Effects of RNA Splicing Inhibitors on Amyloid Precursor Protein Expression

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ABSTRACT: U1 small ribonucleoproteins demonstrate proteopathy in Alzheimer’s disease, and their inhibition modulates the expression of the amyloid precursor protein (APP). We sought to determine whether this effect on the APP expression is a universal result of different kinds of RNA splicing inhibitions. We treated cells with two chemical RNA splicing inhibitors: isoginkgetin (IGK) and spliceostatin A (SSA), in which SSA reduced the APP expression, whereas IGK substantially increased it. The following western blot and reverse transcription polymerase chain reaction analyses showed that the APP expression under the IGK treatment has distinct protein forms, but the total mRNA level was nearly unchanged despite a slight switch within its three major transcripts. Further analysis revealed that the APP-increasing effect of IGK depended on protein translation and might involve inhibition in the degradation system. By immunocytochemistry, the APP likely redistributed from Golgi to endoplasmic reticulum (ER) in cells treated with IGK. When compared to the well-characterized ER-to-Golgi transport inhibitor brefeldin A, IGK showed similar APP expression patterns on the western blot. In summary, we not only determined the diverse effects of RNA splicing inhibition on the APP expression but also found the additional function of IGK on protein subcellular traffic.

INTRODUCTION

Alzheimer’s disease (AD) is an aging-related irreversible neurodegenerative disorder. AD brains are hallmarked by the extracellular amyloid plaques and the intracellular tangles. The major components of the plaques are the Aβ peptides generated from the amyloid precursor protein (APP). Currently, all three AD familial genes (APP, PSEN1, and PSEN2) are directly involved in the Aβ generation, which, in combination with other genetic evidence, confirms the essential role of the amyloid cascade in the AD pathogenesis.

We have discovered the AD-specific, widespread, and early occurring proteopathy of the U1 small ribonucleoprotein (snRNP) complex and the concomitant RNA splicing deficiency in AD. Importantly, U1 inhibition by U1-70K knockdown was intriguingly found to increase APP expression and Aβ production, providing an important clue to the mechanism of Aβ accumulation in AD.

We therefore sought to determine whether this APP-increasing effect is a general result of all different kinds of RNA splicing inhibitions or is only specific to the U1 inhibition. In this study, we used two chemical RNA splicing inhibitors isoginkgetin (IGK) and spliceostatin A (SSA) to treat cells and then examined the APP expression.

RESULTS

Effects of IGK and SSA on the APP Expression. IGK is a RNA splicing inhibitor that arrests spliceosome assembly by retaining pre-mRNA in complex A. SSA is another potent RNA splicing inhibitor that targets the SF3b complex. We first treated HEK293T cells in triplicates with IGK at the dose (30 μM) according to the effective concentration in the report, using the solvent dimethyl sulfoxide (DMSO) as the control for 12 h. In the result of the western blot, the IGK treatment showed a substantial increase of APP expression as compared to the control treatment (Figure 1A). Quantitative analysis of the results from more experiments confirmed the difference between IGK and the control treatments with statistical significance (Student’s t-test, P < 0.05). However, in the SSA treatment (25 nM, HEK293T, for 6 h), the APP expression was largely inhibited and all three independent experiments yielded the similar results (Figure 1B). On the basis of these results, it suggests that the effects of RNA splicing inhibition on the APP expression are not universally consistent. Therefore, the modulation of the APP expression by U1 snRNP inhibition might be unique to itself.

Analysis of APP and RNA Under IGK Treatment. The effect of IGK on the APP expression was intriguing. Because HEK293T is a human kidney cell line, whereas APP/Aβ pathology is mainly related to brain neurons, we therefore repeated the IGK treatment on the SK-N-SH neuroblastoma cells. In the results, both HEK293T and SK-N-SH cells demonstrated remarkable increase of the APP expression in a
dose-dependent manner (Figure 2A), indicating that the effect of IGK also likely remains on neurons.

At the mRNA level, APP has three major transcripts: APP770, APP751, and APP695, which differ in the inclusion of their exons 7 and 8 (Figure 2A).9 At the protein level, APP has three major forms expressed by these mRNA transcripts with posttranslational modifications. The newly synthesized protein APP in endoplasmic reticulum (ER) is "immature" and will move to Golgi for full glycosylation to become "mature".10 On the western blot, APP usually appears in three major protein bands: the immature form of APP695 at the bottom, the mixture of mature APP695 and immature APP751/770 in the middle, and the mature APP751 and APP770 on the top.9

In the IGK treatment, the bottom band remained unshifted but the top and the middle APP bands were replaced with an extra band occurring between them (Figure 2C), suggesting a new protein isoform or distinct modifications. We therefore performed reverse transcription polymerase chain reaction (RT-PCR) using primers targeting the region between exons 6 and 9 to differentiate the three transcripts. We found that the expression of APP770 was reduced and APP695 was increased, consistent with the effect of IGK as a RNA splicing regulator.

However, the total APP RNA level, according to the relative intensities of the three transcript bands resolved on the 1% agarose gel, was not obviously changed. This indicates that the increased APP expression induced by IGK is not directly due to the upregulated mRNA level. Because there seemed no other novel transcripts and all of the three transcripts were still abundant, the APP form change induced by IGK could not be explained by the mechanism of transcript isoform switch.

IGK Inhibits the APP Degradation Process. As a membrane protein, APP is first synthesized in the ER and then transported to Golgi for further processing prior to sorting to the cell membrane.11 When activated, the membrane APP is endocytosed until finally proteolyzed or recycled back into the Golgi (Figure 3A).

We first determined whether the effect of IGK on the APP expression is through protein synthesis using the protein translation inhibitor cycloheximide (CHX). In the experiment, CHX (20 μg/mL) almost completely abolished the APP expression in both the DMSO control and the IGK treatment at different doses (Figure 3B), indicating that the effect of IGK relies on the protein synthesis.
We then examined whether the APP degradation was involved in the effect of IGK. Cells were first treated with 30 μM IGK for 9 h to accumulate sufficient amount of APP, and then the culture medium was refreshed with the addition of 20 μg/mL CHX (a translation inhibitor). By western blotting, we found that the accumulated APP disappeared in about 3 h in the control, whereas MG-132 and baflomycin A1 slowed down this degradation process. IGK similarly reduced the APP degradation with the demonstration of enhanced efficiency (Figure 3C), suggesting additional or other distinct mechanisms being involved. These imply that IGK increases APP possibly through the inhibition of APP degradation.

Figure 3. Effect of IGK on APP is likely through the blockage of protein degradation. (A) Schematic diagram of APP intracellular traffic. Newly synthesized APP from ER is transported to Golgi for processing and sorting to the cell membrane. APP on the cell surface will then be endocytosed or recycled back into Golgi. (B) Protein translation inhibition abolished the IGK’s APP-increasing effect. HEK293T cells were treated with IGK in the presence or absence of 20 μg/mL CHX (a translation inhibitor). (C) Effects of proteasome and lysosome inhibitors on APP degradation. HEK293T cells were first treated by IGK for 9 h to accumulate APP, and then IGK was replaced with the indicated chemicals for 12 h. MG-132, a proteasome inhibitor, 100 μM; Baf A1, baflomycin A1, a lysosome inhibitor that prevents acidification, 0.2 μM. IGK, 30 μM. Ponceau S staining of membrane was used for the demonstration of protein sample loading.

Figure 4. Subcellular redistribution of APP under IGK treatment. APP is likely retained in the ER by IGK. SK-N-SH cells were used. Antibodies: anti-APP (Y188), anti-GM130 (Golgi marker), anti-KDEL (ER marker), anti-M6PR (mannose-6-phosphate receptor, late endosome marker), and anti-EEA1 (early endosome marker). 4′,6-Diamidino-2-phenylindole: fluorescent nucleus marker.
IGK Regulates APP Subcellular Traffic. To see the intracellular distribution of APP within cells under the IGK treatment, we treated SK-N-SH cells with IGK (30 μM) for 6 h and performed double fluorescent immunocytochemistry, using a specific APP antibody (Y188)12 and other antibodies that target Golgi (GM130),13 ER (KDEL),14 early endosome (EEA1), and late endosome (M6PR).12 In the control, APP appeared to be localized in the Golgi with diffusive distribution in the ER, the early and the late endosomes (Figure 4). This is consistent with the APP subcellular distribution and trafficking normally (Figure 3A).11,15 However, when the cells were treated with IGK, the APP became mainly colocalized with the ER staining, suggesting its redistribution from Golgi to ER.

It seems that APP is arrested by IGK in the ER where it starts accumulating. This explains the remarkable increase of APPs and the inhibition of their degradation under the IGK treatment. Because its transport to the Golgi for glycosylation and the inhibition of their degradation under the IGK treatment. Its ER staining suggests its redistribution from Golgi to ER. This is consistent with the APP subcellular distribution and trafficking normally (Figure 3A).11,15 However, when the cells were treated with IGK, the APP became mainly colocalized with the ER staining, suggesting its redistribution from Golgi to ER. It seems that APP is arrested by IGK in the ER where it starts accumulating. This explains the remarkable increase of APPs and the inhibition of their degradation under the IGK treatment. Because its transport to the Golgi for glycosylation and the inhibition of their degradation under the IGK treatment. Its ER staining suggests its redistribution from Golgi to ER. This is consistent with the APP subcellular distribution and trafficking normally (Figure 3A).11,15 However, when the cells were treated with IGK, the APP became mainly colocalized with the ER staining, suggesting its redistribution from Golgi to ER.

IGK Is Similar to Brefeldin A in the Effect on the APP Expression. As IGK is suspected to prevent APP traffic from ER to Golgi, we compared it to the well-established ER-to-Golgi blocker brefeldin A.16 IGK has an overall distinct structure from brefeldin A but might bear some similar chemical groups at certain local regions (Figure 5A). When we treated cells with these two chemicals, a similar time-coursed APP expression pattern was elicited (Figure 5B), suggesting that IGK might have a similar effect of brefeldin A.

■ DISCUSSION

In this study, we investigated the effects of RNA splicing inhibition by IGK and SSA on the APP expression, and they showed diverse results. We also found a unique function of IGK besides its function of RNA splicing inhibition.

The effects of IGK and SSA on the APP expression that we observed in this study are distinct from that of U1 snRNP inhibition. Unlike U1-70K knockdown, SSA inhibited the APP expression directly. Although IGK increased the APP expression, the protein level and forms seemed clearly different from those demonstrated in the U1 inhibition.4 These suggest that the effect of U1 inhibition on the APP expression is unique and thus cannot be extended as a universal result of all general RNA splicing inhibitions. This is consistent with the fact that U1 alteration and APP pathology are unique to AD.

The mechanism of IGK in regulating protein ER-to-Golgi traffic remains unclear. It might act through RNA regulation to downregulate the proteins that are involved in this traffic, such as ADP-ribosylation factors or the protein targets of brefeldin A.17,18 Regardless of the mechanism, it is reasonable to believe that this effect also remains on other membrane proteins. This might be another mode of action of IGK besides its function of RNA splicing inhibition as an anticancer drug.19–21

■ METHODS

Cell Culture and Treatment. HEK293T and SK-N-SH cells were cultured in the Dulbecco’s modified Eagle’s medium (11965-092, Life Technologies) supplemented with 10% fetal bovine serum and antibiotics that included 100 units/mL penicillin and 100 μg/mL streptomycin (15140-122, Life Technologies). Cells were maintained at 37 °C and 5% CO2 in a humidified incubator and subcultured at 20–30% confluent density.

In RNA splicing inhibition experiments, cells were plated at ~40% confluent density and then cultured for 24 h before the addition of chemicals. IGK was purchased (416154, Merck Millipore). Chemicals used in other experiments were CHX (239764, Calbiochem), bafilomycin A1 (B1793, Sigma-Aldrich), MG-132 (1748, Boston Biochem), and brefeldin A (9972S, Cell Signaling Technology).

Western Blotting. In western blotting, cells were lysed in the radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100) with 1× protease inhibitor cocktail (11697498001, Roche). The protein concentration in each sample was quantified by the bicinchoninic acid assay. Sample proteins were separated by NuPAGE 4–12% Bis–Tris precast gels (Life Technologies) or self-made Tris–glycine gels and then transferred to the nitrocellulose membrane (0.2 μm

Figure 5. Similar APP expression pattern by brefeldin A, a protein ER-to-Golgi traffic blocker. (A) Chemical structures of isoginkgetin and brefeldin A. (B) Western blots of time-coursed APP expression in HEK293T cells treated with 30 μM IGK, 5 μM brefeldin A, and DMSO.
pore size, 162-0112, Bio-Rad). Proteins on the membrane were stained by Poncet S (6226-79-5, Sigma-Aldrich) to examine the success of electrophrop and sample loading equality. The membrane was then blocked by 5% nonfat milk in 1× Tris-buffered saline-Tween 20 (TBST; 50 mM Tris—Cl, pH 7.4, 100 mM NaCl, 0.05% Tween-20) for about 15 min and then incubated with primary antibodies in 3% bovine serum albumin (BSA) in 1× TBST for 2 h or overnight. The membrane was then washed by 1× TBST and further incubated with horseradish peroxidase-conjugated secondary antibodies in 2.5% milk. The chemical substrate (SuperSignal West Pico, 34087, Thermo Scientific) was used to develop signals on films.

**Immunocytochemistry.** Cells were grown on coverslips coated by poly-d-lysine (for HEK293T cells) or by collagen (for SK-N-SH cells). They were washed by phosphate-buffered saline (PBS) before being blocked in 10% normal goat serum (50-0622, Life Technologies) for 30 min. After that, the coverslips were incubated with antibodies in 1% BSA in TBS with 0.01% Triton X-100 for 1 h. The cells were permealized by 0.25% Triton X-100 in 1× Tris-buffered saline (TBS) before being blocked in 10% normal goat serum (50-0622, Life Technologies) for 30 min. After that, the coverslips were incubated with antibodies in 1% BSA in TBS with 0.01% Triton X-100 for 1 h. The cells were permealized by 0.25% Triton X-100 in 1× Tris-buffered saline (TBS) before being blocked in 10% normal goat serum (50-0622, Life Technologies) for 30 min. After that, the coverslips were incubated with antibodies in 1% BSA in TBS with 0.01% Triton X-100 for 1 h. The cells were permealized by 0.25% Triton X-100 in 1× Tris-buffered saline (TBS) before being blocked in 10% normal goat serum (50-0622, Life Technologies) for 30 min. After that, the coverslips were incubated with antibodies in 1% BSA in TBS with 0.01% Triton X-100 for 1 h.

**Antibodies.** Antibodies used in this study included: anti-APP (22C11, MAB348, Merck Millipore and Y188, 1565-1, Epitomics), anti-KDEL (10C3, ab12223, Abcam), anti-GM130 (ab52649, Abcam), anti-M6PR (ab2733, Abcam), anti-EEA1 (ab70521, Abcam), and anti-β tubulin (E7, Developmental Studies Hybridoma Bank).

**RT-PCR.** The RNA was extracted from HEK293T cells using an RNeasy Mini Kit (74104, Qiagen) and synthesized into the first DNA strand by SuperScript III reverse transcriptase (18080-051, Life Technologies). PCR was done by Choice Taq Mastermix (CB4070-8, Denville Scientific). The forward primer that targeted APP exon 6 was “AGACAGTGCAAATTCTTA-CCAG”; and the reverse primer targeting APP exon 9 was “AGCGGGAGATCATTGCTCGG”.

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