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The Kunitz-protease inhibitor domain in amyloid precursor protein reduces cellular mitochondrial enzymes expression and function

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ABSTRACT

Mitochondrial dysfunction is a prominent feature of Alzheimer's disease (AD) and this can be contributed by aberrant metabolic enzyme function. But, the mechanism causing this enzymatic impairment is unclear. Amyloid precursor protein (APP) is known to be alternatively spliced to produce three major isoforms in the brain (APP695, APP751, APP770). Both APP770 and APP751 contain the Kunitz Protease Inhibitory (KPI) domain, but the former also contain an extra OX-2 domain. APP695 on the other hand, lacks both domains. In AD, up-regulation of the KPI-containing APP isoforms has been reported. But the functional contribution of this elevation is unclear. In the present study, we have expressed and compared the effect of the non-KPI containing APP695 and the KPI-containing APP751 on mitochondrial function. We found that the KPI-containing APP751 significantly decreased the expression of three major mitochondrial metabolic enzymes; citrate synthase, succinate dehydrogenase and cytochrome c oxidase (COX IV). This reduction lowers the NAD^+/NADH ratio, COX IV activity and mitochondrial membrane potential. Overall, this study demonstrated that up-regulation of the KPI-containing APP isoforms is likely to contribute to the impairment of metabolic enzymes and mitochondrial function in AD.

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1. Introduction

A key neuropathological feature of Alzheimer's disease (AD) is the presence of extracellular amyloid plaques composed of the amyloid β-peptide, which is derived from the transmembrane amyloid precursor protein (APP) [1,2]. APP is encoded by a single 19-exon gene on chromosome 21 [3]. Exons 7, 8 and 15 of the APP gene can be alternatively spliced to produce multiple isoforms. In the brain, there are three major APP isoforms (APP695, APP751, APP770) [4], and APP695 is the predominant splice variant. These transcripts code for protein species containing 695, 751 and 770 amino acids, respectively. Exons 7 and 8 specifically encode a 56-amino acid Kunitz-type Proteinase Inhibitor (KPI) domain and a 19-amino acid domain that shares sequence identity with the OX-2 antigen of thymus-derived lymphoid cells. Both APP770 and APP751 contain the KPI domain, but the former also contain an extra OX-2 domain. APP695 on the other hand, lacks both domains.

Studies have reported that the KPI-containing APP isoforms (APP751/770) are up-regulated in AD brain [5–8]. Several functions have been suggested for the KPI domain on APP [9–11], including the inhibition of serine proteases. Further, cells expressing APP with and without the KPI domain (APP695, APP751) was found to have different susceptibility toward α-and β-secretase cleavage, affecting Aβ production [12,13].

Mitochondrial dysfunction is a prominent feature in Alzheimer's disease (AD) [14,15] and this deficit is likely contributed by defective expression and function of metabolic enzymes, leading to increased production of free radicals [16–21]. However, the cause(s) leading to the down-regulation of these metabolic enzymes is unclear. Furthermore, the functional connection between the increasing generation of the KPI-containing APP isoforms and the detected mitochondrial dysfunction is unknown.

In this study, we have expressed APP695 and APP751 in the APP-null neuronal cell line [22], to compare the effect of these two APP isoforms on mitochondrial function. We found that the KPI-containing APP751 lead to lower expression of major mitochondrial metabolic enzymes. This reduction leads to lower mitochondrial membrane potential, cytochrome c oxidase activity and NAD/NADH ratio in cells expressing APP751.

2. Materials and methods

2.1. Plasmids, cell culture and transfection

The cDNAs for human APP695 (KPI-APP) and APP751 (KPI + APP) bearing the C-terminal Swedish mutations [23] were kindly provided by Dr. Man-Sun Sy (Case Western Reserve

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University, USA) and cloned into the expression vector pcDNA6.2-DEST (Life Technologies).

The APP-knockout (APP-KO) cell line [22] was grown in DMEM supplemented with 10% fetal bovine serum, 5% penicillin–streptomycin-amphotericin B and 5% sodium pyruvate, and maintained at 37 °C in a humidified incubator supplied with 5% CO_2. Expression vectors containing no insert (mock), APP695 and APP751 were stably transfected into the APP-KO cells using Fugene 6 reagent (Roche) according to manufacturer's instructions. Selection for cells containing the required construct was performed in DMEM with 5 μg/ml blasticidin (Life Technologies). Selected clones were maintained in DMEM containing 2.5 μg/ml blasticidin (Life Technologies).

2.2. SDS–PAGE and Western blot analysis

Cells were lysed in ice-cold 1× RIPA buffer (Cell Signaling Technology) containing protease inhibitors cocktail tablet (Roche) before subjecting to brief sonication and centrifugation. Cellular samples (30 or 40 μg of protein) were resolved on 7–15% tris–glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane (Biorad). The membranes were probe with the respective primary antibodies before incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. The reactive protein bands were visualized by chemiluminescence using the SuperSignal® West Dura Substrate (Pierce) system. Pre-stained Precision Plus protein standards (Biorad) were used to calculate the apparent molecular weight of the protein bands.

Immunoblotting of APP was performed using two anti-APP antibodies. Monoclonal antibody 22C11 (Millipore) recognize the N-terminal of human APP (REFS) and 6E10 (Covance) recognizes the C-terminal of human APP (REFS).

Immunoblotting of β-actin using a rabbit polyclonal antibody that binds to the C-terminal of β-actin (Sigma) was included in all Western blot analysis to ensure comparable protein loading. Each immunoblotting was repeated up to three times using different preparations of the same cell line.

2.3. Real-time quantitative PCR

Total RNA was isolated from the cells using TRIzol® reagent (Life Technologies) before conversion to cDNA using the AMV reverse transcriptase (Promega). Expression level of the following human and mouse genes (and the TaqMan Gene Expression Assay catalogue) was investigated using real-time quantitative PCR and TaqMan probe-based chemistry (Applied Biosystems); human APP695 (Hs01562345_m1), human APP751 (Hs01562342_m1), human APP (Hs01552283_m1), mouse citrate synthase (Mm00466043_m1), mouse succinate dehydrogenase (Mm00458268_m1), mouse cytochrome C oxidase (Mm01250094_m1) and mouse β-actin (Mm00607939_s1). These probes span the exon(s) of the targeted

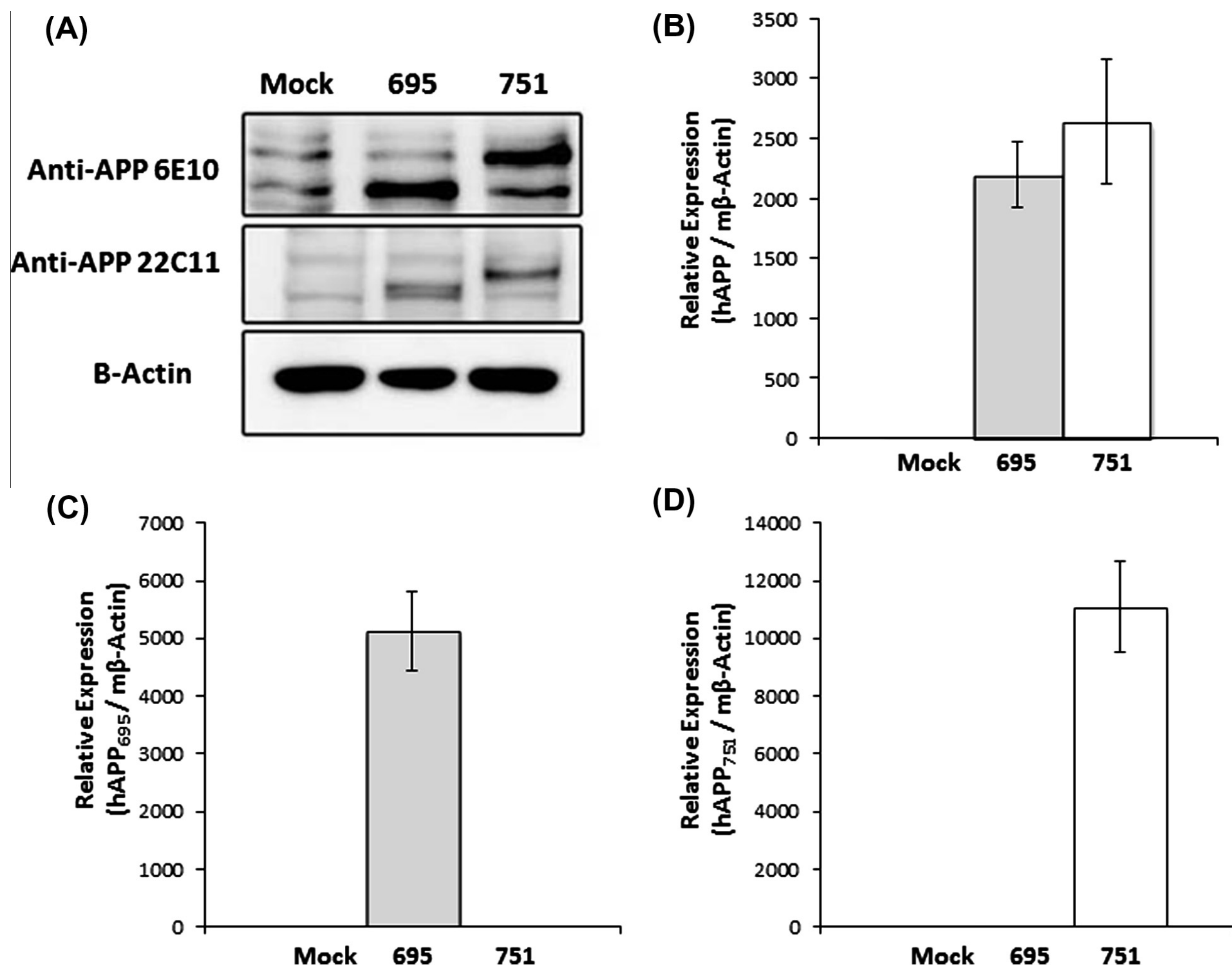

Fig. 1. APP isoforms expression. (A) Immunoblotting of APP in the mock-transfected cell line and transfected cells expressing APP695 and APP751. APP was immune-detected using anti-APP 6E10 and anti-APP 22C11. β-actin was immunoblotted to ensure similar gel loading of the starting material in each sample. Real-time PCR quantification of (B) total APP, (C) APP695 and (D) APP751 expression in the stably-transfected mock, APP695 and APP751 expressing cell lines. The expression of the targeted gene was quantified relative to the endogenous β-actin level for each sample. Each value represents the mean ± SEM of three assays for each cell line sample.

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