Different Eukaryotic Initiation Factor 2Be Mutations Lead to Various Degrees of Intolerance to the Stress of Endoplasmic Reticulum in Oligodendrocytes

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Abstract

Background: Vanishing white matter disease (VWM), a human autosomal recessive inherited leukoencephalopathy, is due to mutations in eukaryotic initiation factor 2B (eIF2B). eIF2B is responsible for the initiation of protein synthesis by its guanine nucleotide exchange factor (GEF) activity. Mutations of eIF2B impair GEF activity at different degree. Previous studies implied improperly activated unfolded protein response (UPR) and endoplasmic reticulum stress (ERS) participated in the pathogenesis of VWM. Autophagy relieves endoplasmic reticulum load by eliminating the unfolded protein. It is still unknown the effects of genotypes on the pathogenesis. In this work, UPR and autophagy flux were analyzed with different mutational types.

Methods: ERS tolerance, reflected by apoptosis and cell viability, was detected in human oligodendrocyte cell line transfected with the wild type, or different mutations of p. Arg113His, p. Arg269* or p. Ser610-Asp613del in eIF2Be. A representative UPR-PERK component of activating transcription factor 4 (ATF4) was measured under the basal condition and ERS induction. Autophagy was analyzed the flux in the presence of lysosomal inhibitors.

Results: The degree of ERS tolerance varied in different genotypes. The truncated or deletion mutant showed prominent apoptosis cell viability declination after ERS induction. The most seriously damaged GEF activity of p. Arg269* group underwent spontaneous apoptosis. The truncated or deletion mutant showed elevated ATF4 under basal as well as ERS condition. Decreased expression of LC3-I and LC3-II in the mutants reflected an impaired autophagy flux, which was more obvious in the truncated or deletion mutants after ERS induction.

Conclusions: GEF activities in different genotypes could influence the cell ERS tolerance as well as compensatory pathways of UPR and autophagy. Oligodendrocytes with truncated or deletion mutants showed less tolerable to ERS.

Key words: Autophagy Flux; EIF2B5 (Eukaryotic Initiation Factor 2Be); Endoplasmic Reticulum Stress; Unfolded Protein Response; Vanishing White Matter Disease

Introduction

Vanishing white matter disease (VWM, OMIM: 603896), an autosomal recessive inherited leukoencephalopathy, is one of the human genetic diseases that directly affect a protein synthesis factor. Clinically, the VWM is classified as congenital, infantile, early childhood, juvenile, and adult type. The early childhood (onset at 2–6 years old) is the most common one. Clinical features of VWM are rapidly neurological deterioration, aggravated by episodes of “stresses” as fever or minor head trauma. Magnetic resonance imaging shows white matter rarefaction and cystic degeneration, which are eventually replaced by fluid-like intensity signal. The disease-causing genes of VWM are EIF2B1, 2, 3, 4, and 5, encoding the five subunits α, β, γ, δ, and ε of eukaryotic initiation factor 2B (eIF2B), respectively.\(^1,2\)

The eIF2B complex is essential for protein synthesis with the function of guanine nucleotide exchange factor (GEF) activity, converting eIF2 from an inactive GDP-bound structure to an active GTP-bound form. Of the five subunits, eIF2B ε is the largest and the most important one, with majority of the mutations reported among all five subunits.\(^3,5\)

The variation in disease severity is extremely wide, which could hardly be explained by genotypic variations.\(^6\) Frameshift mutants that generated truncated protein or mutations that impair eIF2B complex assembly would give rise to a complete loss of eIF2B function, which seriously affected eIF2B function.\(^6,7\)

The pathogenesis of the disease remains poorly understood. Previous studies demonstrated endoplasmic reticulum stress (ERS) participated in the pathogenesis of VWM,\(^8\) and over-activated unfolded protein response (UPR) was found in
Detection of unfolded protein response biomarker
Activating transcription factor 4 is an important component of UPR-PERK pathway. ATF4 could be stimulated from ERS activation indirectly or protein synthesis attenuation by eIF2B mutation directly. ATF4 was determined in human oligodendrocytes transfected with cDNAs of a Wt or mutant eIF2B at basal condition and after ERS induction, detected by Western blot with an antibody against ATF4 full-length protein (#ab23760, Cambridge, England).

Detection of autophagy flux
Autophagy flux reflected an integrated autophagy level. It was measured following the recommendations described by Klionsky et al. LC3-II is the activated form of LC3 (microtubule-associated protein 1 LC3), which maintains the stability of autophagosome by inserting into the extending autophagosome membrane. LC3-II turnover assay (cat #2775, Cell Signaling Technology, USA) with lysosomal inhibitors is taken as the classic measurement for analyzing autophagy level. It was monitored 48 h under baseline condition, and ERS induction in human oligodendrocytes transfected with the Wt or mutant cDNAs. Cells were incubated with three lysosomal inhibitors of Bafilomycin A1 (cat #B1793, Sigma-Aldrich), 200 nmol/L; #P5318 Pepstatin A, 10 µg/ml; and #E8640 E64d, 10 µg/ml for 30 min before ERS stimulation. The rate of LC3-II versus β-actin (Santa Cruz Biotechnology, USA) was quantified.

Statistics analysis
All assays described above were repeated independently three times. Representative FCM, Western blots and quantitative evaluations of those markers were expressed as means ± standard deviation. Statistical differences between two groups were determined by two-tailed unpaired t-test. One-way analysis of variance was for more than two groups. P < 0.05 was considered significantly different.

RESULTS
Oligodendrocytes with truncated or deletion mutants in eukaryotic initiation factor 2Be are less tolerant to endoplasmic reticulum stress
Apoptosis and cell viability assays were performed for the determination of ERS tolerance under baseline condition and ERS induction. Apoptosis rates were tested 48 h in the presence or absence of ERS induction. FCM detected AnnexinV-FITC/PI double-labeled human oligodendrocytes transfected with empty vector, wild-type, and mutations of p. Arg113His (R113H), p. Ser610-Asp613del (Del) and p. Arg269* (R269*). Under a condition without induction of ERS by TG, only the oligodendrocytes transfected with mutation p. Arg269* was undergoing spontaneous apoptosis (t = 4.01, P < 0.05) [Figure 1a, 1c]. When ERS was induced, compared to the cells transfected with the Wt cDNA, oligodendrocytes with mutation p. Arg269* or p. Ser610-Asp613del exhibited higher apoptosis level (p. Arg269* vs. Wt,
Different groups of oligodendrocytes were seeded evenly. The primary cell count is 100% and proliferated at 8 h after ERS induction. Oligodendrocytes transfected with mutation p. Arg269* showed reduction in proliferation at 8 h than other groups ($F = 17.8, P < 0.01$) [Figure 2]. Compared with the Wt, cell viability decreased in cells transfected with the mutations at 24 h after ERS stimulation ($F = 37.8, P < 0.01$). Oligodendrocytes carrying p. Ser610-Asp613del ($t = 11.27, P < 0.001$), but no significant difference was detected for the mutation p. Arg113His [Figure 1b, 1d].

Over activated activating transcription factor 4 (unfolded protein response component) in oligodendrocytes with eukaryotic initiation factor 2B ε truncated or deletion mutants

The expression of ATF4 stayed at a higher level in oligodendrocytes transfected with p. Arg269* or p. Ser610-Asp613del at 48 h under spontaneous culture (Arg269* vs. Wt, $t = 5.43, P < 0.05$; p. Ser610-Asp613del vs. Wt, $t = 9.34, P < 0.05$) [Figure 3a and c]. At 48 h under ERS induction, ATF4 increased the same escalating trend. ATF4 expressed higher in p. Arg269* and p. Ser610-Asp613del, compared with p. Arg113His transfected cells (Arg269* vs. R113H, $t = 4.5, P < 0.05$; Del vs. R113H $t = 14.59, P < 0.05$) [Figure 3b and d].

Depressed autophagy flux in oligodendrocytes with eukaryotic initiation factor 2Be truncated or deletion mutants

Autophagy flux was measured by LC3-II turnover assay in the presence of Bafilomycin A1, Pepstatin A, and E64d at 48 h under basal and ERS condition. Autophagy flux was analyzed by measuring the rates of LC3-II versus β-actin. Increased transformation of LC3-II was observed in oligodendrocytes transfected with empty vector or Wt after ERS induction, but not in cells transfected with mutations ($F = 46.3, P < 0.001$, difference from the mutants and wild-type) [Figure 4]. Both LC3-I and LC3-II expressed at a lower level in the mutants transfected oligodendrocytes after ERS induction. When compared between the three mutation types, LC3-II was lower in cells transfected with the p. Arg269* or p. Ser610-Asp613del, compared with the p. Arg113His after ERS induction (p. Arg269* vs. p. Arg113His $t = 5.06, P < 0.05$; p. Ser610-Asp613del vs. p. Arg113His, $t = 5.81, P < 0.05$) [Figure 4b and d]. In spontaneous culture (without ERS induction), LC3-II transformation was higher in cells transfected with p. Arg269* but lower in other mutant expressions ($t = 4.43, P < 0.05$) [Figure 4a and c].

**DISCUSSION**

The complex of eIF2B is widely expressed in eukaryotic cells and essential for protein synthesis initiation. Assembled eIF2B converts eIF2 from an inactive GDP-bound form to an active GTP-bound form.[3] Gene defects in any subunits of eIF2B α, β, γ, δ, and ε cause VWM. eIF2Be is the largest and the major catalytic GEF domain. Mutations in eIF2B impaired stability of catalytic function and interaction with eIF2.[11] Though eIF2B expresses in all

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**Figure 1:** Cell apoptosis rates in human oligodendrocyte cell line transfected with empty vector (Con), wild-type (Wt), and mutants of p. Arg113His (R113H), p. Ser610-Asp613del (Del) and p. Arg269* (R269*) at 48 h in the presence (b) or absence (a) of TG 1 μmol/L. *P < 0.05, p. Arg269* versus wild-type (Wt); †P < 0.01, p. Arg269* versus Wt; ‡P < 0.001, p. Ser610-Asp613del versus wild-type.
PERK promotes eIF2α phosphorylation. Phosphorylated eIF2α (P-eIF2α) inhibits eIF2B activity to relieve ERS through protein synthesis attenuation. Some stress-inducers as ATF4 are exempt from the inhibition through specific features in their 5' untranslated regions. ATF4 mRNA is specifically translated, followed by the trans-activation of the cascade ERS target genes, leading to programmed cell death.\[23,24\]

How the loss of function in eIF2B explains the elevated UPR components is not fully understood. This may because the increased P-eIF2α potential. Impaired eIF2B activity blocks the transformation from eIF2 to eIF2-GTP. Abundant P-eIF2α molecules are generated from the retained eIF2 factors, followed by the activation of ATF4 under the basal condition and over activated after ERS. In the current study, we measured the expression of ATF4 in oligodendrocytes transfected with different mutants. ATF4 expressed at a higher level in p. Arg269*

**Figure 2:** Endoplasmic reticulum stress induced viability declination. At 8 h: *P < 0.01, the p. Arg269* group versus others. At 24 and 48 h: The wild type versus mutant groups (24 h: P < 0.01; 48 h: P < 0.001); the p. Arg269* versus p. Arg113His (24 h: P < 0.01; 48 h: P < 0.001); the p. Ser610-Asp613del versus p. Arg113His (24 h: P < 0.05; **48 h: P < 0.01).

**Figure 3:** Activating transcription factor 4 (ATF4) was measured in human oligodendrocyte cell line at 48 h under baseline (a and c) or after endoplasmic reticulum stress stimulation (b and d) by western blot. Levels of ATF4 were compared between groups by t-test of variance analysis. *P < 0.05, the mutations of p. Arg269* (R269*) versus the wild type (Wt); †P < 0.05, p. Ser610-Asp613del (Del) versus the Wt; ‡P < 0.05, p. Arg269* versus p. Arg113His (R113H); §P < 0.05, p. Ser610-Asp613del versus p. Arg113His.

body cells, glial cells are selectively involved in VWM. Oligodendrocytes with a heavy protein-folding load in ER are believed to be susceptible to ERS.\[12-15\] It remains unknown how the impaired eIF2B activity affects the destiny of oligodendrocyte and explains this devastating disorder.

Mutation that affected the amount of eIF2B polypeptides or impair the assembly of eIF2B complex would give rise to a complete loss of eIF2B function. Impaired GEF activity varies in different mutations of eIF2Be.\[16-18\] Frameshifts or nonsense mutations generate truncated eIF2Be with serious loss of GEF activity. In our previous study, the truncated mutant (p. Arg269*) and deletion (p. Ser610-Asp613del) that were transfected human embryonic kidney 293 (HEK 293) cells showed a complete loss of GEF functions,\[19\] whereas p. Arg113His confers nearly 50% loss of functions.\[20\] Whether the degree of ERS tolerance varies in different polypeptides caused by the missense mutation p. Arg113H is truncated mutant of p. Arg269* or deletion mutant of p. Ser610-Asp613del is an open question. In the present study, apoptosis and cell viability assay were to reflect ERS tolerance in oligodendrocytes transfected with the different mutant types. The truncated or deletion mutant showed less tolerable to ERS than the missense mutant. The p. Arg269* or p. Ser610-Asp613del transfected cells experienced prominent apoptosis increase after ERS stimulation. The p. Arg269*-transfected cells underwent spontaneous apoptosis, even at the baseline condition (without ERS inducement). Cell viability decreased more rapidly in p. Arg269* transfected cells. This suggested that mutations with a more severe impact on GEF activity of eIF2Be would lead to less tolerance to ERS in oligodendrocytes.

Our next question is what potential mechanisms result in susceptibility to ERS? Intracellular pathways respond to ERS include UPR and autophagy. Previous studies indicated elevated UPR biomarkers in the brain autopsy samples,\[8,9\] fibroblasts\[21\] or lymphoblasts\[22\] from VWM patients. UPR pathway is triggered after ERS induction, beginning with the dissociation of GRP78 from PERK. The dissociated
and p. Ser610‑Asp613del transfected cells at baseline and ERS conditions, which means an over‑activated UPR‑PERK pathway in oligodendrocytes transfected with the truncated or deletion mutant.

Autophagy relieves ERS by delivering the unfolded protein to the lysosome for degradation. The protective role of autophagy has been identified in several unfolded protein accumulating disorders in the nervous system, such as Alzheimer’s disease, Huntington’s disease, multiple system atrophy, amyotrophic lateral sclerosis, and neuronal ceroid lipofuscinosis.\[25‑28\] In our previous research, level of autophagy flux was found depressed in oligodendrocytes transfected with the EIF2B3 mutation compared with the Wt after ERS induction (unpublished data). Whether autophagy level varies in different genotypes of VWM was not analyzed. LC3, expressed in most cell types, is an effective autophagy‑testing marker because the synthesis and process of LC3 are increased after autophagy activation.\[29\] Several autophagy‑related genes (Atgs) participated in the LC3 processing. LC3 is encoded by the mammalian homolog of Atg8. Atg4 promoted the cleavage of LC3 to LC3‑I after autophagy activation. LC3‑II is the membrane bounding form of LC3‑I. Atg3 and Atg7 participated in the transformation of LC3‑II from LC3‑I. In the present study, both of LC3‑I and LC3‑II were found depressed in the mutant cells after ERS inducement, which indicated a damaged reactive autophagy flux and the probable impairment of the expression of Atgs. Truncated or deletion mutant exhibited a lower LC3‑I and II expression than the missense mutation, which could explain the ERS susceptibility. Under baseline condition, LC3‑II turnover was elevated slightly in the p. Arg269* group. It may because the spontaneous apoptosis triggered autophagy degradation in the p. Arg269* group. The autophagic regulation needs to be further studied to explain the difference in autophagy ability with different mutants.

In conclusion, the severity of ERS intolerance was related to the degree of function loss in eIF2Bε with different mutants. The truncated mutant of p. Arg269* or deletion mutant p. Ser610‑Asp613del transfected human oligodendrocytes cell line experienced more obvious apoptosis as well as elevated UPR and depressed autophagy level. The above findings suggested that different impacts on eIF2B polypeptides generated by missense or preliminary terminated mutants resulted in various degrees of ERS susceptibility and stress related compensatory ways as UPR and autophagy pathways. The comparisons between different mutants may provide a better insight into the genotype and pathogenesis correlation analysis. An intact eIF2B function may assist to strength cell resistant ability to ERS in oligodendrocytes.

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