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Regular Article

Neuroprotective effects of aldehyde dehydrogenase 2 activation in rotenone-induced cellular and animal models of parkinsonism

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ABSTRACT

Many studies have shown that mitochondrial aldehyde dehydrogenase 2 (ALDH2) functions as a cellular protector against oxidative stress by detoxification of cytotoxic aldehydes. Within dopaminergic neurons, dopamine is metabolized by monoamine oxidase to yield 3,4-dihydroxyphenylacetaldehyde (DOPAL) then converts to a less toxic acid product by ALDH. The highly toxic and reactive DOPAL has been hypothesized to contribute to the selective neurodegeneration in Parkinson's disease (PD). In this study, we investigated the neuroprotective mechanism and therapeutic effect of ALDH2 in rotenone models for parkinsonism. Overexpression of wild-type ALDH2 gene, but not the enzymatically deficient mutant ALDH2*2 (E504K), reduced rotenone-induced cell death. Application of a potent activator of ALDH2, Alda-1, was effective in protecting against rotenone-induced apoptotic cell death in both SH-SY5Y cells and primary cultured substantia nigra (SN) dopaminergic neurons. In addition, intraperitoneal administration of Alda-1 significantly reduced rotenone- or MPTP-induced death of SN tyrosine hydroxylase (TH)-positive dopaminergic neurons. The attenuation of rotenone-induced apoptosis by Alda-1 resulted from decreasing ROS accumulation, reversal of mitochondrial membrane potential depolarization, and inhibition of activation of proteins related to mitochondrial apoptotic pathway. The present study demonstrates that ALDH2 plays a crucial role in maintaining normal mitochondrial function to protect against neurotoxicity and that Alda-1 is effective in ameliorating mitochondrial dysfunction and inhibiting mitochondria-mediated apoptotic pathway. These results indicate that ALDH2 activation could be a neuroprotective therapy for PD.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorders, which affects 1 to 2% of the population above 65 years of age, and characterized by resting tremors, muscle rigidity, bradykinesia and postural abnormalities (Lees et al., 2009; Olanow et al., 2009). The neuropathological hallmarks of PD are the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the appearance of cytoplasmic inclusions termed as Lewy bodies. Although the molecular pathogenesis of PD is not completely understood, mitochondrial dysfunction, inflammation, excitotoxicity, elevated oxidative stress, aldehyde-related toxicity, and environmental toxins play important roles in the pathogenesis of PD (Thomas and Beal, 2007; Schapira, 2008; Martin et al., 2011; Tsuboi, 2012). Mitochondrial dysfunction and subsequent oxidative stress result in the generation of reactive oxygen species (ROS). These processes contribute to neurodegeneration via lipid peroxidation, which consequently lead to the production of reactive aldehydes, such as 3,4-dihydroxyphenylacetaldehyde (DOPAL), malondialdehyde (MDA), and 4-hydroxy-2-nonenal (4-HNE) (Ohta et al., 2004; Marchitti et al., 2007). DOPAL, which is an intermediate product of dopamine (DA)

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metabolism, has been suggested to contribute to the pathogenesis of PD (Marchitti et al., 2007; Panneton et al., 2010; Goldstein et al., 2013). 4-HNE is one of the most important products of peroxidation of cellular membrane lipids, or circulating lipoprotein molecules during oxidative stress. The presence of 4-HNE is increased in brain tissue in neurodegenerative disorders such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and PD (Zarkovic, 2003). These reactive aldehydes are neurotoxic and form adducts with proteins, leading to neuronal death in PD.

Aldehyde dehydrogenases (ALDHs; EC1.2.1.3), consisting of 19 putatively functional genes with distinct chromosomal locations in human, represent a group of enzymes that are critical for certain life processes and detoxification via the $NAD(P)^+$-dependent oxidation of a wide range of endogenous and exogenous aldehyde substrates to their corresponding carboxylic acids. Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is highly expressed in heart and brain (tissues with a high mitochondrial content) and is found in the dopaminergic neurons of midbrain (McCaffery and Drager, 1994; Zimatkin and Karpuk Yu, 1996). Within dopaminergic neurons, ALDH2 is the principal enzyme involved in detoxifying aldehydes, including DOPAL, 4-HNE and other ROS-induced aldehydes by converting them to a less toxic acid products (Ohta et al., 2004; Marchitti et al., 2007; Chen et al., 2014; Doorn et al., 2014). The prevalent single point mutation of human ALDH2 gene found in a large population of the East Asian countries substitutes a glutamate with lysine at amino acid position 504 (E504K, formerly E487K that the 17-amino acid mitochondrial targeting sequence is not included). The E504K mutant allele, denoted as ALDH2*2, accounts for 35–57% of East Asians (Li et al., 2009). The ALDH2*2 mutation results in a drastic reduction in ALDH2 enzymatic activity to about 1–3% in homozygous ALDH2*2/*2 individuals and 17–38% in heterozygous ALDH2*1/*2 individuals as compared to the wild type individuals (denoted as ALDH2*1/*1) (Yoshida et al., 1984; Lai et al., 2014). Epidemiological studies suggest that ALDH2 E504K mutation increases the risk of Alzheimer's disease among East Asian men (Hao et al., 2011). Transgenic mice over-expressing ALDH2*2 have shortened lifespan and exhibit age-dependent neurodegeneration and hyperphosphorylation of tau in hippocampal neurons (Ohsawa et al., 2008). In addition, $Aldh1a1^{-/-} \times Aldh2^{-/-}$ knockout mice exhibit age-dependent deficits in motor performance responsiveness to levodopa and a significant loss of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra (Wey et al., 2012). These data indicate that ALDH2 likely plays an important role in the pathogenesis of neurodegenerative diseases including PD.

In the present study, we investigated the protective effect of ALDH2 activation in rotenone-induced cellular and animal models for parkinsonism. Our data revealed that over-expression of ALDH2 enzyme confers a neuroprotective effect against rotenone-induced neuronal death and that ALDH2 small molecule activators, like Alda-1, may serve as a therapeutic agent for PD in the future.

Materials and methods

*Stable expression of wild-type (WT) ALDH2 or mutant ALDH2*2 in SH-SY5Y cells*

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium supplemented with Nutrient Mixture F-12 (Ham) (1:1, v/v) (Gibco, Gaithersburg, MD, USA), 2 g/L sodium bicarbonate (Sigma-Aldrich), 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS). Cells were grown at 37 °C in a humidified air with 5% CO_2. SH-SY5Y cells were grown to 80–90% confluence and then sub-cultured into different culture plates. The cDNAs of WT ALDH2 (GenBank ID: BC002967) and (E504K) ALDH2*2 mutant were constructed and subcloned into a mammalian expression vector pcDNA3 (Invitrogen) containing FLAG-tag sequences (DYKDDDDK). Both plasmids were transfected to SH-SY5Y cells using Lipofectamine 2000 (Invitrogen). Two days after transfection, SH-SY5Y cells stably expressing WT ALDH2 and mutant ALDH2*2 were selected in the presence of 1.5 mg/ml G418 (Geneticin sulfate) (Sigma-Aldrich). Positive clones were confirmed by Western blot analysis as described below and maintained in the medium containing 1 mg/ml G418.

Preparation of primary culture of substantia nigra dopaminergic neurons

Primary cultured substantia nigra (SN) dopaminergic neurons were prepared as described previously (Wang et al., 2011). Briefly, substantia nigra was dissected from embryonic days 12 to 15 rats and cultured in DMEM/F12 medium containing pronase (0.5 mg/ml, Roche) and DNase I (0.3 mg/ml, Roche) for 50 min at 37 °C. Tissue fragments were subsequently triturated, and dissociated cells were plated onto poly-L-lysine and collagen-coated six well dishes. SN neurons were cultured in DMEM/F12 medium supplemented with 5% fetal bovine serum, 5% horse serum and GDNF (glial cell line-derived neurotrophic growth factor; 25 ng/ml). 5′-fluoro-2′-deoxyuridine and uridine, which prevent proliferation of glial cells, were added into culture medium after 24 h. Primary neuronal culture of substantia nigra consisted mainly of large oval-shaped dopaminergic neurons and small non-dopaminergic cells. In accordance with our previous studies (Wang et al., 2007, 2011), two subpopulations of neurons, large multipolar or oval-shaped tyrosine hydroxylase (TH)-positive dopaminergic cells (diameter = 25–30 μm), which was confirmed by TH immunostaining (data not shown), and small non-dopaminergic cells (diameter = 15–20 μm), were found in SN neuronal culture.

Immunoblot analysis

Protein samples were prepared by homogenization of SH-SY5Y cells or SN neurons in SDS sample buffer. Subsequently, protein lysate or the immunocomplex was separated on 8 or 10% SDS-polyacrylamide gel and transferred to PVDF membrane. Then, the membrane was incubated at 4 °C overnight with one of the following diluted primary antibodies: (1) Anti-FLAG monoclonal antiserum (Sigma-Aldrich), (2) polyclonal anti-complex IV antibody (Sigma-Aldrich), (3) polyclonal anti-cleaved active caspase 9 antibody (Cell Signaling Technology), (4) polyclonal anti-cleaved active caspase 3 antiserum (Cell Signaling Technology), (5) polyclonal anti-Bax antibody (Cell Signaling Technology), and (6) polyclonal anti-cytochrome c antiserum (Abcam). After washing, the membranes were incubated with horse anti-mouse, anti-donkey or anti-rabbit horseradish peroxidase (HRP) linked secondary antibodies. Then, immunoreactive proteins were visualized by using an enhanced chemiluminescence kit (GE Biosciences). To confirm equal amount of protein sample loading, membranes were stripped and reblotted with monoclonal anti-actin antibody (Chemicon). Gel bands were quantified by a densitometer (Molecular Dynamics Model 375A) and normalized by reprobing the same blot for the actin signals.

Subcellular expression of FLAG-tagged ALDH2

To analyze the subcellular distribution of ALDH2, mitochondrial and cytosolic fractions of SH-SY5Y cells expressing WT or (E504K) ALDH2*2 were prepared. Briefly, stable clones were lysed in ice-cold buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH7.3, 1 mM EGTA, 1 mM DTT, 5 μg/ml pepstain, 5 μg/ml leupetin, 5 μg/ml aprotinin, and 0.3 mM PMSF. Cells were homogenized using a Dounce tissue grinder on ice, and cell lysate was centrifuged at 500 ×*g* for 10 min at 4 °C. The supernatant was centrifuged at 9500 ×*g* for 9 min at 4 °C to pellet the mitochondrial fraction. The resulting supernatant was further centrifuged at 16,000 ×*g* for 20 min at 4 °C, and the final supernatant was used as cytosolic fraction. Protein samples (15 μg) of mitochondrial or cytosolic extracts were separated on a 10% SDS polyacrylamide gel and transferred to PVDF membrane. Then, FLAG tagged ALDH2 on the membrane was visualized by using a ECL protocol as described above. To verify the purity of mitochondrial fraction and