the cellular level is required in order to establish the role of KGDHC in the pathophysiology of AD and to identify appropriate novel therapeutic targets. Therefore, in the present study, we examined the consequences of the reduced KGDHC activity on mitochondrial protein signaling using a very specific inhibitor of KGDHC, the carboxy ethyl ester of succinyl phosphonate (CESP) (Bunik et al., 2005) in the human neuroblastoma cell line, SH-SY5Y. We hypothesized that the consequences of mild impairment of KGDHC would be amplified by alterations in protein signaling that control the fusion/fission and mitophagy/autophagy. Our findings suggest the regulation of mitochondrial dynamism by the intracellular KGDHC activity by recruitment of different players of autophagy/mitophagy, and altered protein signaling may be critical in the neurodegenerative process.

2. Materials and methods

2.1. Cell culture

SH-SY5Y human neuroblastoma cells obtained from American Type Culture Collection (Manassas, VA, USA) were maintained at 37 °C in a humidified incubator under 5% CO_2 and 95% air in equal volumes (1:1) of Eagle's Minimum Essential Medium (EMEM) and F12K supplemented with 10% fetal bovine serum (FBS) (Life Technologies, CA, USA). For regular maintenance, cells were grown in 100 mm tissue culture plates. On reaching 70–80% confluence, the cells were trypsinized in 2 ml of 0.05% trypsin-EDTA for 2 min, followed by centrifugation at 200g for 10 min and suspension of the resulting cell-pellet in fresh medium. For subsequent maintenance, the cells were split and 0.5 x 10^6 viable cells, counted by trypan blue method (Banerjee et al., 2014), were seeded in freshly obtained sterile 100 mm tissue culture plates. The culture medium was changed every 2–3 days. All experiments were done using cells with passage numbers less than 20. Unless otherwise mentioned, all materials for cell culture were obtained from Corning, NY, USA.

2.2. Antibodies

Mouse monoclonal antibodies used in the study were anti-LC3 (MBL International, MA, USA), anti-translocase of the inner membrane (TIM23 or anti-Tim23, BD Transduction Lab, CA, USA), Parkin (Santa Cruz, TX, USA) and Drp1 (Santa Cruz, TX, USA), while rabbit polyclonal antibodies used were β-actin (Cell Signaling Technology, MA, USA) and cytochrome c (Santa Cruz, TX, USA). Secondary antibodies used were Licor fluorescent antibodies (Licor, NE, USA).

2.3. Cytochemistry assay for measurement of KGDHC activity in SH-SY5Y cells

SH-SY5Y cells were seeded (0.25×10^5) in 24-well-plates and grown for three days in 1:1 EMEM:F12K medium containing 10% FBS. Cells were rinsed with 1:1 EMEM:F12 media without FBS. Cells were then incubated in the latter media with or without CESP (100 μM) and incubated for 0, 1, 2, 5 or 24 h at 37 °C in a 5% CO_2 incubator. Cells were subsequently rinsed once with a buffered balanced salt solution (BSS) and once with BSS containing 0.05% (v/v) Triton X-100. Cells were incubated for 1 h in incubation buffer (1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.05 mM EDTA, 0.2% Triton X-100, 0.3 mM thiamine pyrophosphate, 5 μg/ml Rotenone, 35 mg/ml Polyvinyl alcohol, 50 mM Tris HCl, pH 7.6) with (experimental group) or without (blank) α-ketoglutarate (KG) and coenzyme A (CoA) for both the control and the CESP treated groups. The final