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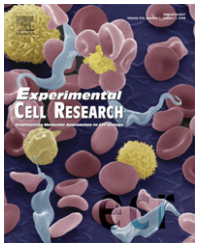

Research Article

Increased AICD generation does not result in increased nuclear translocation or activation of target gene transcription

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ABSTRACT

A sequence of amyloid precursor protein (APP) cleavages culminates in the sequential release of the APP intracellular domain (AICD) and the amyloid β peptide (Aβ) and/or p3 fragment. One of the environmental factors favouring the accumulation of AICD appears to be a rise in intracellular pH. Here we further identified the metabolism and subcellular localization of artificially expressed constructs under such conditions. We also co-examined the mechanistic lead up to the AICD accumulation and explored possible significances for its increased expression. We found that most of the AICD generated under pH neutralized conditions is likely cleaved from C83. While the AICD surplus was unable to further activate transcription of a luciferase reporter via a Gal4-DNA-binding domain, it failed entirely via the endogenous promoter regions of proposed target genes, APP and KAI1. The lack of a specific transactivation potential was also demonstrated by the unchanged levels of target gene mRNA. However, rather than translocating to the nucleus, the AICD surplus remains membrane tethered or free in the cytosol where it interacts with Fe65. Therefore we provide strong evidence that an increase in AICD generation does not directly promote gene activation of previously proposed target genes.

Introduction

Alzheimer's disease remains one of the most prevalent brain disorders of our time. The disease itself is characterized by the extracellular accumulation of Aβ plaques within the brain parenchyma and cerebral blood vessels [1]. The amyloid precursor protein (APP) is the parent protein to the toxic Aβ peptide. APP undergoes a set of sequential cleavages undertaken by a group of enzymes, collectively known as secretases. α- and β-secretase cleave APP to release its large extracellular domain with the concomitant generation of membrane anchored stubs [2–6]. These stubs can be subsequently processed by γ-secretase to release Aβ and/or p3 (γ-cleavage) [7,8]. In addition, this cleavage event liberates the APP intracellular

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domain (AICD) (ε-cleavage), which might contain a few amino acids from the transmembrane region (TMR) followed by the cytoplasmic tail of APP [9,10]. Cleavage of Notch is highly analogous to that of APP and the release of its intracellular domain (NICD) is critical for the transcription of genes involved in early embryonic development [11,12]. This prompted the notion that the AICD may also play such an important functional role [13]. Indeed, it has been shown that AICD can be stabilized by the nuclear adaptor protein Fe65 [14]. The latter two proteins together with Tip60, a histone acetytransferase, form a nuclear complex, which regulates the expression of artificial expression constructs in transfected cells [13]. It was later shown that translocation of AICD to the nucleus was in fact not necessary for transcriptional activation [10]. The authors rather showed that membrane tethered AICD, as opposed to AICD free in the cytosol, recruits and activates Fe65 and upon cleavage by γ-secretase both, AICD and active Fe65, translocate to the nucleus [10]. Although many putative target genes for AICD have been documented, much controversy surrounds the part played by AICD in the transcription of these genes [15–20].

Mounting evidence suggests that the sequential cleavage events liberating Aβ/p3 and AICD can be differentially regulated [21–24]. A better understanding of the regulators governing these cleavage events could point to alternative routes for the use of γ-secretase inhibitors, perhaps in combination with other therapeutic strategies. The study of Aβ versus AICD production is hampered by the high liability of AICD, which is why most investigators employ in vitro assays [14,25]. These assays involve membrane isolation and incubation at temperatures optimal for enzyme activity. While these assays have been useful in determining the ability of a compound to inhibit γ-secretase, other interpretations should be considered cautiously, because they are conducted outside the cell and therefore do not address what happens inside an intact cellular environment.

Strong evidence supports the endosomal to lysosomal degradation route as key compartments leading to Aβ generation [26]. Moreover, these compartments are likely organelles where AICD is also generated. It was recently shown that AICD accumulates while Aβ levels are reduced under alkalizing conditions [27]. The authors later showed that APP and the accumulating catabolites, AICD and APP C-terminal fragments (CTFs) were present in luminal vesicles of multivesicular endosomes and also in exosomes [28]. In our study, we sought to explain the mechanistic build up to the AICD accumulation and its potential functional activity in target gene induction. We found that the AICD surplus was likely that cleaved from C83 and not C99 but did not result in an overall increase in transcriptional activity. In our hands the AICD surplus was still membrane tethered or free in the cytosol where it interacted with Fe65. The evidence presented here suggests that an overall increase in AICD level would not be detrimental with regard to its potential to further induce transcription of certain genes since we were unable to observe any APP or KA1 promoter activity. Additionally we re-examined multiple postulated AICD target genes, which in our hands did not respond to different AICD concentrations. The data documented here may rather point to altering the environmental working conditions of γ-secretase as an attractive method for therapeutic targeting.

Materials and methods

Antibodies, cell lines and cell culture

Polyclonal anti-APP antibody CT15, which reacts with the cytoplasmic domain of APP, has been described previously and was used for the detection of full length APP, APP-CTFs, AICD and APP Gal 4 metabolites [29]. Monoclonal Flag antibody against the flag epitope sequence was purchased from Sigma and the monoclonal 9E10 antibody against the myc epitope sequence was produced from a hybridoma cell line. Monoclonal antibody 26D6 which reacts with the ectodomain of APP used to detect secreted Aβ and APPsα has been described previously [30]. We used CHO cells either stably over-expressing wild type human APP_{751} (7WD10) alone or stably co-expressing human mutant PS1 (M146L) (7WML). CHO K1 cells which were a kind gift from Dr. S. Leppla. C99 was inserted into the pLHCX retroviral expression vector and transferred into the HEK GP2 packaging cell line. After infection with recombinant viruses, CHO K1 cells were selected with hygromycin. CHO cells were cultured in α-Dulbecco's modified medium (Cambrex Bio Science Verviers, Belgium) containing 10% fetal bovine serum (FCS) (Gibco), 1 mM MEM sodium pyruvate (Gibco) and 1× antibiotics/antimycotics (penicillin G, sodium, streptomycin sulphate, amphotericin B) (Gibco) in a humidified 5% CO_2 incubator at 37 °C.HEK 293 cells stably co-expressing an N-terminally flag-tagged and a C-terminally myc-tagged mNotchΔE construct and APP Swedish (FNEXTsw) were maintained in Dulbecco's modified medium (Gibco) supplemented with 10% FCS, 1 mM MEM sodium pyruvate, and 1× antibiotics/antimycotics. Cell lines were transfected when 90% confluent with equal amounts of DNA for 24 h using Lipofectamine 2000 according to standard protocols (Invitrogen, Carlsbad, CA, USA).

cDNA constructs

pCDNA3-Fe65 has been described previously [6]. Plasmids encoding the Gal4 DNA-binding domain (pMST), the Gal4 DNA-binding domain engineered into the APP695 cytoplasmic tail (pMST-APP), the APP Gal 4 fusion protein with a mutation in the NPTY motif of the APP cytoplasmic tail pMST-APP*, and a Gal4 reporter plasmid encoding firefly luciferase (pG5E1B-luc) have been described and were a kind gift from Dr. T. Sudhof (Dallas, Texas). Plasmid pRL-TK encoding *Renilla* luciferase was from Promega (Madison, WI, USA), and pcDNA3 was purchased from Invitrogen (Carlsbad, CA, USA). p12-C99 has been described previously and was a kind gift from Dr. S.F Lichtenthaler [31]. The C99 fragment of this construct was cloned by PCR into the pLHCX vector that was digested with Hind III/Cla I. The identity of the construct obtained by PCR was confirmed by DNA sequencing. pGL3-APP-Luc, the regulatory promoter region of APP, pGL3-KAI1-Luc, the KAI1 promoter and pGL3-luciferase empty vector (that is, no promoter) were used as described before [18]. Estrogen receptor α (ERα) was a kind gift from Dr. Manthey [32].

Treatments

To investigate the effect of a pH neutralized environment of APP metabolites, media from cells was replaced with fresh media containing either vehicle (H_2O) or 10 mM NH_4Cl and