Involvement of nicotinamide phosphoribosyltransferase in both amyloidogenic and non-amyloidogenic pathways in hippocampus

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Involvement of nicotinamide phosphoribosyltransferase in both amyloidogenic and non-amyloidogenic pathways in hippocampus

Directed by Professor Eosu Kim

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이 글을 쓰기까지 수많은 난관과 인고의 시간을 견딜 수 있도록 무한한 믿음과 용기, 그리고 스승의 사랑과 질책을 아낌없이 주신 지도교수 김어수 교수님께 이 장을 빌어 진심으로 감사의 뜻을 전하려 합니다. 학위과정 동안 지속적인 관심과 조언을 통하여 어려운 상황에서도 흔들리지 않도록 도와주신 남궁기 교수님, 심사위원 위촉 허락과 더불어 졸업 논문 전반적인 틀을 잡아주신 안용호 교수님, 바쁘신 일정 속에서도 자세한 부분에서의 지속적인 조언을 주신 김세훈 교수님께도 감사 드립니다. 또한, 학위 과정의 시작부터 실험의 기틀을 잡을 수 있도록 해 준 박민선 박사에게도 감사 드립니다.

많은 실험 일정에 쫓겨 무리한 부탁을 해도 항상 잘 챙겨주신 이수경 선생님, 학위 실험에 바쁜 부족한 사수를 만나 정신없는 시간을 보내면서도 불평 불만 없이 잘 따라준 후배 김은우와 장은화에게 고마움과 미안함을 전합니다. 또한 412 호 식구라는 이유 하나만으로 바쁘신 와중에도 부족한 저에게 항상 아낌없는 조언과 해결책을 제시해 주신 약리학 교실 조아련 박사님, 내과학 교실 황혜진 박사님, 그리고 건국대에 계시는 최지원 박사님께도 이 곳을 통하여 감사함을 전하며 앞으로의 연구 활동에 무궁한 발전이 있기를 기원합니다.

반복되는 실험실 생활에 지칠 때마다 친형과 같이 자신감과 힘을 실어 준 주현 형, 지난 5 년간 가난한 학생 친구에게 밥과 술을 챙겨 준 친구 상길, 성빈, 민혁, 그리고 바쁜 병원 일정에도 말동무가 되어 준 후배 지호에게도 감사함을 전합니다.

늦은 나이에 새로운 길을 걸어가고자 하는 아들을 묵묵히 지켜보시며 힘이 되어주신 아버지, 바쁜 탓에 얼굴도 제대로 못 보는 아들에게 항상 용기를 주신 어머니께 감사와 사랑을 전합니다. 바쁜 사위와 늘어난 손자들 때문에 고생하심에도 단 한번 내색하지 않고 대학원 생활에 전념할 수 있는 시간을 만들어주신 아버님, 어머님께도 이 곳을 빌어 감사함과 죄송함을 표현하고자 합니다.

지난 5 년의 시간 동안 항상 아내라는 이유만으로 무한한 믿음과 사랑을 주고, 많은 시간 힘든 육아와 직장 생활을 병행하면서도 무사히 학위를 마칠 수 있도록 꼼꼼히 챙겨주고 묵묵히 곁에 있어 준 아내 최은정에게 사랑한다고, 그리고 고맙다는 말을 전하려 합니다. 그리고, 아빠로써 많은 시간을 함께 해 주지 못했음에도 항상 가족의 소중함을 느끼게 해 주는 세 아들 영훈, 영찬, 영준에게도 사랑을 전합니다.

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정 지 현

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ABSTRACT

Involvement of nicotinamide phosphoribosyltransferase in both amyloidogenic and non-amyloidogenic pathways in hippocampus

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(Directed by Professor Eosu Kim)

AMP-activated kinase (AMPK) and silent mating-type information regulator 2 homolog 1 (SIRT1) are the two representative enzymes involved

in energy homeostasis. Concomitant action of AMPK and SIRT1 mediates various beneficial effects including suppression of Alzheimer's disease (AD) pathophysiology. Even though these two enzymes activate each other, metformin, the most widely prescribed anti-diabetic drug, has shown to exert neurotoxic effects via AMPK activation. Therefore, I aimed to examine the effect of metformin on neuronal SIRT1 activity, as well as on AMPK. Normally, metformin-induced AMPK activation would result in concomitant SIRT1 activation via activating AMPK/nicotinamide phosphoribosyltransferase (Nampt)/nicotinamide adenine dinucleotide (NAD) pathway. However, I found that metformin could not activate neuronal SIRT1 despite AMPK activation. Rather, metformin reduced the levels of Nampt and NAD, which accounts for the failure of SIRT1 activation. Metformin-induced AMPK activation increased the levels of beta-site amyloid precursor protein cleavage enzyme 1 (BACE1) and secreted beta-amyloid (Aβ). In addition, metformin-induced Nampt suppression was associated with decreased expression of tumor necrosis factor alpha converting enzyme (TACE) which promotes non-amyloidogenic pathway. These adverse effects of metformin were also identified in the brains of diabetic db/db mice. Overexpression of Nampt rescued metformin-induced neurotoxicity *in vitro*. Also, donepezil treatment reversed the negative effects of metformin *in vitro* and *in vivo*, by promoting Nampt/NAD pathway. Data from the current study implicate the importance of the role of Nampt in regulating amyloidogenesis; AMPK activation by metformin might be neurotoxic because it could not induce concomitant action of AMPK and SIRT1 owing to the lack of Nampt

involvement in the brain contrasted with other peripheral tissues. Co-treatment of donepezil could be a proper measure to protect the brain from potential risk of promoting AD pathogenesis in diabetic patients taking metformin.

key words: Nampt, SIRT1, AMPK, Aβ, Amyloidogenesis, Metformin, BACE1, TACE, Donepezil

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I. INTRODUCTION

1. Nicotinamide phosphotransferase (Nampt)

Nampt has been referred to as pre-B-cell colony-enhancing factor (PBEF) or visfatin. Originally, this was first discovered as a putative cytokine, and so gained the name as PBEF.[1] In 2002, Rongvaux et al. first identified that

PBEF has a key role of nicotinamide adenine dinucleotide (NAD) biosynthesis.[2] After that, it was reported that PBEF gene could encode a Nampt. And also, another function of Nampt/PBEF was identified as a visceral fat-derived hormone (visfatin),[3] so this protein has been called as Nampt, PBEF or visfatin after all.

In NAD biosynthesis, Nampt is the rate-limiting enzyme converting nicotinamide (NAM) to nicotinamide mononucleotide (NMN).[4,5] NAD has fundamental role in energy metabolism such as redox metabolism in citric acid cycle and oxidative phosphorylation.[6,7] NAD is required in DNA repair and telomere maintenance in ADP-ribose transfer reactions via poly(ADP-ribose) polymerases (PARP).[8] NAD is also consumed by histone deacetylase action of silent mating type information regulation 2 homolog 1 (sirtuin or SIRT1),[9] and sufficient NAD level is thus required for normal functioning of SIRT1. NAD biosynthesis have two metabolic pathways, one is *de novo* pathway and another is NAD salvage pathway (Fig. 1). In *de novo* pathway, NAD is made from tryptophan through several cascade steps.[10] Separately *de novo* pathway, NAD salvage pathway is operated in cell organisms, which make and use precursors of NAD.[11] Especially, NAD salvage pathway was also known as an essential mechanism for NAD supply in humans. In NAD salvage pathway, NAD is transferred to NAM, which then is converted to NMN by Nampt. If action of Nampt is insufficient in this step, NAD would be only exhausted, and after all, cellular respiration, oxidative phosphorylation and other NAD-dependent mechanisms will be disintegrated.[10,11]

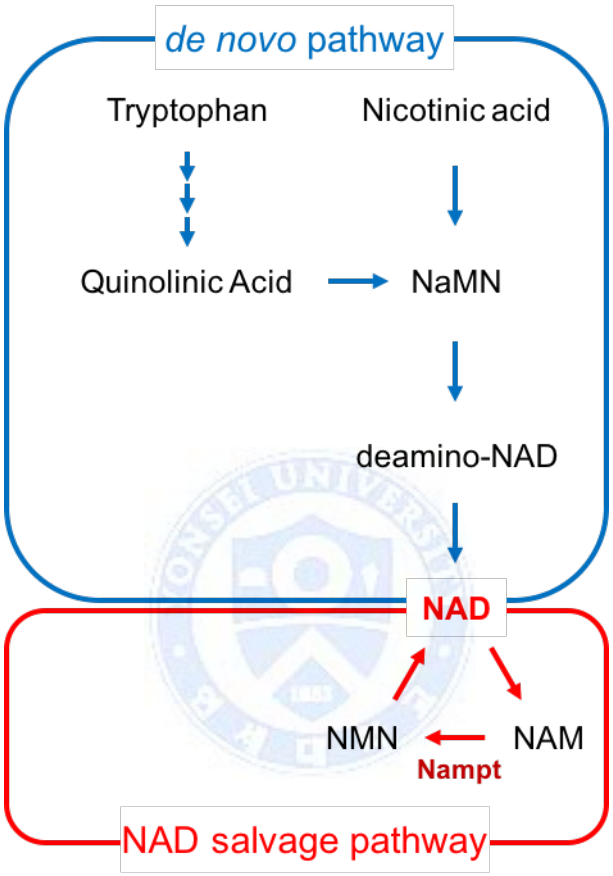

Figure 1. NAD biosynthetic pathway in mammals. There are two pathways in NAD biosynthesis. One is *de novo* pathway using tryptophan or nicotinic acid, another is NAD salvage pathway which is controlled by Nampt, the rate-limiting enzyme of NAD synthesis. NaMN, nicotinic acid mononuclotide; NAM, nicotinamide; NMN, nicotinamide mononucleotide.

2. Alzheimer`s Disease

Alzheimer`s disease (AD) is the most common form of senile dementia,[12] constituting about 70% of all cases. According to World Alzheimer Report 2014, there are estimated 44 million patients with dementia worldwide. And the number of AD patients is predicted to increase more than triple by 2050 so that 1 of 85 people would suffer from AD.[13]

AD is a neurodegenerative disease, leading to memory loss, cognitive and functional decline and death. The molecular mechanisms of AD were investigated in many previous studies. Nevertheless, there has been no cure for AD yet; there are just a few mitigating treatments at the moment. Additionally, overt AD pathology can only be found once neurodegenerative processes are progressed to a degree. Therefore, the preventive healthcare via understanding AD mechanism is the key role of reducing and protecting AD. There are several hypotheses for AD pathogenesis, among which are beta-amyloid (Aβ),[14] tau[15] and cholinergic hypothesis.[16]

A. Amyloid cascade hypothesis

Extracellular amyloid plaque (also called as senile plaque) in the central nervous system is the hallmark of AD pathology.[17] Amyloid plaques lead to inflammatory responses in neurons and tau pathology,[18,19] another etiological factors of AD. Plaques are formed by accumulation of Aβ peptides, which are cleaved fragments of amyloid precursor protein (APP).[14]

The enzymes associated with APP cleavage are α-secretase, β-secretase and γ-secretase. APP cleavage pathways are classified according to two cleavage enzymes, α-secretase and β-secretase. These two enzymes cleave each specific sequential sites of APP, making different fragments of APP with another cleavage enzyme, γ -secretase. First, amyloidogenic pathway is composed of two cleavage steps. APP is cleaved by beta-site amyloid precursor protein cleavage enzyme 1 (BACE1), which makes sAPP-β and C99. Then, C99 is digested to Aβ and amyloid precursor protein intracellular cytoplasmic/c-terminal domain (AICD) by γ-secretase (Fig. 2).[20] On the other hand, α-secretase induces non-amyloidogenic pathway through

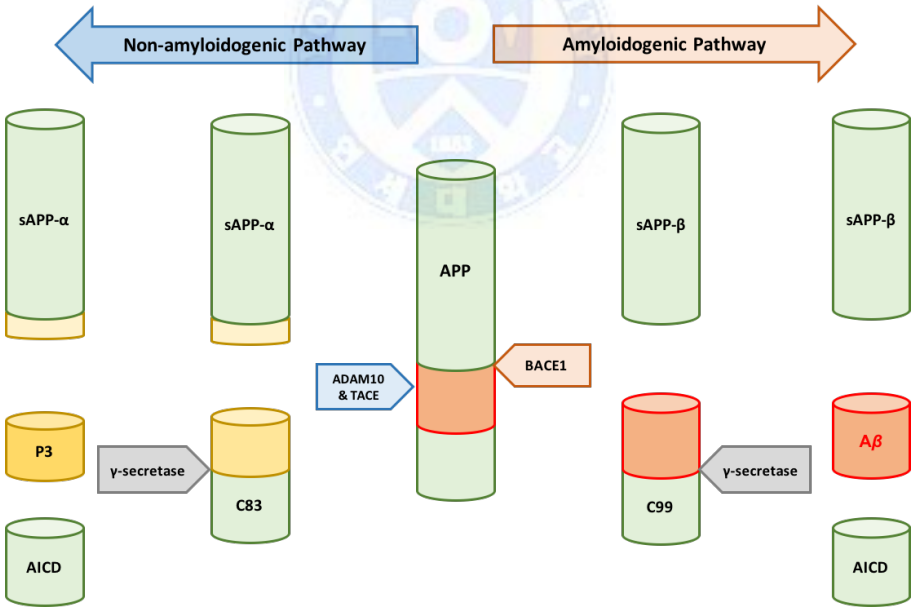

Figure 2. Amyloidogenic and non-amyloidogenic pathways. There are two pathways in amyloid cascade, and these pathways are occurred via α-secretase and β-secretase competitively.

cleaving different site of APP (Fig. 2). A disitegrin and metalloproteinase (ADAM) family was known as the representative α-secretase, and ADAM10 and tumor necrosis factor alpha converting enzyme (TACE, also known as ADAM17) are the typical α-secretases.[21,22]

Amyloid plaque is found in early stage of AD, and Aβ peptide oligomers themselves are the cause of acute synaptotoxic effects.[14,23] In these regard, Aβ has been a priority target of AD therapeutic strategies such as modulation of Aβ production, inhibition of Aβ aggregation and enhancement of Aβ degradation.

B. Tau hypothesis

Another hallmark of AD is neurofibrillary tangles (NFTs), which are formed by hyperphosphorylated tau proteins (Fig. 3).[15,24] Tau, soluble microtubule-associated protein, is found mostly in, neurons than other peripheral tissues. Main functions of tau are the stabilization of axonal microtubules and the growth factor of cytoskeletal elements.

NFTs lead to damage of cytoplasmic functions and interruption of axonal transport, and it brings neuronal cell about death in the end. Actually, hyperphosphorylated tau is the pathology in a number of other neuronal disorders such as corticobasal degeneration, motor neuron diseases and traumatic brain injury.[25] Additionally, NFTs via hyperphosphorylated tau is found in frontotemporal dementia, and also cognitive dysfunction, which is the typical symptom of AD, is correlated with NFTs.[26] Furthermore, recent

studies revealed that tau was secreted extracellularly via the release of exosome in AD.[27]

Tau protein is known as the factor of various neuronal diseases, however the correlation of tau and AD is investigated in recent studies.[28] Also, it revealed that amyloid plaques increased tau hyperphosporylation via AMPK.[18] So tau pathology has received attention as much as Aβ in AD pathogenesis.

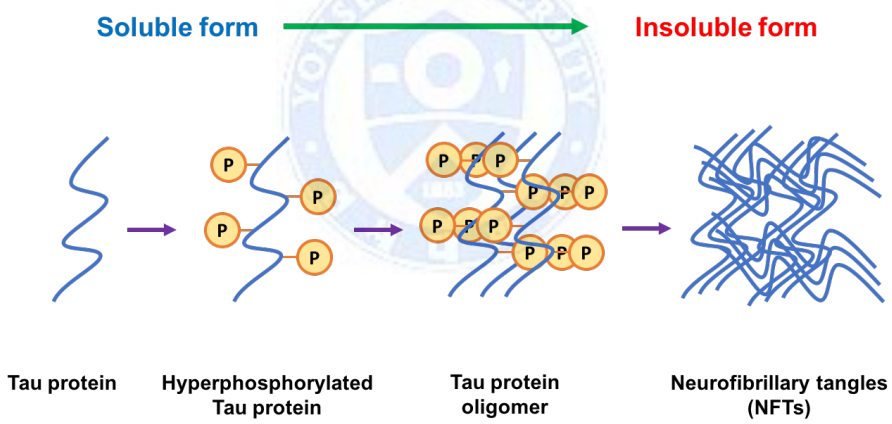

Figure 3. Tau pathway in AD pathogenesis. Soluble tau protein is changed to insoluble form via hyperphosphorylation and aggregation through cascade pathway. After all, tau oligomer is changed to NFTs which lead to neurodegeneration.

C. Cholinergic hypothesis

In 1970s, Drachman et al. showed that synthesis of acetylcholine (Ach) was deficient in the brains of AD patients, and that this shortage led to neurochemical abnormality via presynaptic cholinergic deficit.[29] Following this preliminary study, "cholinergic hypothesis of AD" was established based on emerging role of Ach in learning and memory system.[30]

Ach is a neurotransmitter which is involved in cognitive function. Actually, deficits of Ach and cholinergic transmission have been also found in various neurodegenerative disorder.[31] Besides, other study investigated that Ach level was deficient in the brain of normally aging animals. So, cholinergic hypothesis was controversial regarding AD pathogenesis. However, recent studies established that Ach was strongly related with AD pathogenesis, especially Aβ synthesis. The metabolism of Aβ was regulated by stimulation of muscarinic or nicotinic cholinergic receptors. And also, it was found that cholinergic deficits led to be a secondary effect of Aβ toxicity.[16,32]

Cholinergic deficiency may not be a direct factor but could be a metabolic reason for initiation of amyloid cascade pathway. On this basis, increase in synthesis and synaptic activity of Ach was focused on as a therapeutic strategy on neuropharmacology,[32] and cholinersterase inhibitors such as donepezil, rivastigmine and galantamine, are being used for the treatment of AD.[33,34]

3. AD and Diabetes

According to the National Statistical Office (NSO), the world already has entered into aging society, and is progressing towards super-aged society. An aging society incurs many severe problems, which are the labor shortage, slowdown of economic growth, and increase of support expense. In particular, elderly people may be prone to various diseases, and this is the major cause of increment of support expense. Cognitive and metabolic disorders are among the most representative geriatric diseases, such as AD and diabetes.[35] Intriguingly, many studies have suggested that AD and diabetes were correlated from epidemiologic and mechanistic perspective.[36,37]

A. Epidemiologic interaction

Diabetes, especially Type 2 diabetes mellitus (T2DM), is connected with AD, and many previous studies showed that diabetes is a high risk factor of AD and other dementias.[35,36] However, from other previous epidemiologic findings, correlation of AD and diabetes has been controversial. Some investigators showed that although diabetes had increased risk for development of AD and dementia, the effects of risk factors, especially insulin resistance, were independent rather than linked between dementia and AD in longitudinal survey.[38] Also in other study, people who had lower body mass index (BMI) were faced in higher risk for developing AD than higher BMI.[39]

B. Mechanistic interaction

Mechanistic correlation between AD and T2DM has been studied by many investigators. Actually, T2DM and obesity share a lot of factors increasing dementia risk, such as peripheral insulin resistance, oxidative stress, increment of pro-inflammatory cytokines, chronic hyperglycemia and cerebral microvascular disease.[39,40] However, direct correlation of T2DM and AD was controversial via some studies.[41,42]

A previous study showed that old adults with T2DM had some risk factors of AD, including hippocampal atrophy, and lacunes in brain via neuroimaging. Also, another study showed that T2DM was occurred more frequently in AD patients than non-AD patients.[41] However, other study investigated that T2DM and increase of NFTs were not correlated, and suggested that T2DM was not sufficient factor of increase of AD.[42]

In a recent study, correlation between T2DM and AD was shown regarding to histopathological, molecular and biochemical abnormalities using high-fat diet feeding mice and human studies.[43] Moreover, these investigators referred AD as “type 3 diabetes mellitus” and asserted that AD might be treatable, preventable, or curable by anti-diabetes medications.

4. Metformin

Metformin has been used as first-line medication for T2DM for over 50 yr.[44] Metformin has some advantages including safety profile and weight reduction effects in cardiovascular disease.[45,46] Additionally, metformin has shown some possibilities to reduce cancer risk in diabetic patients.[47] It has been also used in other insulin resistance diseases such as gestational diabetes and polycystic ovary syndrome.[48,49] On the basis of these advantages and various usages, metformin was selected as a WHO Model List of Essential Medicines by World Health Organization (WHO).

A. History and molecular action

Metformin belongs to the biguanide class which was used as folk medicine in the past. In 1922, metformin was first come up in a scientific report as a product of synthesis of *N, N-dimethylguanidine* via reaction of dimethylamine hydrochloride and 2-cyanoguanidine with heating (Fig. 4-A).[50,51] However, for over 30 years, metformin had been forgotten because of insulin usage for diabetes. In 1957, metformin was first used for humans having diabetes as named "Glucophage" by French physician, Jean Sterne. After that, metformin was permitted usage for treatment of diabetes by British National Formulary in 1958, and approved by Canada in 1972. In 1994, metformin finally was recognized by U.S. Food and Drug Administration (FDA).[52]

Metformin suppresses blood sugar level by gluconeogenesis inhibition and reducing hepatic glucose output in terms of anti-diabetic effects,[50] and metformin is also known as AMPK activator in energy metabolic aspects. Guanidine-containing complexes had been known for reducing hyperglycemia before metformin was developed, however they had a toxicity.[53] Many pioneer investigators, who were interested in the guanidine effects, tried to reduce toxicity, and finally found the methods of suppressing the toxicity via composed of two guanidines. Metformin was found before the generation of target-specific drug discovery, so its mechanism has been investigated since clinical usage, and is currently underway. Up to date, a major anti-hyperglycemic mechanism of metformin in liver cells, including that of other biguanides, has been known as inhibition of mitochondrial electron transport chain, complex I, which leads to reduction in ATP: AMP ratios regarding cellular energy depletion.[54,55] Increase of AMP concentration inhibits directly gluconeogenesis, and also suppresses activation of glucagon via inhibition of adenylate cyclase.[56] This cascade pathway is the main anti-diabetic mechanism of metformin. Additionally, increment of AMP activates AMPK, and it leads many positive effects in energy metabolism (Fig. 4-B).

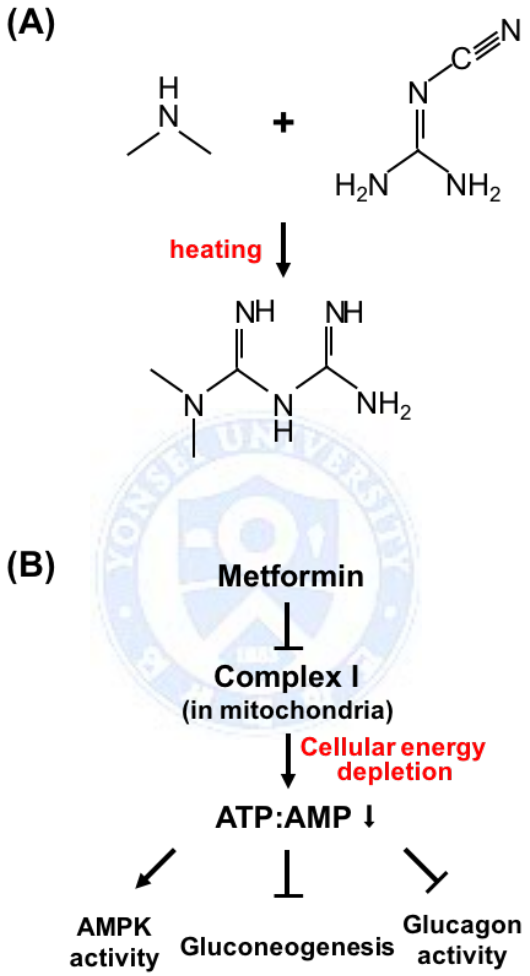

Figure 4. Synthesis and molecular action of Metformin. (A) Metformin was synthesized via reaction of dimethylamine and 2-cyanoguanidine. (B) Metformin activates AMPK and inhibits gluconeogenesis and activation of glucagon via suppressing of mitochondria complex I.

B. Metformin and AD

As mentioned above, metformin activates AMPK via increase of AMP concentration in hepatocytes. AMPK activation is critical mechanism of energy metabolic regulation, and previous studies showed that metformin-induced AMPK activation modulated various effects of AMPK-dependent energy metabolic pathways; decrease of sterol regulatory element-binding protein-1 (SREBP-1),[57] suppression of lipid synthesis in liver[58] and increase of fatty acid oxidation in hepatocytes.[54]

On the other hand, in 2009, it was reported that metformin induced adverse effects on AD pathway.[59] Chen et al. demonstrated that metformin increased Aβ production via AMPK activation *in vitro* and *in vivo*. They elucidated that metformin-induced AMPK activation increased the activity of BACE1 promoter, causing increase in Aβ levels in the brain. Given the general expectation that anti-diabetic medication may exert beneficial effects on AD pathogenesis, the findings from Chen et al. study have raised issues on the role of AMPK in AD pathogenesis.

5. AMPK and SIRT1

A. AMPK

In neurons, AMPK is known as the important factor for maintenance of energy balance and healthy aging. Caloric restriction (CR) is well known to extend lifespan, and such effect was linked to increase of AMPK activity.[60]

Moreover, AMPK increases the autophagy via the mammalian target of rapamycin (mTOR) pathway, and enhances stress resistance through reducing of forkhead box protein O1 (FOXO1) and nuclear factor erythroid 2-related factor 2 (Nrf2) activity. AMPK is also regarded as a critical target molecules to rescue or prevent neurodegeneration diseases.[61] Resveratrol, a kind of natural polyphenol, was known to exert a positive effect on Parkinson`s disease, amyotrophic lateral sclerosis, and AD through AMPK activation. Especially, resveratrol attenuated AD by modulating Aβ- and tau-related pathogenesis via AMPK.[62]

B. SIRT1

In a previous study with resveratrol, Price et al. showed another key factor to gain therapeutic effects other than AMPK activation, it was SIRT1. It was known as a deacetylase enzyme.[63] SIRT1 is linked to numerous cellular processes, including gene silencing, DNA repair, metabolic regulation, and suppression of neurodegeneration or aging via both direct and indirect deacetylation of various transcription factors.[64,65,66] In previous studies, SIRT1 was shown to have beneficial effects against AD under CR. In SIRT1-deficient condition, calorie restricted mice did not reduce Aβ plaque.[67] In contrast, SIRT1 activation led to prevent amyloidogenesis in CR condition mice.[68] Also, SIRT1 overexpression mice were rescued from AD phenotype similar to CR condition.[69]

C. Concomitant action of AMPK and SIRT1

Recent investigations have revealed that concomitant action of AMPK and SIRT1 is critical for their beneficial effects on metabolism since experimental manipulations to activate only either of the two have shown failures in deriving expected outcomes.[70,71] Nevertheless, AMPK and SIRT1 normally activate each other. Thus, activation of either of the two would be expected to induce their concomitant action. Indeed, resveratrol, as a SIRT1 activator, has shown to activate AMPK via SIRT1/liver kinase B1 (LKB1) pathway in hepatocytes (Fig. 5).[62,72] 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMPK activator, is known to activate SIRT1 via Nampt/NAD pathway or promoting beta-oxidation (Fig. 5).[73] However, such a dynamic relationship between AMPK and SIRT1 has not been fully elucidated in neurons compared to other peripheral tissues.

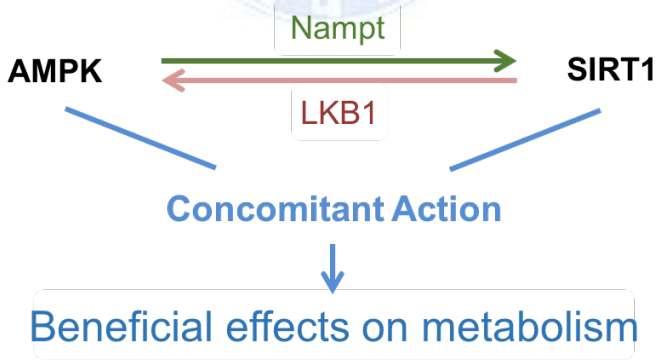

Figure 5. The scheme of concomitant action of AMPK-SIRT1. Many recent studies show that AMPK and SIRT1 activate each other via Nampt and LKB1, respectively. Resultantly achieved, concomitant activation lead to beneficial effects on metabolism in several peripheral tissues.

D. AMPK, SIRT1 and Nampt

Nampt is the key molecule of NAD synthesis, and thus SIRT1, NAD+ dependent deacetylase, is directly affected by Nampt.[74] Additionally, SIRT1 converts NAD to NAM in NAD salvage pathway, thus depletion of Nampt leads to lack of NMN directly, and shortage of NAM indirectly.[9] Moreover, previous study showed that AMPK could activate SIRT1 using NAD.[73] Therefore, Nampt may be a critical mediator of beneficial effects on metabolism of concomitant action of AMPK-SIRT1.

6. Aims of this study

Metformin has been still first line of anti-diabetic medication, and this trend may continue for a while. Moreover, diabetes is a chronic disease which needs life-long cares and treatments. So, a potential of metformin to induce AD pathogenesis is a highly serious problem to patients with diabetes who may have already a tentative risk of AD.

AMPK activation is the pivotal mechanism of metformin, and thus is thought to be related to various beneficial effects of this drug. According to recent findings, however, activation of neuronal AMPK might have a negative, rather than positive, role in AD pathogenesis. Neuronal AMPK activation by various compounds including metformin has been shown to induce BACE1 upregulation and synaptotoxicity via AMPK-dependent tau

phosphorylation.[18,28] These findings are highly unexpected because AMPK has been known to increase SIRT1 activity through Nampt/NAD pathway at least in peripheral tissues,[73,75] and concomitant action of AMPK and SIRT1 has been shown to suppress AD pathologies.[70] However, to my best knowledge, effects of metformin on SIRT1 activity have not been directly examined in neuronal tissue, which is known to have relatively low levels of Nampt expression and NAD depletion.[76,77]

Here I show that metformin increased activity of neuronal AMPK, but failed to induce concomitant activation of SIRT1 in neuronal tissues. Such failure in SIRT1 activation was attributed, at least in part, to metformin-induced downregulation of Nampt which mediates AMPK-dependent SIRT1 activation via the NAD salvage pathway. In addition, Metformin-induced suppression of Nampt was found to decrease the expression of TACE which decreases Aβ production by promoting non-amyloidogenic pathways.[21] Interestingly, the most widely prescribed anti-dementic drug, donepezil, reversed such adverse effects of metformin by activating Nampt and thus rendering the concomitant activation of AMPK and SIRT1.

II. MATERIALS AND METHODS

1. Cell culture and transfection

Neuro2A (from ATCC, Manassas, VA, USA), the mouse neuroblastoma cell lines, was grown in Dulbecco`s Modified Eagle`s Medium (GE healthcare, South Logan, UT, USA) with 10% fetal bovine serum (GE healthcare) in a humidified incubator containing 5% CO_2, at 37°C. NAMPT and SIRT1 DNA (Addgene, Cambridge, MA, USA) were amplified by PCR and sub-cloned into pcDNA3.1 vector. Neuro2A cells were transfected with cloned DNA supplement 10ug jetPRIME (Polyplus Transfection, Illkirch, France) for 24hr before the start of the experimental incubations. For stable cell line, Human-APP_{695}, sub-cloned into pcDNA3.1 vector, was transfected into Neuro2A cells. And transfected cells were selected geneticin (200ug/ml, Gibco, Grand Island, NY, USA) for 48hr, then only selected cells were sub-cultured by DMEM-OptiMEM (Gibco) mixed media supplemented 10% fetal bovine serum with 200ug/ml geneticin. Stable cell line was fast freezing and stocked in liquid nitrogen tank.

2. Animal experiments

Eight-week-old male db/db mice (Central lab, Animal Inc. Seoul, Korea) were treated metformin (2mg/ml in drinking water)[59] and donepezil (5mg/kg) ad libitum. Water intake and body weight of the animals were monitored every day. After 7days of treatment, mice were sacrificed and their hippocampi were snap-frozen on dry ice, and used for Western blot analysis and qRT-PCR. All mice were housed with a 12hr light-dark cycle in Yonsei University College of Medicine Animal Care Facilities approved Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were approved by the Animal Care Committee of Yonsei University college of Medicine with NIH guidelines.

3. Reagents and antibodies

Metformin (1,1-bimethylbiguanide hydrochloride) and FK866 were purchased from Sigma Aldrich (St. Louis, MO, USA). Compound C, an AMPK inhibitor, was from Merck Millipore (Darmstadt, Germany). Anti-BACE1, pAMPK (Thr172), AMPK, HRP-rabbit and HRP-mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-

SIRT1 antibody was from Merck Millipore. Anti- TACE, ADAM10, GAPDH, and β-actin antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA).

4. Western blot analysis

Cells were lysed in ice-cold RIPA buffer (Thermo, Rockford, Illinois, USA), addition of 1mM NaF, 1mM Na_3VO_4, 1.15mM Na_2MoO_4, 2mM $C_3H_2N_4$, 4mM $C_4H_4Na_2O_6 \cdot 2H_2O$ (Sigma Aldrich), and complete protease inhibitor cocktail (Roche, Indianapolis, Indiana, USA). All lysates were measured with the BCA Protein Assay Kit (Thermo). I made samples with equal amounts of protein and boiled at 97°C for 10min to denaturation of protein structure. Each protein sample was separated on 4-12% bis-Tris gel or 10% Tris-Glycine gel for the molecular weight of the protein of interest. And proteins were transferred to nitrocellulose membranes (GE healthcare) using wet transfer systems (Mighty Small Transphor, GE healthcare). Finally, protein bands were detected with Amersham ECL western blotting reagent (GE Healthcare) using the LAS mini system (Fuji Film, Tokyo, Japan). All band intensities were measured using Image J software (http://rsbweb.nih.gov/ij/).

5. Quantitative Real-time PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Equal amounts of RNA were reverse transcribed using Tetro cDNA Synthesis Kit (Bioline, London, UK). Quantitative PCR was performed using StepOne plus systems (Applied Biosystems, Foster City, California, USA) with a SensiFAST SYBR Hi-ROX kit (Bioline). All RNA-level expressions were calculated using the ΔΔCt method and normalized to GAPDH. The sequences of the sense (Forward; F) and antisense (Reverse; R) primers were as follows; BACE-F (5`-GATGGTGGSCAACCTGAG-3`), BACE-R (5`-CTGGTA-GTAGCGATGCAG-3`), TACE-F (5`-ATCTGAAGAGTTTGTTCGTCG-AG-3`), TACE-R (5`-TCCACGGCCCATGTATTTAT-3`), ADAM10-F (5`-CCATGCTCATGGAAGACAGTT-3`), ADAM10-R (5`-CCTTCTTCACC-ATAAATATGTCCA-3`), Nampt-F (5`-GGTCATCTCCCGATTGAAGT-3`), Nampt-R (5`-TCAATCCAATTG-GTAAGCCA-3`), GAPDH-F (5`-GGC-ATTGCTCTCAATGACAA-3`) and GAPDH-R (5`-ATGTAGGCCATGA-GGTCCAC-3`).

6. Enzyme-linked Immunosorbent Assay

The quantitative assessment of A$\beta_{1\text{-}42}$ of secretion was performed using the Human Amyloid β42 ELISA (HS) High sensitivity Kit (EMD Millipore Corp. MA. USA) according to manufacturer`s instruction. N2A-APP$_{695}$ cells were cultured in 6well plate and treated metformin and donepezil for 24hr, and 100ul of culture medium was used for measurement. All results are normalized to the value of protein assay with BCA protein Assay Kit (Thermo) using cell lysates.

7. NAD/NADH ratio measurements

The measurement of NAD/NADH ratio was performed with whole cell extracts of Neuro2A cells using the NAD/NADH Quantitation kit (Biovision, Milpitas, CA, USA) according to the manufacturer`s instruction. This colorimetric assay measured NADt, which was the sum of NAD and NADH, and NADH only. So I could calculate the value of NAD with NADt and NADH levels. All assays were measured at 450nm absorbances. All values of NAD and NADH were normalized to the protein content of the cell lysate determined using the Pierce BCA Protein Assay kit (Thermo).

8. SIRT1 activity assay

Cells were lysed as described in Western blot analysis. After measurement of proteins, 10ug of total protein was used in SIRT1 activity assay using Colorimetric SIRT1 Activity Assay Kit (Abcam) according to the manufacturer`s instruction. The kit included specific substrate that was deacetylated by SIRT1, and measured the levels of deacetylation with specific antibody. Each sample was performed via triplet assay and the values are calculated on average and compared to control value.

9. Statistical Analysis

All results are expressed as the mean ± S.E. All data were analyzed with Student's t-test and one-way ANOVA followed by Dunnett post hoc test. Significance was set at P value less than 0.05.

III. RESULTS

1. Metformin increases BACE1 expression via AMPK

I first examined the effects of metformin-induced AMPK activation on BACE1 expression in Neuro2A cells. The protein and mRNA levels of BACE1 were significantly elevated at 10mM treatment of metformin (Fig 6-A and B). Also I found that the treatment of 10mM metformin increased phosphorylation of AMPK (Fig. 6-A). However, cotreatment of compound C, an AMPK inhibitor, with metformin suppressed BACE1 upregulation (Fig. 6-C and D), suggesting that metformin-induced BACE1 upregulation is AMPK dependent. Next, to confirm that the production of Aβ is indeed affected by metformin-induced BACE1 expression, I measured the amount of secreted Aβ in culture medium of N2A-APP_{695} stable cell lines treated with metformin by ELISA. As expected, ELISA results showed that the secretion level of Aβ was significantly increased at 10mM metformin treatment (Fig. 6-E).

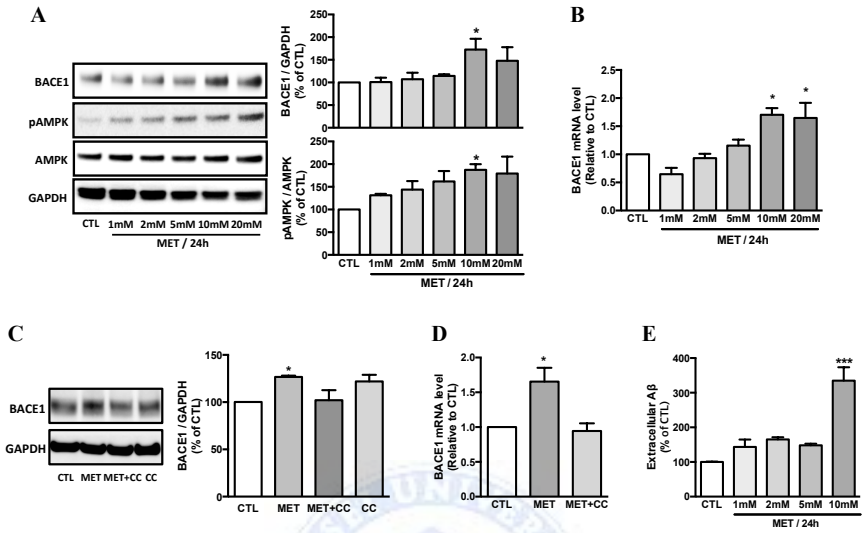

Figure 6. Metformin increases BACE1 expression. (A) BACE1 protein levels and AMPK activation levels were determined using Western blot analysis. (B) BACE1 mRNA levels were measured by qRT-PCR. Neuro2A cells were treated with multiple dose of metformin (MET) for 24hr. (C, D) Neuro2A cells were exposed with Compound C (CC) for 6hr after 10mM metformin treatment. BACE1 protein levels and mRNA levels were analyzed with western blotting and qRT-PCR. (E) Extracellular Aβ in N2A-APP$_{695}$ cells was measured using ELISA. **P*<0.05 and ****P*<0.001 compared with control (CTL).

2. Metformin decreases TACE expression while increasing BACE1

In Aβ synthesis, cleavage of APP via BACE1 was the first and critical step.[20] However, cleavage APP via α-secretase prevented the Aβ production, and these two events occur competitively. The representative α-secretases are "a disintegrin and metalloproteinase" (ADAM) family, especially ADAM10 and TACE, that cleaved specific site of APP to make non-amyloidogenic fragments.[22] I therefore assessed ADAM10 and TACE expression levels in Neuro2A cells, and found that metformin reduced protein and mRNA levels of TACE (Fig. 7-A and B). To confirm *in vivo* results, I analyzed the expression levels of α- and β-secretases in the hippocampal tissues of db/db mice treated with metformin (2mg/ml, 7days) in drinking water. Increase in BACE1 expression and decrease in TACE expression also were identified *in vivo* (Fig. 7-C and D). These results showed that metformin affects TACE as well as BACE1 expressions, and eventually promote AD-related pathogenesis.

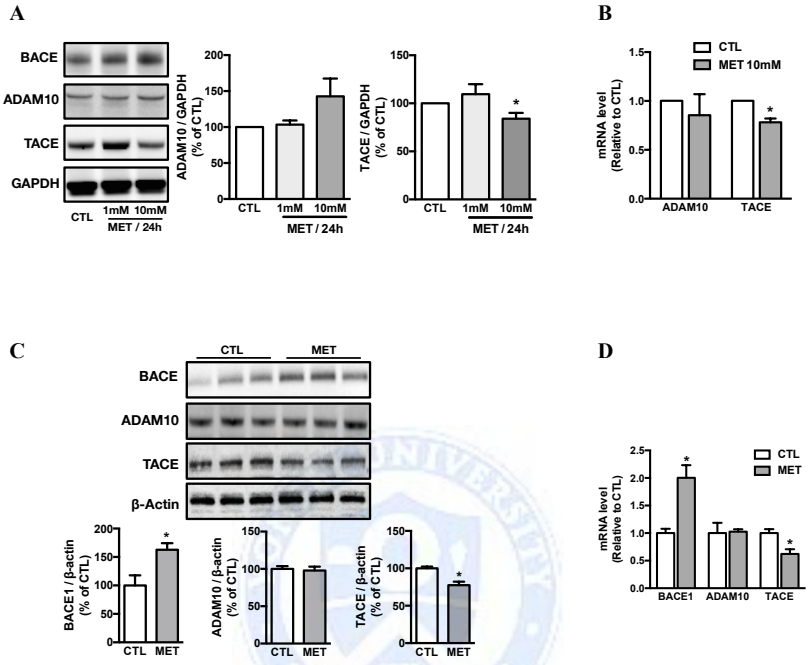

Figure 7. Metformin decreases TACE expression *in vitro* and *in vivo*. (A-B) Metformin-induced α-secretases (ADAM10 and TACE) expressions were analyzed western blot and qRT-PCR. (C-D) The hippocampi of db/db mice (8 week-old, n=3) treated 2mg/ml of metformin for 7days were used for analyze of expression of α- and β-secretases. All mice were fed metformin on water ad libitum. **P*<0.05 compared with control (CTL).

3. Metformin decreases activity and expression of SIRT1 by reducing NAD/NADH ratio

The activation of AMPK can lead to activation of SIRT1. Activation of both AMPK and SIRT1 then can reduce BACE1 expression level and Aβ production.[78,79] Nevertheless, the previous study and data from the current study demonstrated that metformin increased BACE1 expression via AMPK activation in neurons. Therefore, I sought to the effect of metformin on SIRT1 in neuro2A cells. Protein level of SIRT1 was decreased by metformin in Neuro2A cells (Fig. 8-A). Furthermore, metformin significantly suppressed activity of SIRT1 at 10mM (Fig. 8-b), the same concentration at which metformin increased BACE1 expression and AMPK activation (Fig. 6-A and B). These data revealed that, in neurons, metformin decreased the expression and activity of SIRT1 in contrast with previous findings with other peripheral tissues. AMPK is known to activate SIRT1 by increasing NAD/NADH ratio.[73,75,80] So I measured NAD level and NAD/NADH ratio in Neuro2A cells exposed to metformin. Metformin reduced the level of NAD and NAD/NADH ratio (Fig. 8-C). Taken together, these results suggested that metformin reduces SIRT1 activity via reducing NAD/NADH ratio despite AMPK activation in neurons.

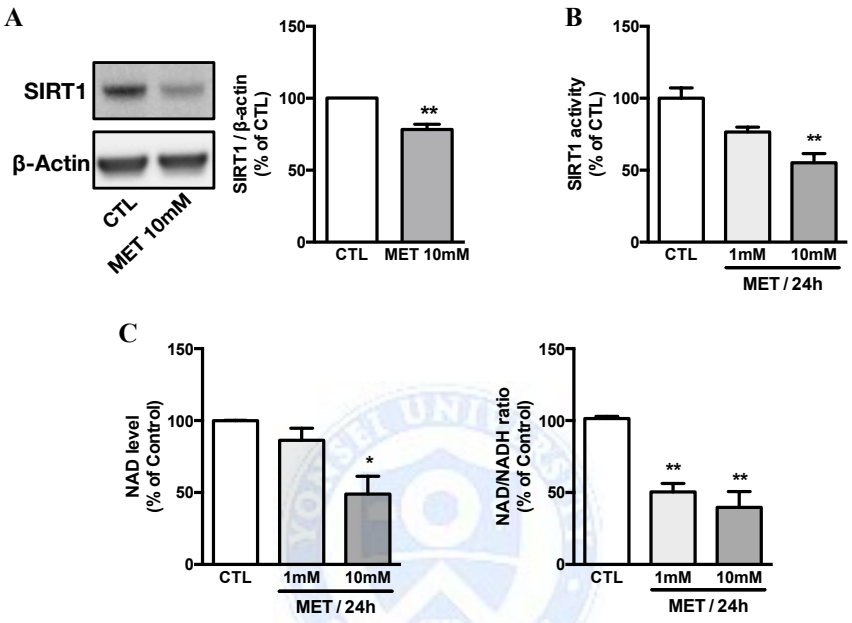

Figure 8. Metformin decreases SIRT1 activity and expression. (A) Metformin-induced changes in SIRT1 expression was analyzed using western blot. (B) SIRT1 activity was measured using SIRT1 activity kit at 1mM and 10mM metformin treatment. (C) The cellular levels of NAD and NADH were evaluated at 450nm. All experiments were performed at 24hr treatment condition and were repeated three times. **P*<0.05 and ***P*<0.01 compared with control (CTL).

4. Metformin-induced Nampt suppression accounts for reduced SIRT1 activity and NAD levels

SIRT1 has been reported to be activated through AMPK/Nampt/NAD pathway.[81] Inhibition of Nampt decreases the cellular level of NAD.[82] However, results from the current study showed that the expression and activity of SIRT1 was decreased despite the activation of AMPK by metformin. I then asked whether metformin affects the levels of α- and β-secretases via Nampt. First, I examined the effect of metformin on Nampt expression. In Western blot analysis and qRT-PCR, expression level of Nampt was decreased upon metformin treatment (Fig. 9-A and B). Next, I observed the changes in BACE1 and TACE levels upon Nampt overexpression in Neuro2A cells. Although Nampt was reduced by metformin even in overexpression condition, BACE1 and TACE levels were unaffected by metformin (Fig. 9-C and D). These results suggest that sufficient amount of Nampt could suppress metformin-induced aberrations in Aβ metabolism by supplying NAD needed to SIRT1 activation.

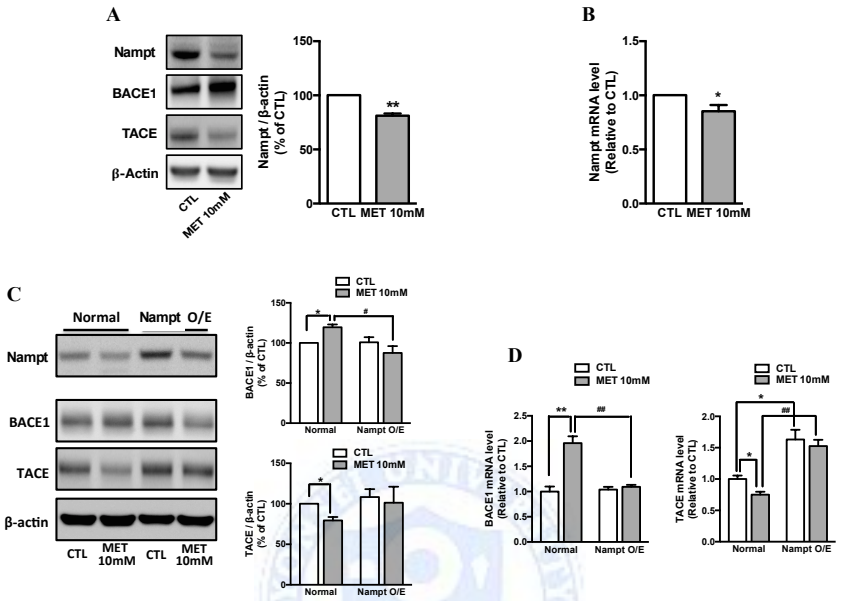

Figure 9. Metformin decreases Nampt expression. (A) Metformin-induced Nampt expression was analyzed using western blot. (B) Nampt mRNA expression was measured using qRT-PCR upon 10mM metformin treatment. (C-D) In Nampt overexpression condition, BACE1 and TACE expressions were analyzed by western blot and qRT-PCR. All experiments were performed at 24hr treatment condition and were repeated three times. **P*<0.05 and ***P*<0.01 compared with control (CTL). $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared with metformin (MET 10mM).

5. Donepezil reverses metformin-induced TACE suppression via Nampt/NAD/SIRT1 *in vitro* and *in vivo*

Donepezil is the most widely prescribed anti-dementic drug, which has been also identified as an AMPK activator.[83] I thus intended to examined if donepezil could suppress (as an anti-dementic drug) or aggravate (as an AMPK activator) the observed adverse effects of metformin on neurons. First, I analyzed the amount of secreted Aβ using ELISA in Neuro2A cells treated metformin alone and metformin plus donepezil. Interestingly, Aβ was reduced upon the cotreatment compared to treatment of metformin alone (Fig. 10-A). I also examined the changes in the levels of secretases, and found that expression levels of secretases were reversed by donepezil cotreatment compared to metformin alone (Fig. 10-B and C). Especially, mRNA levels of BACE1 and TACE were significantly changed towards the direction of reducing Aβ production. Next, I investigated whether donepezil regulates Nampt/SIRT1 pathway as well. In donepezil cotreatment condition, NAD level and Nampt expression were increased (Fig. 10-B to D), while SIRT1 expression was not changed (data was not shown), suggesting that donepezil might enhance the activity of SIRT1 via Nampt/NAD pathway. Thus, I aimed to confirm the involvement of Nampt in actions of donepezil by using FK866, a Nampt inhibitor. FK866 mitigated

the effects of donepezil on mRNA levels of TACE (Fig. 10-E and F). Finally, I intended to confirm the effect of donepezil *in vivo*. I treated db/db mice for 7days with either of donepezil (5mg/kg) plus metformin (2mg/ml) or metformin alone. The reason for use of db/db mice was to recapitulate the context-relevant situations in real clinical realms where metformin is prescribed. I found that donepezil also reversed adverse effects of metformin on BACE1 and TACE expression in the hippocampal tissues of db/db mice (Fig. 11-A and B). Taken together, these results suggest that donepezil treatment could be an effective option to suppress the adverse effects of metformin on TACE and BACE1 expression via Nampt/NAD/SIRT1 pathway.

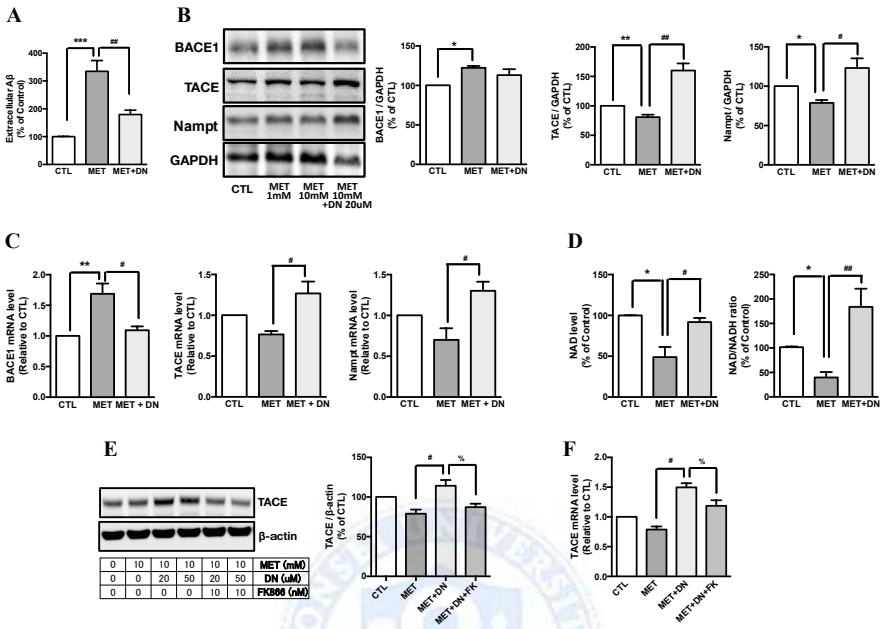

Figure 10. Donepezil reverses metformin-induced amyloidogenesis *in vitro*. (A) Conditioned medium of Neuro2A cells that were treated with 10mM metformin (MET) and 20uM donepezil (DN) for 24hr were used for measurement of secretion of Aβ via ELISA. (B) Protein levels of α- and β-secretases of which Neuro2A cells were treated metformin (10mM) and donepezil (20uM) for 24hr were determined using western blot. (C) BACE1 and TACE mRNA levels were measured using qRT-PCR. (D) The cellular NAD level and NAD/NADH ratio were evaluated using NAD/NADH Assay Kit. (E-F) The TACE expressions of Neuro2A cells treated with10mM metformin, 20uM donepezil and 10nM FK866 (FK) were measured by Western blot and qRT-PCR. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared with control (CTL). $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared with metformin (MET 10mM). $^{\%}P$<0.05 compared with cotreatment of metformin and donepezil (MET+DN).

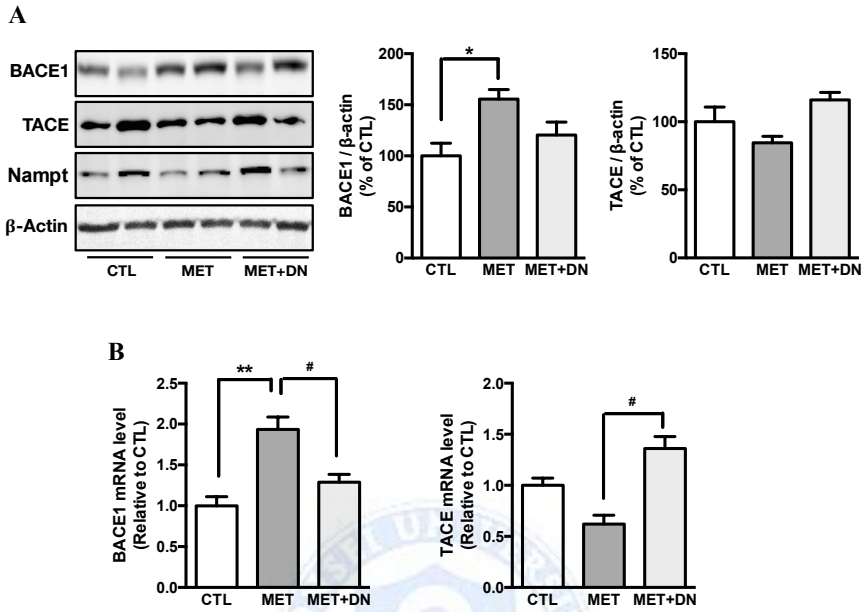

Figure 11. Donepezil reverses metformin-induced amyloidogenesis *in vivo*. (A-B) The expressions of α- and β-secretases of hippocampi in which db/db mice (n=3) were treated donepezil (5mg/kg) with metformin for 7days were analyzed using Western blot and qRT-PCR. **P*<0.05 and ***P*<0.01 compared with control (CTL). $^{\#}$*P*<0.05 compared with metformin (MET 10mM).

IV. DISCUSSION

The role of metformin, having been primarily used for DM over the past 50yr,[45] has been still controversial regarding AD-related pathologies. In various mechanistic studies, actions of metformin have been related to its ability to activate AMPK.[84] In addition, a recent study has shown that metformin can activate SIRT1 independently of AMPK pathway in hepatocytes.[80] Many previous studies reported that the activations of both AMPK and SIRT1 are beneficial for energy metabolism in various organs including the brain.[61,85,86] Whereas, metformin was reported to increase of Aβ via upregulation of BACE1 in neurons. Therefore, I confirmed that metformin increases BACE1 expression and Aβ production via AMPK in neurons, consistent with previous findings.[59] Contrary to previous finding from other peripheral tissues,[86] I found that metformin decreased the expression and activity of SIRT1 via suppression of Nampt/NAD pathways even while activating AMPK. I found that AMPK activation without SIRT1 activation by metformin caused amyloidogenesis in neurons (Fig. 12-A). Additionally, I identified that donepezil could reverse the adverse effects of metformin within the framework of translational research using diabetic model mice (Fig. 12-B).

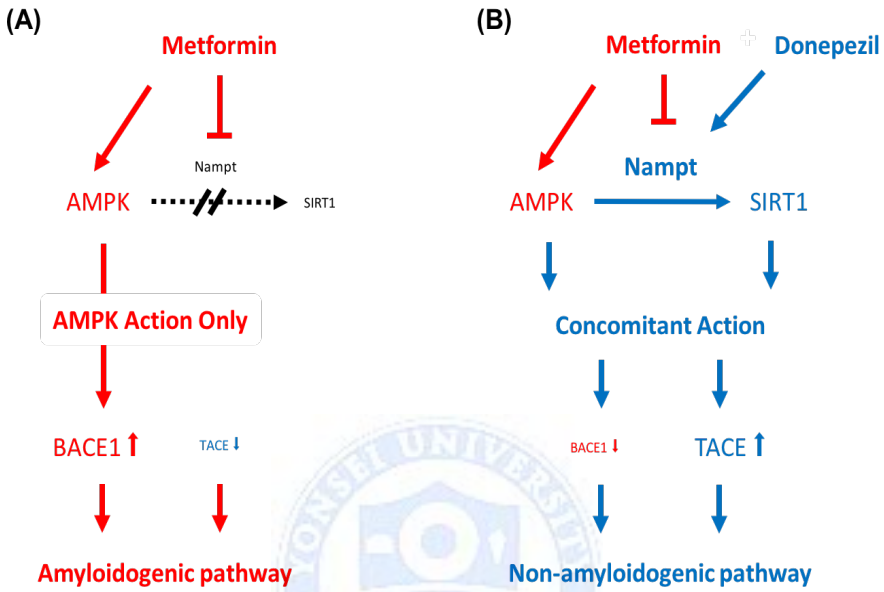

Figure 12. Working hypothesis for actions of metformin and donepezil in amyloidogenesis. (A) Metformin can activate only AMPK but fails to activate SIRT1 due to its inhibitory action on Nampt. This action results in enhancement of amyloidogenic pathway leading to both increase in BACE1 and decrease in TACE. (B) Donepezil increases Nampt so that AMPK and SIRT1 can work concomitantly, leading to enhancement of non-amyloidogenic pathway.

The cerebral aggregation of Aβ is one of main pathogenic events in AD.[17] Aβ is generated from APP by actions of two enzyme, BACE1 and γ-secretase.[14,20] In contrast to BACE1, α-secretases, such as ADAM10 and TACE, forestall Aβ production through cleavage of APP at other specific site (Fig. 2).[21,22] The result of this study showed that metformin can augment the production of Aβ via not only increasing BACE1 but also decreasing TACE. I also measured ADAM10 protein and mRNA level. ADAM10 expression was not significantly changed by metformin treatment (Fig. 7-A and B). Recently, TACE has also been focused on regarding Aβ metabolism.[87,88] Yoshida et al. showed that activating TACE via SIRT1 suppressed the production of Aβ in human astrocytoma cells.[89] Above these previous reports, findings from the current study also suggest that metformin reduced TACE via suppression of Nampt was important in amyloidogenesis.

In a previous study, Chen et al. showed only increment of BACE1 via AMPK activation. In fact, the role of AMPK was controversial in neurons. AMPK activation could increase the tau phosphorylation.[18] Additionally, it was reported that tau phosphorylated by AMPK mediates synaptotoxic effects of Aβ oligomers.[28] I demonstrated that metformin increases amyloidogenesis in neurons via inactivation of SIRT1 by Nampt suppression while AMPK was activated (Fig. 7 and 8) .

Finally, I intended to see if an anti-dementic drug could offset adverse effects of metformin as a translational approach. Donepezil, the acetylcholinesterase inhibitor, is being most widely prescribed for AD patients worldwide. Donepezil reduced Aβ induced-neurotoxicity via GSK-3β,[90] and decreased Aβ plaque density.[91] Data from the current study showed that donepezil suppresses the Aβ secretion increased by metformin *in vitro* (Fig. 10-A). Interestingly, donepezil significantly enhanced the levels of NAD and expression of TACE (Fig. 10-C and D). Upon treatment of FK866, an Nampt inhibitor, donepezil failed to increase TACE expression (Fig. 10-E and F), indicating that Nampt is participated in non-amyloidogenic mechanism via donepezil. Moreover, I confirmed that donepezil could reverse the adverse effect of metformin via modulating BACE1 and TACE *in vivo* (Fig. 11).

In a previous study, it was identified that β-oxidation, rather than Nampt, mediates AMPK-induced enhancement in NAD/SIRT1 pathway in the skeletal muscle cells.[55] However, considering that β-oxidation is not favored energy metabolism in neurons, metformin-mediated suppression of Nampt could be the main reason for the failure of SIRT1 activation in neurons upon AMPK activation. Current experiments used db/db mice, a well-defined diabetic model, considering that metformin is an anti-diabetic medication. In

previous studies, diabetic mice have been used to examine the effects of diabetes on Aβ secretion, tau protein level and cognitive function.[59,92] However, for I only examined short-term effects of treatment of medications (for 7days), long-term experiments are further required to confirm the translational meanings of findings from this study, since diabetic patients need to take medicines for a long time.

Results from the current study demonstrated that metformin suppressed neuronal Nampt expression which is crucial for SIRT1 activation. Moreover, to the best of my knowledge, this is the first thesis demonstrating that metformin reduced TACE via suppression of Nampt. Although I did not investigate the interaction of metformin and γ-secretase, metformin-mediated decrease in TACE expression was confirmed as reason for the increase in amyloidogenesis by this drug in addition to BACE1 upregulation. Finally, these results about cotreatment of metformin and donepezil suggested the possibility of pharmacological modulations that prevent the adverse effect of metformin in neurons.

V. CONCLUSION

This study identified that Nampt was the critical target of metformin-induced adverse effects on amyloidogenic pathway. Also, I showed that donepezil could reverse the metformin-induced negative effects in neurons. What I have found through this study is,

1. Metformin activated AMPK, however reduced Nampt in neurons.

2. Metformin-mediated suppression of Nampt caused a decrease in SIRT1 activity, and so the failure in the concomitant action of AMPK and SIRT1.

3. AMPK activation without SIRT1 activation increased BACE1 expression and decreased TACE1 expression, which increased amyloidogenesis.

4. Donepezil could reverse the adverse effects of metformin on amyloidogenic pathway by rendering possible the concomitant action of AMPK and SIRT1 via enhancing Nampt expression.

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ABSTRACT (In Korean)

Hippocampus 내 아밀로이드 생성과 비생성 경로에서의
nicotinamide phosphoribosyltransferase 의 관여 기전

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정 지 현

최근 신경세포에서 metformin 의 AMP-activated kinase (AMPK) 활성화가 Alzheimer`s disease(AD)와 관련, 아밀로이드 생성 기전 활성을 유발하는 것으로 보고되었다. 1950 년대부터 2 형당뇨의 1 차약제로 사용되고 있는 Metformin 의 주요기전은 AMPK 를 활성화 시켜 포도당 생성 억제, 포도당 흡수촉진 및 나아가 대사증후군 억제를 하는 것으로 알려져 있다. 기존 말초조직의 연구에서 AMPK 의 활성화는 Nicotinamide phosphotransferase (Nampt)/NAD 기전을 통하여 silent mating-type information regulator 2 homolog 1 (SIRT1) 의 활성화를 유도, AMPK-SIRT1 동시작용을 유도하는 것으로 보고되어 있다. AMPK 와 SIRT1 의 동시작용은 생체 에너지 항상성과 관련하여 다양한 효능이 있는 것으로 알려져 있다. 또한, 최근 연구를 통하여 간조직에서 metformin 은 SIRT1 을 활성화

시키는 것으로 보고되었다. 본 연구에서는 신경세포에서 metformin 이 AMPK 를 활성화 시킴에도 불구하고 아밀로이드 생성 경로에서의 부정적 효과를 가져오는 원인과 해결 방법을 찾고자 하였다. 우선적으로 신경모세포종에서 metformin 에 의한 세포 내 SIRT1 의 활성화 및 발현의 억제를 발견하였다. 그리고, SIRT1 활성 억제 원인으로 metformin 의 Nampt 감소에 따른 신경세포 내 NAD 공급을 억제하기 때문인 것으로 확인하였다. 또한, metformin 이 beta-site amyloid precursor protein cleavage enzyme 1 (BACE1) 을 증가시켜 아밀로이드 생성 경로를 활성시키는 것 외에, Nampt 감소를 통하여 tumor necrosis factor alpha converting enzyme (TACE) 발현을 억제, 아밀로이드 비생성 경로를 억제하는 것을 Nampt 과발현 세포 모델을 통하여 검증하였다. 마지막으로 중개연구의 관점에서 세포 및 동물 모델을 통하여 donepezil 이 metformin 의 신경독성을 억제하는 결과를 확인하였다.

본 연구는 metformin 의 치매병리에서의 신경 독성 유발 관련, SIRT1 과 Nampt 를 통하여 에너지 대사의 측면에서 신경세포의 특이성과 그 원인을 밝혔다. 그리고 metformin 에 의한 아밀로이드 생성경로의 주요 타겟으로서 BACE1 외에 TACE 가 관련됨을 규명하였다. 또한, donepezil 이 metformin 복용으로 인한 치매병리의 발병을 억제할 수 있는 가능성을 가지고 있음을 보여줌으로서 metformin 의 신경세포 내 부작용의 원인 및 그 해결 방법을 제시하는 데 본 연구의 중요한 의의가 있다고 본다.

핵심되는 말: Nampt, SIRT1, AMPK, Aβ, Amyloidogenesis, Metformin, BACE1, TACE, Donepezil