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| 学 位 論 文 名 | 優性 WFS1 変異体の in vitro 機能解析とその機能レスキューに関する検討 |
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論文内容の要旨

1 研究目的

Some rare forms of DM result from one or more defects in a single gene and are called monogenic. So far, more than 20 genetic subtypes have been detected in patients with monogenic diabetes, accounting for approximately 1–6.5% of cases of diabetes in children. Wolfram syndrome (WS) is a rare disorder caused by pathogenic variants in the gene encoding wolframin (WFS1) and is characterized by diabetes mellitus, optic atrophy, sensorineural deafness, diabetes insipidus, and neurodegeneration. This disease is usually inherited as an autosomal recessive manner, but an autosomal dominant form has been reported. *WFS1* encodes a protein called wolframin. Wolframin is a 100 KDa protein with nine transmembrane segments that localizes to the endoplasmic reticulum (ER) and maintain the ER homeostasis. Despite the underlying significance of ER dysfunction in WS, molecular mechanisms linking the ER to β -cell death have not yet been clarified. Because there is currently no effective treatment for WS, symptoms of WS deteriorate with age and patient with WS lead to death. Thus, understanding the molecular mechanism of pancreatic and neuronal cell death in WS may aid the search for treatments for this disease. We speculated dominant WFS1 pathogenic variants were thought to increase ER stress-related cell apoptosis and the increased ER stress could be reduced by 4-phenylbutyrate (PBA) and valproate (VPA) reduce ER stress. The purpose of our study is to elucidate the molecular mechanism of pancreatic β -cell death by dominant WFS1 pathogenic variants *in vitro*. In addition, we analyze the effects of PBA and VPA on dominant pathological variants.

2 研究方法

We selected pathogenic variants of p.His313Tyr, p.Trp314Arg, p.Asp325_Ile328del, p.Glu809Lys, and p.Glu864Lys as dominant type and p.Pro724Leu and p.Arg629Trp as recessive type. Human wild-type WFS1 cDNA was inserted into a pcDNA3.1 expression vector. The plasmids for WFS1 pathogenic variants were created by site-directed

mutagenesis using a Gene-Art Mutagenesis Kit (Invitrogen, Carlsbad, CA). We determined whether dominant WFS1 variants (p.His313Tyr, p.Trp314Arg, p.Asp325_Ile328del, p.Glu809Lys, and p.Glu864Lys) increased ER stress by a luciferase assay of ER stress response element (ERSE) using HEK-293 or MIN6 cells respectively. We also examined the effect of dominant variants on the ATF6 α promoter activity. Furthermore, dominant negative effect of each variant was also analyzed. Luciferase activity of ERSE and ATF6 α promoter was determined by Luciferase Reporter Assay System (Promega, Madison, WI). The subcellular localization of dominant WFS1 variants was examined by confocal microscopy (FLUOVIEW FV1000; Olympus, Tokyo, Japan) in HeLa cells. Moreover, the rescue of cell apoptosis induced by dominant WFS1 variants following treatment with PBA or VPA was determined by quantitative real-time PCR of C/EBP homologous protein (CHOP) mRNA expression.

3 研究成果

WFS1 plays a role in the suppression of ER stress-mediated cell death by preventing the hyperactivation of the ER stress response. Thus, we determined whether each WFS1 variant increased ER stress. All of the dominant WFS1 variants activated ERSE reporter activity significantly more than wild-type WFS1, however recessive variants did not statistically increase.

Moreover, when cells were equally co-transfected with wild-type and dominant or recessive WFS1 expression plasmid, ER stress was still elevated by all dominant variants but not recessive variants. These results indicated that each dominant WFS1 variant has a dominant negative effect on the wild type, but recessive variants do not have a dominant negative effect. Next, we analyzed whether each dominant WFS1 variants activated ATF6 α promoter activity. All dominant WFS1 variants activated ATF6 α promoter activity significantly more than wild-type WFS1, but recessive variants did not. We determined the expression levels of CHOP mRNA. Quantitative real-time PCR showed that all dominant WFS1 variants induced a significant increase of CHOP mRNA. Regarding subcellular localization, GFP-tagged wild-type WFS1 showed a diffuse reticular pattern that colocalized with the ER in HeLa cells. In contrast, all dominant WFS1 variants showed a punctate staining pattern in the ER. These findings suggested that these dominant WFS1 variants may be misfolded and aggregate in the ER, particularly p.Glu809Lys and p.His313Tyr generated a highly punctate staining pattern in the ER. Moreover, after treatment with PBA and VPA, ER stress and cell apoptosis were reduced in each dominant variant in in vitro condition.

4 考察

WS is usually inherited in an autosomal recessive manner and many of the reported recessive variants are dispersed throughout all of the domains of WFS1. In contrast, six dominant variants of WS have been reported to date. It is of note that all of the dominant

variants located in the first transmembrane domain (p.His313Tyr, p.Trp314Arg, and p.Asp325_Ile328del) are closely clustered. The close localization of the mutations in the first transmembrane domain and the specific amino acid changes in the ER lumen domain may be related to the formation of the dominant variants. In present study, ER stress have been induced by all dominant WFS1 variants and each dominant WFS1 variant demonstrated exerted dominant negative effect similar to previous studies of p.His313Tyr and p.Asp325_Ile328del. Because WFS1 could forms multimer, which composed of dominant WFS1 variant and wild-type monomers are not structurally competent or fully functional, these variants exert the dominant negative effects. Our study has also shown that ATF6 α promoter activity was activated in dominant WFS1 variants. It is thought that the interaction between ATF6 α and the dominant variants may be impaired, resulting in increased ER stress. This may be one of several mechanisms of increased ER stress by dominant WFS1 variants. Regarding cell apoptosis, the expression levels of CHOP mRNA increased compared with wild-type WFS1, suggesting that the enhanced ER stress by these dominant variants led to cell apoptosis. In addition, we tested whether recessive variants activate ERSE luciferase activity, ATF6 α promoter activity and exert a dominant negative effect. By contrast recessive variants did not increase ER stress significantly and did not have a dominant negative effect in agreement with previous studies. Based on these results, ER stress may be able to be caused by dominant variants themselves, rather than the effect of overexpression by transfection of expression vector. Regarding the effect of PBA and VPA, these agents alleviated ER stress and cell apoptosis caused by dominant variants. Indeed, all dominant variants localized to the ER as punctate granules of misfolded protein; thus, PBA may improve the accumulation of misfolded WFS1, thereby reducing ER stress. As a future step, it is necessary to further investigate the administration of PBA and VPA to Wfs1 knockout mice.

5 結論

Our present study demonstrated the increased ER stress and dominant negative effect of each dominant WFS1 pathogenic variant. Moreover, PBA and VPA could reduce the ER stress and cell apoptosis caused by dominant WFS1 variants *in vitro*. Thus, these molecules may be effective to regulate ER stress-related cell apoptosis and could be used to delay the progression of WS and WS-like disorders caused by dominant WFS1 variants and other diseases associated with ER dysfunction.

論文審査の結果の要旨

本学位論文では Wolfram 症候群の原因遺伝子 WFS1 の遺伝子変異について機能解析を行い、遺伝子変異がドミナントネガティブ効果によってテトラマーとして働く正常遺伝子産物の機能を阻害し ER ストレスを亢進させ、膵 β 細胞のアポトーシスを引き起こす可能性を明らかにしました。更にバルプロ酸ナトリウムや 4-フェニルブチル酪酸などの ER ストレス阻害剤が遺伝

子変異の ER ストレスを低減する可能性を *in vitro* で示しました。本研究の主要部分は既に論文として出版されており、示された実験の方法や結果は信頼できるもので、学位授与に十分値すると思います。ただし、優性遺伝病の主要な三つの発症メカニズム（**toxic effect**、**dominant negative effect**、**haploinsufficiency**）を考慮した上で、本疾患においてそれぞれの寄与がどの程度かということを示す実験を今後積み重ねた方がよいと考えられました。本論文の新規性は **Wolfram** 症候群の治療薬としてバルプロ酸ナトリウムや 4-フェニルブチル酪酸の可能性を示したことにありますが、**Wolfram** 症候群の原因遺伝子変異の機能にどう影響を与えるのか今後の検討課題と思われます。最後に英文として論文にふさわしくない言い回しや文法上の誤りがあり、英文校正を行うことを強く勧めます。

最終試験の結果の要旨

- ・ 申請者の発表は研究の背景・目的・方法・結果・考察について必要十分な要素が含まれていたと思われます。
- ・ また、今後の研究の方向性についてもいくつかの具体的アイデアも示されました。
- ・ 申請者は質疑についてその内容を概ね正しく理解して応答できたと考えられます。
- ・ 以上より本申請者の研究能力及び科学的素養・態度は学位に値するものと判断致しました。