Figure 2. Effects of NOEV on human skin fibroblasts from patients with β-galactosidase deficiency. **A:** Cells were incubated in the absence or presence of NOEV for 96 hr and the β-gal activity in lysates were measured (I51T/Y316C; patient #24 and G190D/G190D; patient #18). **B:** Fibroblasts from patient with R201C/R201C were cultured with or without NOEV for 96 hr and cell lysates were subjected to Opti-prep fractionation as described in Materials and Methods. Each fraction was assessed for β-gal activity. Each bar represents the mean ± SEM of three determinations each done in triplicate. *$P < 0.05$, statistically different from the values in the absence of NOEV. **C:** Cells were cultured with or without NOEV for 96 hr and subjected to BODIPY-Cer and anti-golgin97 labeling. Representative images were obtained under confocal microscope. Bar = 20 μm.

Moreover, upregulation of ubiquitinated proteins in lysate from R201C astrocyte was significantly ameliorated after NOEV treatment (Fig. 5E).

Discussion

To date, more than 130 mutations in *GLB1* have been identified as disease causing [Brunetti-Pierri and Scaglia, 2008; Hofer et al., 2009, 2010; Suzuki et al., 2008], of which approximately 75% cause amino acid substitutions. Some of the mutant proteins have normal or near-normal activity, but they are unstable at neutral pH in the ER/Golgi apparatus because of inappropriate molecular folding, and are rapidly degraded by intracellular quality control

systems [Fan et al., 1999; Suzuki et al., 2009]. Because most of the novel mutations (except for S54I, E131K, and L608P) identified in this study caused premature termination or frame shift, they showed no response to NOEV.

In our initial study, we identified NOEV as a potent inhibitor of human β-gal, and confirmed its chaperone effect [Iwasaki et al., 2006; Matsuda et al., 2003; Suzuki et al., 2007]. In this study, we characterized the effect of NOEV on lysosomal trafficking of mutant R201C protein and on lipid trafficking in patients' fibroblasts. A novel compound, DLHex-DGJ, which is an N-alkaylated derivative of 1-deoxygalactonojirimycin, was recently reported to act as a chaperone for mutant β-gal at much higher concentrations than NOEV [Fantur et al., 2010].
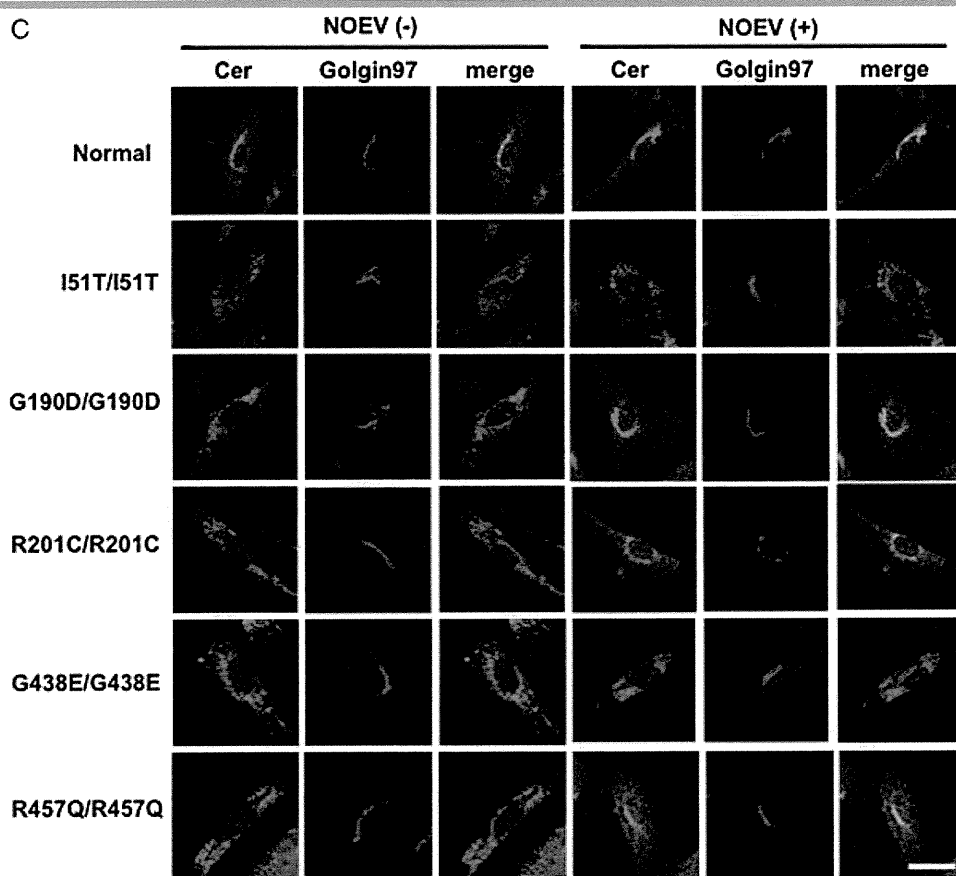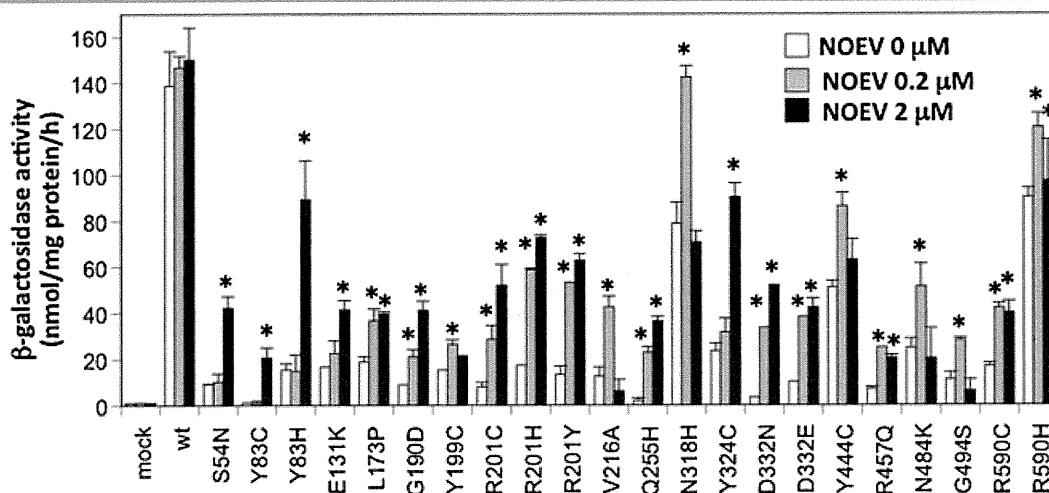
C

Figure 2. Continued.

Figure 3. Screening of NOEV effects on human mutant β-galactosidase. β-Gal-deficient mouse fibroblasts were transiently transfected with human normal or mutant β-gal cDNA and incubated with or without NOEV for 48 hr. Cell lysates were assessed for β-gal activity. Mock tranfection was used as a control. Each bar represents the mean ± SEM of three determinations each done in triplicate. *$P<0.05$, statistically different from the values in the absence of NOEV.

To evaluate NOEV effects on different mutations, we performed transient transfection experiments and found that 23% of the mutants examined were responsive to NOEV. Many of these mutations were found in juvenile and infantile form of G_{M1}-gangliosidosis. We estimate that NOEV therapy would be effective in approximately 30–40% of the patients. Although several mutants (D214Y, W273R, N318H, Y347C, and R590H) showed relatively high residual activities, this is most likely
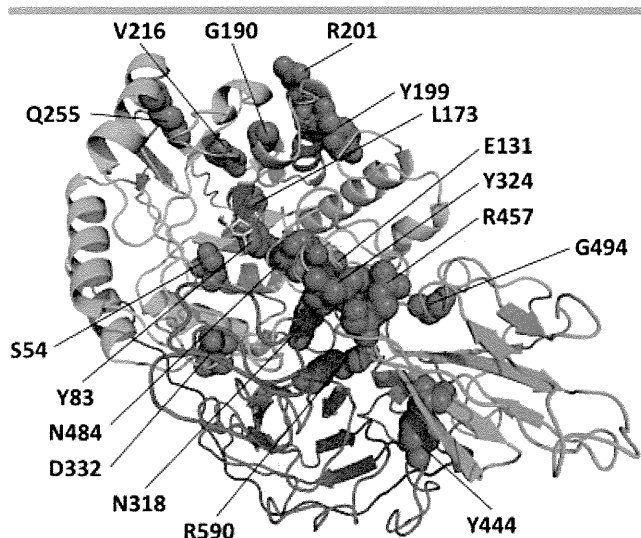
Figure 4. Structural model of human β-galactosidase protein and its interaction with NOEV. The tertiary structure of human β-gal protein and its interaction with NOEV were predicted by computational analyses as described in Materials and Methods. NOEV is colored in yellow. Red circles represent location of residues responsive to NOEV. A blue region represents a typical TIM barrel including the active site.

because of the overexpression of the mutant proteins. Both human skin fibroblasts and transfected cells will be necessary for future screening of chaperone effects [Iwasaki et al., 2006].

The structure of lysosomal enzyme proteins has been determined either by crystal diffraction analysis or homology modeling [Dvir et al., 2003; Garman and Garboczi, 2004; Kang and Stevens, 2009; Lemieux et al., 2006; Rempel et al., 2005]. Molecular interaction of some lysosomal enzymes with their respective chaperones has been resolved [Flanagan et al., 2009; Jo et al., 2010; Lieberman et al., 2009]. These studies revealed that chaperone compounds bind and restore protein conformation of N370S mutant β-glu protein [Lieberman et al., 2009; Lin et al., 2004; Sawkar et al., 2002]. In the current study, we used a predicted structural model and thus we have not obtained the statistical significance. A crystal structure of the β-gal protein would be warranted for further study.

Various pathophysiological changes including impaired calcium homeostasis, elevated levels of inducible nitric oxide synthase, activation of inflammation cascades, accumulation of undegraded proteins, and elevation of ER stress have been shown in the brain of G_{M1}-gangliosidosis model mouse [Jeyakumar et al., 2003; Sano et al., 2009; Tessitore et al., 2004], and alteration in lipid trafficking has been shown in fibroblasts from G_{M1}-gangliosidosis patients [Marks and Pagano, 2002; Puri et al., 1999]. Then we examined the relevance of NOEV on the pathophysiology at the cellular level, and found that NOEV clearly reduced G_{M1} accumulation in R201C astrocytes. Besides, accumulations of p62 and ubiquitinated proteins in R201C astrocytes were effectively suppressed by NOEV providing further evidence on the efficacy of NOEV.

In summary, we found 22 missense mutations responsive to NOEV chaperone effects. We also confirmed that NOEV restored several of cellular functions in the affected cells. These results provide further evidence that NOEV is a promising chaperone compound for β-gal deficiency.
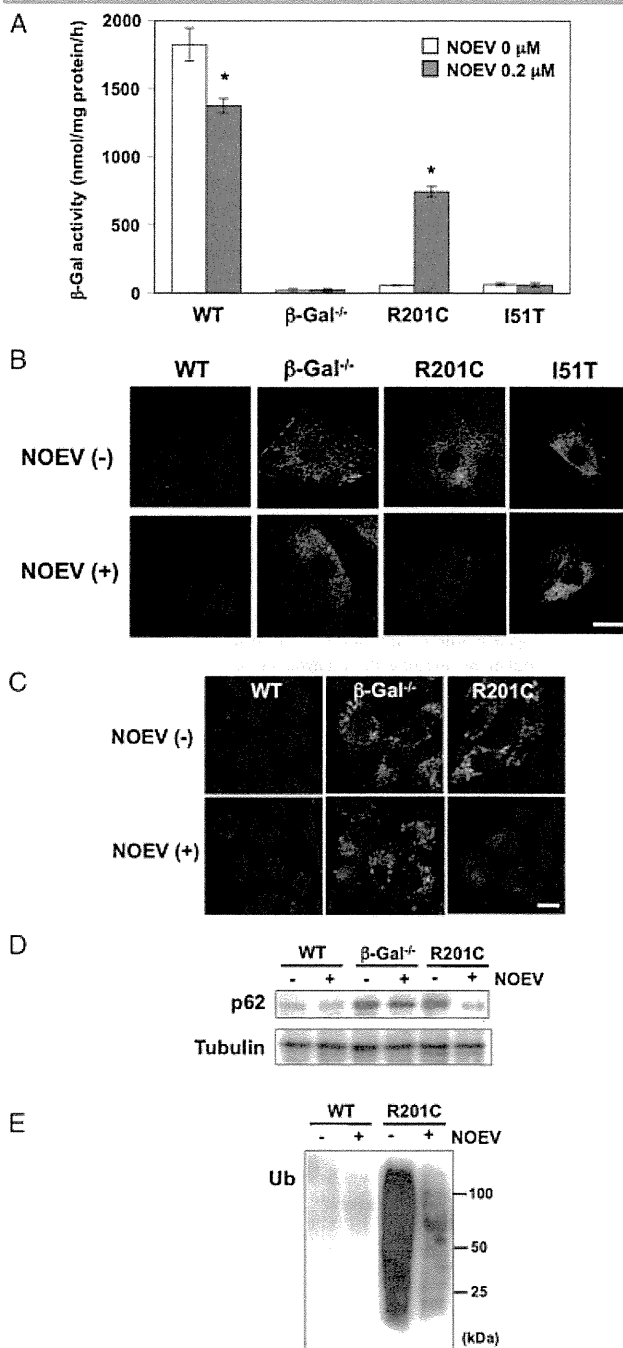

Figure 5. NOEV reduces G_{M1} and ubiquitin accumulation in primary astrocyte from R201C mouse brain. **A:** Chaperone effect of NOEV on R201C primary astrocytes. Each point represents means of triplicates obtained in at least three independent experiments. $^*P<0.05$, statistically different from the value in the absence of NOEV. **B:** Immunostaining with anti-G_{M1}. Cytoplasmic lysosomal accumulation of G_{M1} was observed in R201C astrocytes and it was diminished by NOEV. **C:** Immunostining with anti-p62. Bar = 20 μm. **D** and **E:** Immunoblotting with anti-p62 and tubulin (D), and with anti-ubiquitin (E).

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—Note—

Use of Sample Mixtures for Standard Curve Creation in Quantitative Western Blots

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Abstract: For accurate protein quantification when using quantitative western blot analysis with chemiluminescence reagents, standard curves are needed because of the narrow quantifiable ranges. However, they are often difficult to obtain because authentic proteins are not always available. Here we present our original and convenient method using a sample mixture as a scale to create standard curves. This method allowed us to determine the quantifiable range of target and loading control proteins, making quantitative comparisons among independent blots more reproducible. Our results indicate that using a sample mixture to create standard curves is a practical method that guarantees the accuracy and reproducibility of quantitative western blot analysis.
Key words: chemiluminescence, quantitative western blots, standard curve

Quantitative western blot analysis with chemiluminescence is often used to compare protein expressions in various biological samples [2]. For precise protein quantification by western blot, linear ranges, i.e., ranges of protein levels linearly related to signals on western blots, have to be confirmed in advance by creating standard/calibration curves because the linear ranges quantified by chemiluminescence are quite narrow [1, 3]. However, authentic proteins for making such curves are not always available. Therefore, it is important to find alternative materials with which to generate the standard curve. In this paper, we propose our original and convenient method using a sample mixture as a scale for creating standard curves for comparative protein quantification using western blots. To the best of our knowledge, sample mixtures have not been utilized for standard

curve creation in quantitative western blotting.

Luteinizing hormone receptors (LHR) and α-tubulin in mouse ovaries were used as target and loading control proteins, respectively. Ovarian proteins were extracted from ovaries of 4-week-old A/J females using a Ready-Prep® sequential extraction kit (Bio-rad, Hercules, CA, USA). Protein fraction #2 from each ovary was used for western blots. The protein concentration of each sample was measured using an EZQ® protein quantification kit (Invitrogen, Carlsbad, CA, USA). All of the ovarian protein aliquots from each female (a total of 36 females) were mixed and the sample mixture was used to create a standard curve. Protein samples and various volumes of the sample mixture were separated using SDS-PAGE with NuPAGE® 4–12% Bis-Tris gels and NuPAGE® MES SDS Running Buffer (Invitrogen). The proteins
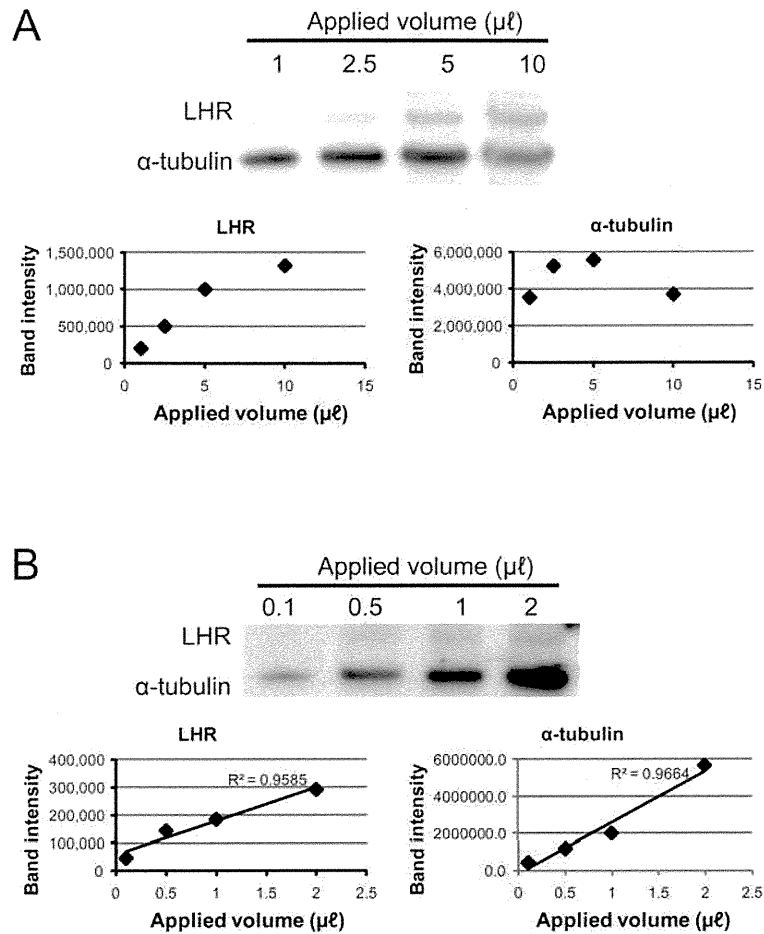
Fig. 1. Determination of linear ranges in quantitative western blots with chemiluminescence using a sample mixture. Linear ranges of band chemiluminescence for LHR and α-tubulin were determined with various volumes of sample mixture (~1.5 μg/μl): A) 1, 2.5, 5, and 10 μl, B) 0.1, 0.5, 1, and 2 μl. Band intensity of α-tubulin reached a plateau at 2.5 μl (~3.8 μg protein) of the sample mixture (A). Plots of band intensities versus applied volumes for two molecules indicate that linear ranges for both proteins were less than 2 μl (~3 μg protein). High correlation coefficients (R^2) confirmed good linearities for both proteins (B). At 10 μl (~15 μg protein) of the sample mixture, the α-tubulin band was a so-called "ghost" band due to an excess of target protein.

were transferred onto polyvinylidene fluoride (PVDF) membranes (Pall, Ann Arbor, MI, USA). LHR (~60 kDa) and α-tubulin (~55 kDa) proteins were visualized by immunoblot analysis using a SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA) with primary antibodies against mouse LHR (sc-26343, goat antibodies, Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-tubulin (rabbit antibodies, Rockland Immunochemicals, Gilbertsville, PA, USA), and horse radish peroxidase (HRP)-conjugated secondary antibodies against goat and rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Antibody-bound bands were visualized by chemiluminescence (ECL-plus, GE Healthcare, Buckinghamshire, UK) and images were captured with a charge-coupled device (CCD) camera (LAS-3000, Fujifilm, Tokyo, Japan). The densitometry of each band was performed using Multigauge V1.0 software (Fujifilm). The linear ranges for LHR and
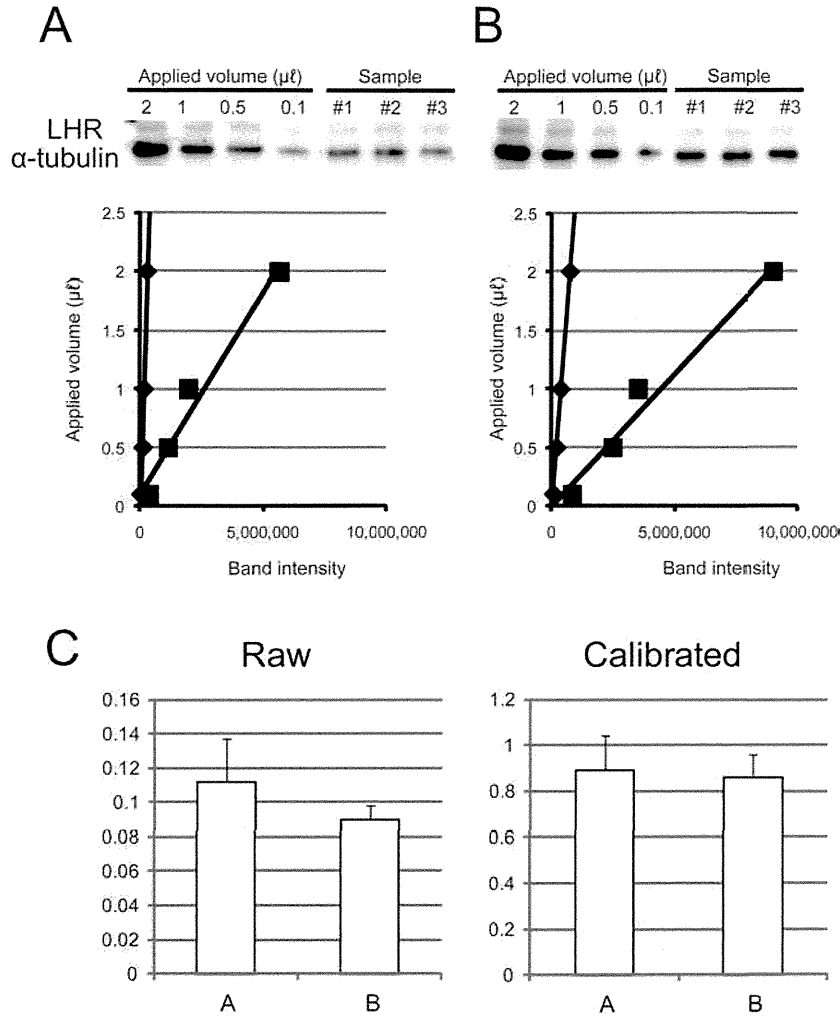
Fig. 2. High reproducibility of comparative protein quantification based on standard curves created with a sample mixture. (A and B) Standard curves from chemiluminescence intensities using various volumes of sample mixture for α-tubulin (◆) and LHR (■) were created for two independent western blots with the same sample set (three samples per set). (C) Comparison of LHR/α-tubulin ratios for two blots (mean ± SD). Higher similarity was achieved in LHR/α-tubulin ratios between blots (A and B) when the ratios were calculated using relative sample mixture volumes converted by standard curves (Calibrated) than directly with chemiluminescence intensities (Raw).

α-tubulin proteins were determined by creating a standard curve based on various volumes of sample mixture (Fig. 1). The correlation coefficients were calculated once good linearities (R^2 values) were found. To ensure reproducibility, two independent assays were performed using the standard curves made from the same sample mixture (Fig. 2). A set of three samples was measured using two separate quantitative western blots. Standard curves for both proteins were obtained as linear ap-proximations for each blot, using various volumes of the sample mixture. The concentrations of LHR and α-tubulin protein were calculated as relative amounts of the corresponding proteins in the standard mixture using linear approximations for each blot (1 unit=amount of the corresponding protein in 1 μl of the sample mixture). LHR protein levels were normalized with those of α-tubulin proteins. Aliquots from the same tubes of the sample mixture were used for creating standard curves

for all blots in Figs. 1 and 2.

This method allowed us to determine linear ranges for both target and internal control proteins in quantitative western blots (Fig. 1). The linear ranges may be different between the two proteins due to the different affinities of the antibodies. With standard curves, reaction conditions can be adjusted so that both target and loading control proteins show similar linear ranges. In addition, out-of-range errors (i.e., saturated immunoreactions) can be determined through standard curve analysis. For instance, the band intensity of α-tubulin in 5 μl of the sample mixture was almost the same as that in 2.5 μl even though the actual amount of α-tubulin in the former lane was twice as much as that in the latter lane (Fig. 1A). In this study, various amounts of the sample mixture using the same immunoblot conditions were analyzed to determine the linear ranges. Alternatively, the linear range common to both proteins can be found by adjusting immunoblot conditions such as antibody concentrations. The level of target and loading control proteins in the sample mixture can be interpreted as the average protein content of each sample. Therefore, the appropriate volume (i.e., total protein content) of sample for each gel lane for electrophoresis can be estimated so that all of the samples fit within the linear ranges. In practice, however, the volume of each sample should be adjusted to fit within the linear ranges through preliminary experiments prior to performing quantitative western blot analysis.

The protein content of multiple blots can be quantitatively compared by creating standard curves using the same sample mixture for each blot (Fig. 2). Precise comparisons of protein content among blots are difficult when the content is calculated directly by band intensity without generating a standard curve. This irreproducibility is mainly due to variation in chemiluminescence reactions across blots (Raw in Fig. 2C). On the other hand, samples can be compared among different blots because the difference in the chemiluminescence reaction is corrected when the values are calculated

based on the standard curve for each blot (Calibrated in Fig. 2C). When the supply of sample mixture is depleted, a newly prepared sample mixture can be used in the same experiment if the results are compared and normalized by generating a new standard curve. Thus, the standard mixture is a useful tool for generating an accurate standard curve for measuring protein expression.

Although a standard curve based on a sample mixture cannot determine absolute protein concentration as can a standard curve based on authentic proteins, it is a practical tool for comparing the protein expressions of multiple samples via western blot analysis. Moreover, generating a standard curve based on a sample mixture is uncomplicated because authentic/standard proteins are not needed. Thus, we propose that sample mixtures can be used as an alternative to authentic/standard proteins to determine the linear range of standard curves for western blot quantification. In experiments using laboratory animals, researchers are required to obtain comparable amounts of information from fewer animals. With our method described here, protein quantification in animal samples would be more accurate and reproducible, resulting in better data acquisition with reduced animal testing.

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Role of Insulin Signaling in the Interaction Between Alzheimer Disease and Diabetes Mellitus: A Missing Link to Therapeutic Potential

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Abstract: Diabetes mellitus (DM) is one of the major non-genetic risk factors for Alzheimer disease (AD). However, the mechanism by which DM increases the risk of AD has not been elucidated. Here, we summarize recent findings to address this question. Whereas neuropathological studies in humans suggest that DM does not increase Aβ accumulation in the brain (a major hallmark of AD), earlier works in animal models show that Aβ does accumulate. Therefore, alternate mechanisms might exist. Recent studies using the human brain indicate that insulin signaling is impaired in the AD brain. In neurons, this insulin signaling plays a key role in modulating synaptic function and neuronal senescence besides regulating tau phosphorylation, another hallmark of AD. On the other hand, in cerebrovessels, DM causes vascular remodeling, which involves increased RAGE (receptor for advanced glycation endproducts) expression, and AD is associated with cerebrovascular amyloid angiopathy (CAA). Our recent study involving AD mice with DM has revealed that a vicious circle underlies the interaction between AD and DM. Interestingly, in our mouse model, AD increased RAGE expression, and DM worsened CAA. The contribution of vascular factors such as RAGE expression and CAA to the impairment of insulin signaling will be discussed. This impaired insulin signaling might be a possible link between AD and DM.

Moreover, insulin signaling is also involved in the mechanism of aging, decreasing with an increase in age. An identification of the mechanism whereby DM modifies the pathological condition of AD through the modulation of insulin signaling is required to develop potential therapeutics for AD not only with but also without DM.

Keywords: Alzheimer disease, diabetes mellitus, insulin signaling, Aβ, vascular factor.

1. INTRODUCTION

Alzheimer disease (AD) is a progressive neurodegenerative disorder. Although the global prevalence of AD is set to rise to more than 35 million people [1], current clinical therapy for AD is limited to choline esterase inhibitors and *N*-methyl-D-aspartate activated (NMDA) receptor antagonists. Recently, the Aβ cascade hypothesis that modifies the disease, has been exploited for its therapeutic potential however, this is not yet available for use in clinical settings. To enable the future society care for large numbers of the elderly, alternative strategies to treat the disease should be encouraged. It is well known that a specific set of genetic and non-genetic risk factors contributes to the onset of AD [2]. Non-genetic risk factors include age, duration of education, DM [3], and hypertension [4]. Numerous epidemiological studies have demonstrated that patients with DM have a significantly higher risk of developing AD [3, 5]. As 285 million people battle DM worldwide [6], the mechanism by which DM increases the risk of AD should be understood in order to improve public health, but progress in this regard has been slow.

Cognitive dysfunction is the major symptom observed in AD patients. It is reported that raising plasma insulin levels through intravenous infusion and keeping plasma glucose at a fasting baseline levels improves cognitive function in patients with AD [7]. Furthermore, taking advantage of the fact that insulin receptors are also localized in olfactory areas [8], it is reported that intranasal insulin improved cognitive function in AD [9] and healthy adults [10]. These findings are also supported by the results from animal models [11,12]. On the other hand, two clinical studies demonstrate that an anti-DM drug, pioglitazone (a member of thiazolidinediones {TZD}), which are peroxisome proliferator activated receptor (PPAR) γ agonists [13], improves cognitive function in patients with AD and mild cognitive impairment (MCI) with DM [14, 15]. Consistent with these findings, in AD model mice, pioglitazone normalized the cerebral glucose utilization response to increase neuronal activity [16]. Although larger double-blind, randomized and placebo-controlled studies are required, it is now becoming clear that a therapeutic potential might emerge if the missing link between AD and DM is explored.

In our recent studies, novel animal models mirroring the pathologic conditions of AD and DM helped us investigate the pathophysiological interaction between these two diseases. AD transgenic mice (APP23) were crossed with two types of DM mice (*ob/ob* and Nagoya-Shibata-Yasuda (NSY) mice), and their metabolic and brain pathological characteristics analyzed [17]. *Ob/ob* mice are leptin-deficient

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mice exhibiting obesity and DM, whereas NSY mice are established as an inbred animal model with spontaneously developing DM [18, 19]. Novel AD mouse models with DM manifesting early onset of cognitive dysfunction were developed [17]. The results of this study will be adopted in this review to discuss the relationship among insulin signaling, AD, and DM.

In addition to DM, aging is an important non-genetic risk factor for AD [20, 21]. Many of the central nervous system (CNS) changes observed in patients with DM and animal models of DM are reminiscent of the changes seen during normal aging [22]. Furthermore, insulin signaling is involved in the mechanism of aging, and insulin resistance is associated with aging [23-25]. Therefore, an identification of the mechanism by which DM modifies the pathological condition of AD might help develop potential therapeutics for AD not only with but also without DM. In this review, recent findings are summarized to clarify the role of insulin signaling in the relationship between AD and DM. In the future, this can prove beneficial for the identification of potential therapy for AD.

2. DIABETES MELLITUS AND ALZHEIMER DISEASE

Numerous epidemiological studies have shown that patients with DM have a significantly higher risk of developing AD [3, 5, 26]. In the Rotterdam study, DM almost doubled the risk of dementia and AD [3]. However, neuropathological studies in patients with AD suggested that DM did not increase AD pathological changes always [27], though earlier work using animal models suggests that it does [28, 29]. This suggests alternate mechanisms might exist. Consistent with clinical observations, it was recently found that the onset of DM exacerbates AD-like cognitive dysfunction without an increase in brain Aβ burden in APP$^+$-*ob/ob* mice [17]. Furthermore, feeding a high-fat diet caused severe memory deficit in APP$^+$-NSY mice, without any increase in brain Aβ loads [17].

APP$^+$-*ob/ob* mice also showed an accelerated DM phenotype as compared to *ob/ob* mice, suggesting that the pathological amyloid in AD might aggravate the DM phenotype. Similarly, APP$^+$-NSY fusion mice also showed a more severe glucose intolerance as compared to NSY mice [17]. These findings suggest that a link underlying the association between AD and DM. In these mice, it was also found that brain insulin signaling is impaired [17]. Therefore, in the following sections, we summarize the physiological function of insulin signaling in the brain, in order to consider the role of impairment of insulin signaling in this crosstalk.

3. ROLE OF INSULIN SIGNALING IN CNS

3.1. Introduction

Insulin signaling plays significant roles in glucose metabolism in the periphery as also in brain function. It is presumed that insulin-related signaling systems evolved millions of years ago, predating the appearance of vertebrates [30]. In vertebrates, insulin-related signaling includes insulin-like growth factor (IGF) I and II. To fulfill its function, insulin binds to the α subunit of the insulin recep-

tor, which activates tyrosine kinase in the β subunit. Subsequently, IRS-1 protein undergoes tyrosine phosphorylation and binds phosphatidylinositol 3-kinase (PI3K), acting as a multisite docking protein to bind signal-transducing molecules [31]. Insulin signal transduction also involves the PI3K-AKT pathway, with downstream involvement of glycogen-synthase kinase-3 (GSK3)β [32-34]. GSK3β is recognized as an integrator of many signaling pathways [35] and has been implicated in AD [36]. IRS-1 and IRS-2 exhibit a high degree of structural homology, are expressed in the brain, and are thought to be responsible for transmitting the insulin signal from the insulin receptor to the intracellular effectors. Phosphorylation of serine residues on IRS proteins is a key step in the negative feedback control of insulin signaling under both physiological and pathological conditions [37]. However, both IRS-1 and IRS-2 function in a distinct manner to regulate glucose homeostasis [38]. Indeed, in IRS-1-deficient mice, IRS-2 provides signal transduction to major pathways of insulin signaling [39]. On the other hand, disruption of IRS-2 causes DM owing to insulin resistance [40, 41], without a compensatory mechanism *via* IRS-I. However, cross-breeding of IGF-I transgenic mice with IRS-1-deficient mice reduces IGF-1-stimulated brain growth [42], suggesting a significant role of IRS-1 in IGF-1 signaling.

3.2. Existence of Insulin Signaling in CNS

Growing evidence suggests that insulin receptors are ubiquitous in the CNS [8, 43, 44]. Staining with radioactive insulin shows that the brain, including the cortex and the hippocampus, is well supplied with insulin receptors [8, 44]. Circulating insulin accesses the brain by crossing the blood-brain barrier (BBB) [45] and executes pivotal functions in the CNS [46, 47]. In addition to insulin from the circulation, locally synthesized insulin in the brain also exists [48], though in small amounts [45]. These reports suggest that the key molecules of insulin signaling exist in the brain.

3.3. Role of Insulin Signaling in Synaptic Function

The role of insulin signaling in synaptic function has recently received much attention [49-52]. Importantly, the insulin receptor is a component of CNS synapses in the post-synaptic density [52]. Intranasal insulin delivery in mice evokes a robust phosphorylation of Kv1.3, a voltage-gated potassium channel, and its association with the insulin receptor at the postsynaptic site in the olfactory bulb besides increasing object memory recognition [12]. In humans, as mentioned before, insulin improves cognition in AD [7, 9]. These data suggest that insulin can modulate cognitive function.

Among glucose transporters in the brain, glucose transporter 4 (GLUT4) is an insulin-regulated glucose transporter [53] and is also expressed by numerous neurons in the brain, including the cortex and the hippocampus. Intracerebroventricular administration of insulin stimulates a translocation of GLUT4 to the plasma membrane in the rat hippocampus, causing hippocampal neurons to rapidly increase glucose utilization during increases in neuronal activity [54]. For learning and memory, translational control at the synapse is also an important process [55]. Translational control *via* the mammalian target of rapamycin (mTOR) pathway is espe-

cially critical for this process in neurons [56-63]. The effects of insulin on mTOR are observed in the brain [64, 65] and peripheral organs [66, 67]. Insulin actually stimulates post-synaptic density-95 protein translation *via* the PI3K- protein kinase B (PKB)/AKT- mTOR signaling pathway in the hippocampus [68].

Similarly, IGF-I and its receptor also exist in the CNS [69-71]. IGF-1 is a well-known key regulator of energy metabolism and growth [72]. In addition to functions in the periphery, its function in the CNS has emerged. IGF-1 plays a key role in mediating environmental enrichment effects on retinal development [73]. Interestingly, an analysis of brain-specific insulin receptor knockout mice, with a complete loss of insulin-mediated activation of phosphatidylinositol-3 kinase (PI3K), shows no alteration of neuronal survival, memory in the Morris water maze test, and basal brain glucose metabolism [74]. One possible explanation for this observation might be the compensation of IGF receptor signaling for insulin signaling.

Conversely, memory formation is associated with changes in expression of insulin signaling molecules at a specific site. After long-term memory consolidation following a water maze, gene expression of the insulin receptor is up-regulated in the CA1 [75]. It is well known that physical activity preserves cognition in the aging brain. Physical activity also reportedly modifies the effects of learning on the expression of genes involved in insulin signaling [76]. Thus, memory formation might be associated with expressional changes of insulin signaling molecules.

Even in invertebrates, the effects of DAF-2 (insulin/IGF-1 receptor) mutations modulate learning behavior in Caenorhabditis elegans (C. elegans) [77]. In Drosophila, the insulin receptor also functions in axon guidance as a guidance receptor [78]. Taken together, these findings indicate that insulin signaling exists in the CNS and plays key roles to modulate synaptic function and memory formation.

4. LEVEL OF INSULIN SIGNALING IN AGING AND DISEASE

4.1. Level of Insulin Signaling in Normal Aging

Clinically, in the human brain, insulin concentrations decrease with aging, as does brain insulin receptor density [79]. Old animals exhibit impaired *in vivo* insulin receptor kinase activity in the liver [80]. In peripheral organs, old animals show impaired insulin receptor kinase activity [80], PI3K activity [81] and IRS-2 tyrosine phosphorylation [82]. Age-dependent impairment of insulin-insulin receptor signaling system appears to be observed throughout the body, including the brain [79].

Epigenetic modifications, such as DNA methylation and the post-translational modification of histone proteins, regulate many aspects of genome function, including gene expression [83]. Disturbances within the epigenetic landscapes during aging can potentially influence cellular function such as memory formation [83, 84]. Thus, it might be safe to speculate that the expressional changes in insulin signaling molecules by age-related variations during epigenetic modification contribute to aging-associated memory impairment [83, 85].

4.2. Level of Insulin Signaling in AD

The levels of insulin and IGF-I were significantly reduced in advanced AD relative to controls [86]. On the other hand, the insulin receptor reportedly increases in AD [79], but decreases in advanced AD [86] relative to controls. In contrast, the IGF-I receptor levels remain unchanged in AD [79]. Interestingly, tyrosine kinase activity, a signal transduction mechanism common to both receptor systems like IRS-1, IRS-2 and PI3K-AKT, is reduced in AD relative to controls [79, 86]. Lowered insulin-induced PI3K activation is also found in peripheral blood mononuclear leukocytes from patients with AD [87]. Patients with very mild AD in whom dementia had progressed, had marked decreases in insulin and hyperglycemic memory facilitation [7, 88]. Taken together, these findings indicate that insulin signaling is impaired in the AD brain.

5. MECHANISMS OF IMPAIRED INSULIN SIGNALING IN AD AND DM

5.1. Previously Reported Mechanisms of Impaired Insulin Signaling in AD and DM

Thus far, as mentioned, the mechanism by which insulin signaling is impaired in AD, has not yet been elucidated. In postmortem brains, insulin-degrading enzyme activity, which also degrades Aβ, is higher in AD brains than in controls [89]. An increase of insulin degrading enzyme (IDE) activity may decrease insulin levels in the brain [90], and thereby impair insulin signaling in the AD brain. This is one possible explanation for impaired insulin signaling in the AD brain.

Another possible explanation is based on impaired brain insulin transport. Increased peripheral insulin is associated with cognitive dysfunction [91, 92]. It is reported that AD patients have lower CSF insulin, increased peripheral insulin, and a reduced CSF-to-plasma insulin ratio compared with controls [93]. Increased peripheral insulin might affect brain insulin transport from plasma to the CNS. Higher peripheral insulin induced by a high-fat diet is associated with reduced brain insulin transport from plasma to the CNS in experimental animals [94]. Interestingly, in mice with increased peripheral insulin from a high-fat diet, intranasal insulin delivery is no longer effective in increasing long-term object memory recognition [12]. This study suggests that a high-fat diet causes brain insulin signaling dysfunction.

5.2. Another Possible Mechanism of Impaired Insulin Signaling in AD and DM

Another possible mechanism responsible for the impairment of insulin signaling in AD with DM is proposed. We have reported that APP⁺-*ob/ob* mice show cerebrovascular inflammation and severe amyloid angiopathy [17]. In humans, vascular changes in the brain are observed in AD and DM [95-97]. In AD, cerebral amyloid angiopathy (CAA) is one of the major characteristics observed in the brain. In DM, vascular remodeling is a major feature [98-101], and expression of receptor for advanced glycation endproducts (RAGE) increases in such remodeled vasculature. Here, we introduce CAA and RAGE first, and then discuss their relation to inflammation.

Although the incidence of CAA increases with age [102, 103], being observed in one third of the brains in elderly subjects [104], CAA is especially associated with cerebral hemorrhage [105, 106] and AD [103, 107-110]. Electron microscopy reveals that CAA shows extensive arteriolar deposition of amyloid filaments with an apparent destruction of the media and intact endothelium [111, 112]. Till date, the source of CAA is unknown. However, APP23 mice with an APP-null background develop similar degrees of both plaques and CAA, providing evidence that a neuronal source of Aβ is sufficient to induce CAA [113]. Exacerbation of CAA is indeed associated with cognitive impairment in APP mice with DM [17]. Vascular remodeling induced by DM might contribute to the increase of CAA in these mice.

Vascular remodeling is associated with the expression of specific proteins. Chronic hyperglycemia stimulates the formation of advanced glycation endproduct (AGE). Interaction of aldoses such as glucose and ribose with proteins initiates a chain of nonenzymatic reactions leading to the covalent addition of AGE to proteins [114,115]. AGE accumulates in vascular tissue at accelerated rates in diabetes [116]. RAGE is a cell surface receptor for AGE and a member of the immunoglobulin superfamily of receptors [116, 117] and also mediates amplification of inflammatory responses [118-122]. Interestingly, Aβ is also one of the ligands for RAGE [122, 123]. An experimental study also suggests that intracerebral Aβ interaction with RAGE at the blood-brain barrier (BBB) causes circulating T cell infiltration [118] and increases expression of proinflammatory cytokines [122]. RAGE expression and inflammation are indeed enhanced in the vasculature of APP Tg mice [17] and APP/Presenilin-1 double Tg [124] with DM at a young age.

Notably, inflammatory changes are also observed in the AD brain. A clinical study reveals that patients with DM and dementia have an increased cortical interleukin-6 (IL-6) concentration [125]. IL-6 is also an independent predictor of DM and correlates with insulin resistance [126]. In our ADxDM mice, IL-6 was upregulated around the cerebral vasculature [17]. Moreover, insulin signaling is also impaired in these mice [17]. The cross talk between inflammatory signaling and insulin signaling has been intensively investigated, especially in peripheral systems. For example, IL-6 induces insulin resistance in adipocytes [127, 128] hepatocytes [129, 130], skeletal muscle [131] and endothelial cells [132, 133]. In turn, insulin antagonizes IL-6 signaling in adipocytes [134]and hepatocytes [135]. Another inflammatory cytokine, TNF-α, also induces insulin resistance [136]. In ADxDM mice, CAA [17] and RAGE expression [17, 124] is enhanced. Interaction between Aβ and RAGE might lead to inflammation and subsequent modulation of insulin signaling (Fig. 1). This cross talk between insulin signaling and inflammatory signaling might also contribute to the exacerbation of cognitive function in AD with DM.

6. INSULIN SIGNALING, TAU, DM, AND AD

6.1. Tau Pathology in AD, DM, and Normal Aging Brain

In AD, paired helical filament (PHF)-tau is one of the major pathological findings. While normal tau promotes the assembly and stabilization of microtubules, abnormally hyperphosphorylated tau sequesters normal tau and disrupts

microtubules [137, 138]. In PHF-tau, 19 phosphorylation sites have been identified; all the phosphorylation sites except for one site are localized in the amino- and carboxyl-terminal flanking regions of the microtubule-binding domain [139]. Even in nondemented patients aged over 75 years, neurofibrillary degeneration with PHF-tau is present in variable amounts in the hippocampal regions [140- 144]. Similarly, age-related phosphorylation of tau is also observed in the mouse brain [145]. In summary, these findings indicate that tau phosphorylation increases with age.

Interestingly, phosphorylation of tau at some of the abnormally hyperphosphorylated sites in AD is increased in the DM brain [146]. In experimental db/db mice, tau phosphorylation is also increased in the cortex and the hippocampus compared with controls [147]. In addition, just over 16 weeks of a high-fat diet in mice significantly increased the level of tau [148]. The mechanism by which DM promotes hyperphosphorylation of tau is yet to be elucidated, but it might involve impairment of insulin signaling.

6.2. Insulin Signaling in Tau Metabolism

Mounting evidence suggests a role of insulin signaling in tau phosphorylation. Insulin signal transduction involves the PI3K-AKT pathway, with downstream involvement of glycogen-synthase kinase-3 (GSK3)β [32-34]. Insulin and IGF-1 inhibit tau phosphorylation in neurons through the inhibition of GSK3β *via* the PI3K-AKT signaling pathway [149]. Conversely, loss of either insulin [150], the insulin receptor [74], or IRS-2 [151- 153] results in the hyperphosphorylation of tau. Together, these findings indicate that impaired insulin signaling might increase tau phosphorylation, partly *via* the activation of GSK3β. In general, protein phosphorylation is regulated by protein kinases and phosphatases. In the case of tau, protein phosphatase 2A (PP2A) dephosphorylates tau at multiple sites [154]. Disruption of the IRS-2 gene also downregulates PP2A [154]. These findings indicate that impaired insulin signaling might induce tau phosphorylation, mainly *via* activation of GSK3β and inactivation of PP2A.

In an experimental model, streptozotocin (STZ) causes DM *via* decreased autophosphorylation of the insulin receptor-kinase in lower doses [155] as also by cytotoxicity resulting from DNA damage in the pancreatic β-cells *via* the glucose transporter 2 in higher doses [156]. The low doses of STZ in the brain and high doses in the periphery downregulate expression of insulin receptors in the brain [157, 158]. Besides genetic models, dysfunction of the brain insulin system in both STZ-treated mice [158, 159] and rats [157] also generates hyperphosphorylated tau protein without any formation of PHF-tau. STZ exacerbates tau pathology only in a transgenic mouse model that over-express the P301L mutant human tau [160]. Collectively, the reduction of insulin signaling increases tau phosphorylation, but this increase is not enough to lead to the formation of PHF-tau.

7. ROLE OF INSULIN SIGNALING IN NEURONAL SENESCENCE AND FOLDING AND ASSEMBLY OF PROTEIN

There is a notion that insulin signaling might modulate neuronal senescence. Insulin/IGF-like signaling is an evolutionarily conserved pathway determining the life span in
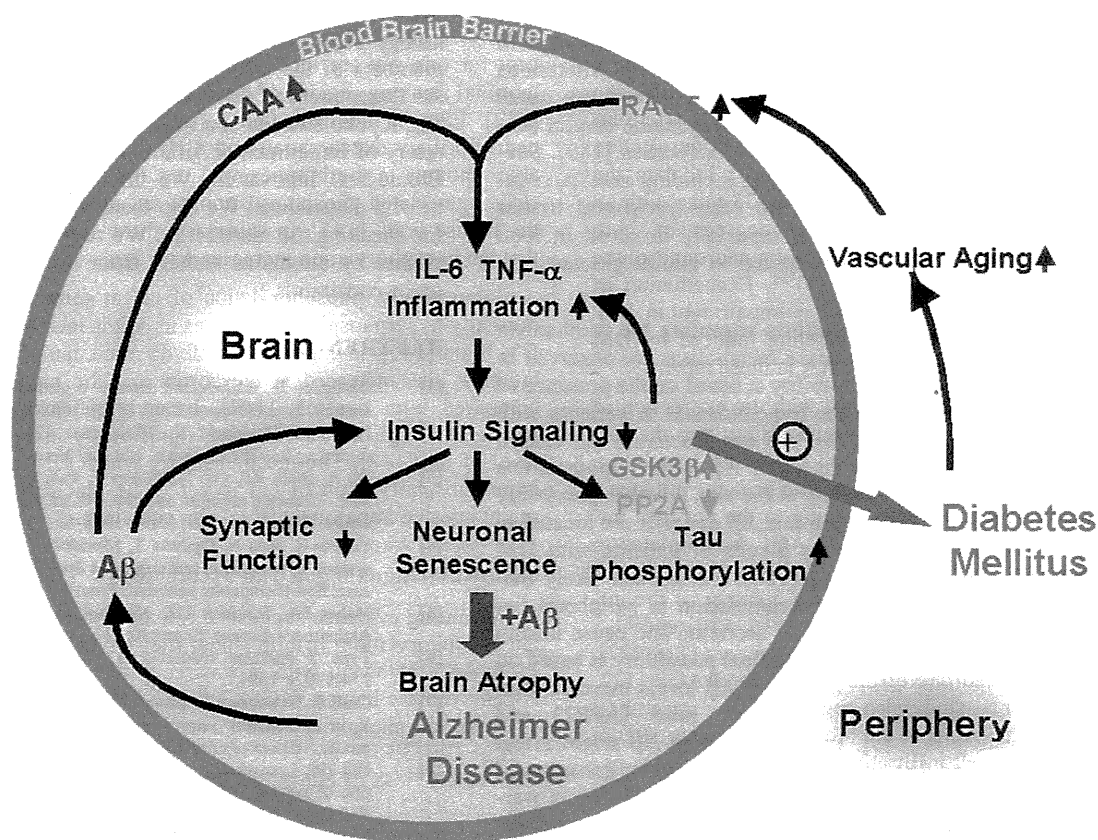
Fig. (1). Possible link among insulin signaling, diabetes mellitus (DM), and Alzheimer disease (AD)
A vicious circle underlies the interaction between AD and DM. In AD and DM, insulin signaling is reduced in the brain. Insulin signaling also plays a pivotal role in the pathological interactions between AD and DM. Impaired insulin signaling might affect synaptic function and neuronal senescence and increase tau phosphorylation, accelerating the predisposition to AD. Under such circumstances, Aβ can easily drive the development of AD in patients at risk. DM is associated with vascular remodeling, which involves increased RAGE expression, and AD is associated with cerebrovascular amyloid angiopathy (CAA). AD increases RAGE expression, and DM worsens CAA. Inflammation caused by vascular factors such as RAGE expression and CAA might play significant roles in impairing insulin signaling. GSK3β, glycogen-synthase kinase-3β, PP2A, protein phosphatase 2A.

yeast, Drosophila, C. elegans and mouse [161, 162]. In C. elegans, neuronal DAF-2, the homolog of IR/IGF-1R, controls life span [163,164]. Loss of CHICO, a Drosophila IRS, also extends life span [165]. Furthermore, a loss of IRS-2 in only the CNS extends life span in mice [166]. These data indicate that loss of insulin signaling in the brain extends life span *in vivo*.

Moreover, loss of DAF-2 reduces Aβ42 toxicity, by decreasing soluble Aβ oligomers [167]. In mice, reduced IGF signaling also protects against Aβ-associated behavior impairment, by decreasing soluble Aβ oligomers, while increasing Aβ aggregates [168]. This result suggests that IGF signaling might affect protein folding and assembly. On the other hand, Freude *et al.* report that a reduction of IGF signaling decreases Aβ deposition [153]. More notably, a loss of IRS-2 is reported to reduce Aβ deposition [152, 153]. Insulin/IGF signaling is reportedly also involved in Aβ generation, clearance, and trafficking [49, 90, 169-173]. However, ADxDM mice manifest a reduction in insulin signaling, but unchanged brain Aβ levels, with increased Aβ deposition in the cerebral vasculature [17]. In ADxDM mice, the reduced

insulin signaling might affect protein folding and assembly and increase clearance from the brain through the BBB. Because DM induces vascular remodeling, Aβ might easily be captured by the vasculature. Thereby, CAA might be aggravated in ADxDM mice.

Notably, earlier work using Tg2576 on high fat diets suggests that DM increases Aβ load in the brain [28, 29]. Discrepancies between those experiments and ours [17] might be explained by the degrees of impairment of insulin signaling, which would control Aβ levels in the brain [152, 153].

8. ROLE OF INSULIN SIGNALING IN WORSENING OF DM BY AD

It was also observed that APP+-*ob/ob* mice show an accelerated DM phenotype as compared to *ob/ob* mice, suggesting that pathological changes in amyloid in AD can aggravate the DM phenotype. Similarly, APP+-NSY fusion mice showed a more severe glucose intolerance as compared with NSY mice [17]. In patients with AD, alterations in glu-

cose metabolism are also reported [174, 175]. Recent evidence points to a crucial role of the central nervous system in controlling glucose homeostasis [176, 177]. Alterations within this hypothalamic/peripheral organ circuit can cause increased plasma glucose levels [178], systemic insulin sensitivity [179], and pancreatic β cell proliferation [180]. Several hormones in the hypothalamus, including insulin, regulate metabolism in the liver and other peripheral tissues [181-184]. Insulin signaling is especially involved in food intake [183] besides being required to inhibit glucose production [184].

There are several possibilities regarding the mechanism by which alterations in glucose metabolism are observed in patients with AD. One possibility is based on the presence of amorphic Aβ plaques in the hypothalamus in patients with AD [185]. It is reported that Aβ can inactivate the insulin receptor substrate in neurons [186]. Aβ in the hypothalamus might impair insulin signaling at the site. Another possibility takes into account the finding that Aβ deposits are located in degenerating pancreatic islets β-cells of patients with DM [187] besides skeletal muscle [188]. Yet another likely theory is that of enhanced Aβ accumulation in peripheral tissues, which might impair insulin secretion and cause insulin resistance in patients with AD. A final possibility is based on our recent findings wherein plasma Aβ levels increase after glucose loading in AD transgenic mice (APP23 and APP/presenilin-1) [189]. Increased plasma Aβ might affect insulin signaling directly in peripheral tissues or enhance Aβ accumulation in these tissues. Further investigation is warranted to elucidate the mechanism of alterations in glucose metabolism in DM with AD.

9. CONCLUSION

To conclude, insulin signaling plays a pivotal role in the pathological interaction between AD and DM. Impaired insulin signaling might affect synaptic function and neuronal senescence besides increasing tau phosphorylation, thus accelerating the predisposition to develop AD. Under such conditions, Aβ can easily drive the development of AD in patients at risk. Interestingly, the loss of insulin signaling decreases the level of Aβ in the brain. The question that remains is whether or not impaired insulin signaling in AD is a compensatory mechanism to reduce the level of Aβ in the CNS. If such is the case, impaired insulin signaling might also spur the development of AD through increased tau phosphorylation. To determine a therapeutic strategy for AD, the role of insulin signaling in Aβ metabolism is an essential issue to be resolved. Of note, a vicious cycle might underlie the interaction between AD and DM. Insulin signaling plays an important role in this cycle.

Moreover, insulin signaling is involved in the mechanism of aging, decreasing with age. Identifying the mechanism whereby DM modifies the pathological condition of AD through the modulation of insulin signaling, might contribute to the development of potential therapy for AD not only with but also without DM.

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A Potent Inhibitor of SIK2, 3, 3′, 7-Trihydroxy-4′-Methoxyflavon (4′-O-Methylfisetin), Promotes Melanogenesis in B16F10 Melanoma Cells

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Abstract

Flavonoids, which are plant polyphenols, are now widely used in supplements and cosmetics. Here, we report that 4′-methylflavonoids are potent inducers of melanogenesis in B16F10 melanoma cells and in mice. We recently identified salt inducible kinase 2 (SIK2) as an inhibitor of melanogenesis via the suppression of the cAMP-response element binding protein (CREB)-specific coactivator 1 (TORC1). Using an *in vitro* kinase assay targeting SIK2, we identified fisetin as a candidate inhibitor, possibly being capable of promoting melanogenesis. However, fisetin neither inhibited the CREB-inhibitory activity of SIK2 nor promoted melanogenesis in B16F10 melanoma cells. Conversely, mono-methyl-flavonoids, such as diosmetin (4′-O-metlylluteolin), efficiently inhibited SIK2 and promoted melanogenesis in this cell line. The cAMP-CREB system is impaired in A^y/a mice and these mice have yellow hair as a result of pheomelanogenesis, while $Sik2^{+/-}$; A^y/a mice also have yellow hair, but activate eumelanogenesis when they are exposed to CREB stimulators. Feeding $Sik2^{+/-}$; A^y/a mice with diets supplemented with fisetin resulted in their hair color changing to brown, and metabolite analysis suggested the presence of mono-methylfisetin in their feces. Thus, we decided to synthesize 4′-O-methylfisetin (4′MF) and found that 4′MF strongly induced melanogenesis in B16F10 melanoma cells, which was accompanied by the nuclear translocation of TORC1, and the 4′-O-methylfisetin-induced melanogenic programs were inhibited by the overexpression of dominant negative TORC1. In conclusion, compounds that modulate SIK2 cascades are helpful to regulate melanogenesis via TORC1 without affecting cAMP levels, and the combined analysis of $Sik2^{+/-}$ mice and metabolites from these mice is an effective strategy to identify beneficial compounds to regulate CREB activity *in vivo*.

Introduction

Melanin plays an important role in animals by preventing the cellular damage induced by ultraviolet (UV) light. When keratinocytes in the skin are exposed to UV irradiation, alpha-melanocyte stimulating hormone (alpha-MSH), a peptide hormone, is processed from the precursor peptide proopiomelanocortin and is secreted as a paracrine factor [1,2,3,4]. Secreted alpha-MSH subsequently binds to its receptor, the melanocortin 1 receptor, on the membrane of melanocytes and activates adenylyl cyclase, resulting in increased levels of intracellular cAMP. cAMP then activates protein kinase A (PKA), which phosphorylates the transcription factor cAMP response element (CRE)-binding protein (CREB) at Ser133, initiating the transcriptional cascades of the melanogenic program, *e.g.*, the induction of microphthalmia-associated transcription factor (*Mitf*) expression [5,6]. Finally, MITF induces the expression of tyrosinase, which initiates the catalysis of melanin from tyrosine by the sequential hydroxylation [7].

Flavonoids are polyphenolic compounds that are widely distributed in vegetables and fruits and protect organisms from damage caused by UV exposure and reactive oxygen species [8,9]. Flavonoids consist of two parts: one is a basic skeleton having three rings (A, B, and C) with one or two oxygen molecules (*e.g.*, flavan or flavone, respectively), while the other part consists of modified side chains, *e.g.*, hydroxy, methoxy, and O-glycosyl groups [10].

Based on the health-promoting effectiveness of flavonoids and their low levels of toxicity, they are used as supplements to prevent disease, such as cancer and metabolic syndromes. In addition, flavonoids, *e.g.*, procyanidins [11] and quercetin [12], are added to cosmetic products to suppress melanogenesis by inhibiting tyrosinase. However, other flavonoids have been reported to have the opposite effect on melanogenesis. For example, nobiletin [13]

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