Master's Thesis

석사 학위논문

AMP-activated protein kinase as a regulator of autophagic cell death in hippocampal neural stem cell.

Seol-Hwa Jeong (정 설 화 鄭 雪 花)

Department of Brain and Cognitive Sciences

뇌·인지과학전공

DGIST

2016

Master's Thesis

석사 학위논문

AMP-activated protein kinase as a regulator of autophagic cell death in hippocampal neural stem cell.

Seol-Hwa Jeong (정 설 화 鄭 雪 花)

Department of Brain and Cognitive Sciences

뇌·인지과학전공

DGIST

2016

AMP-activated protein kinase as a regulator of autophagic cell death in hippocampal neural stem cell.

Advisor : Professor Seong-Woon Yu

Co-Advisor : Professor Ji-Woong Choi

by

Seol-Hwa Jeong

Department of Brain and Cognitive Sciences

DGIST

A thesis submitted to the faculty of DGIST in partial fulfillment of the requirements for the degree of Master of Science in the Department of Brain and Cognitive Sciences. The study was conducted in accordance with Code of Research Ethics[1)]

12. 04. 2015

Approved by

Professor Seong-Woon Yu (Signature)
(Advisor)
Professor Ji-Woong Choi (Signature)
(Co-Advisor)

[1)] Declaration of Ethical Conduct in Research: I, as a graduate student of DGIST, hereby declare that I have not committed any acts that may damage the credibility of my research. These include, but are not limited to: falsification, thesis written by someone else, distortion of research findings or plagiarism. I affirm that my thesis contains honest conclusions based on my own careful research under the guidance of my thesis advisor.

AMP-activated protein kinase as a regulator of autophagic cell death in hippocampal neural stem cell.

Seol-Hwa Jeong

Accepted in partial fulfillment of the requirements for the degree of Master of Science

12. 04. 2015

Head of Committee _____(Signature)

Prof. Seong-Woon Yu

Committee Member _____(Signature)

Prof. Ji-Woong Choi

Committee Member _____(Signature)

Prof. Eun-Kyoung Kim

MS/BS
201425008

정 설 화. Seol-Hwa Jeong. AMP-activated protein kinase as a regulator of autophagic cell death in hippocampal neural stem cell.
Department of Brain and Cognitive Sciences. 2016. 27 pages.
Advisors Professor Seong-Woon Yu. Professor Ji-Woong Choi.

Abstract

AMP-activated protein kinase (AMPK) is a well-studied molecule for the control of cellular metabolism. In addition, AMPK can affect autophagy to sustain homeostasis. Adult hippocampal neural stem (HCN) cells undergo autophagic cell death (ACD) without apoptotic features following insulin withdrawal, therefore, insulin-deprived HCN cells are regarded as a genuine model for studying autophagy and cell death-related signaling mechanisms. In this study, we investigated the role of AMPK on autophagy regulation in HCN cells following insulin-withdrawal. Genetic and pharmacological activation of AMPK promoted ACD, while inhibition of AMPK yielded the opposite outcomes. These data suggest that AMPK can be a positive regulator of ACD in HCN cells following insulin withdrawal.

Keywords: Autophagy, Adult hippocampal neural stem cells, Autophagic cell death, Insulin, AMPK

Contents

Abstract i

List of contents..... ii

List of figures..... ii

1. Introduction.....1

2. Materials and methods

2.1 Cell culture 5

2.2 Antibodies and reagents 5

2.3 Cell death assay.....5

2.4 GFP-LC3 puncta assay.....6

2.5 Western blot.....6

2.6 Transfection.....6

2.7 Sh-RNA Lentiviral system.....7

2.8 Statistical analysis.....7

3. Results

3.1 AMPK activator, A769662 increased cell death following insulin withdrawal. 8

3.2 Ectopic expression of AMPK constructs modulated cell death.....8

3.3 Silencing of AMPK reduced ACD in insulin-deprived HCN cells.....9

3.4 Cell death mode induced by genetic and pharmacological activation of AMPK was ACD, not apoptosis 9

3.5 Suppression of autophagy related gene, Atg7 prevented ACD.....10

4. Discussion.....11

5. Figure legends.....13

6. Figures.....16

Reference.....24

Abstract in Korean.....26

Acknowledgement.....27

List of figures

Figure 1. The process of autophagy.

Figure 2. Model for AMPK activation.

Figure 3. Increase of cell death with A769662 following insulin withdrawal.

Figure 4. Ectopic expression of AMPK constructs effected on cell death.

Figure 5. Silencing of AMPK decreased ACD in HCN cells following insulin withdrawal.

Figure 6. Cell death mode induced by genetic and pharmacological activation of AMPK was ACD, not apoptosis.

Figure 7. Suppression of autophagy related gene, Atg7 prevented cell death in insulin-deprived HCN cells.

Figure 8. Schematic drawing of suggested autophagy regulation by AMPK in HCN cells.

1. Introduction (Background and significance)

‘Neural stem cells (NSCs)’ is defined by the capacity of self-renewal and differentiation to neural cell types such as astrocytes, neurons, and oligodendrocytes. In the brain, dentate gyrus and subventricular zone have stem cells during development and adulthood. Since the discovery of stem cells even in adult brain during lifetime, stem cell therapy got spotlight as a new strategy to cure neurodegenerative diseases. However, stem cell therapy such as transplantation was not successful and faced several ethical issues and technical problems. Rather, manipulating endogenous NSC can be a promising alternative ways. However, factors causing neurodegeneration also affect the endogenous NSCs, and thereby induce PCD of the endogenous NSCs and limit their regenerative capacity. Therefore, it is imperative to understand how NSCs respond to cellular stress and PCD of NSCs is regulated.

Programed cell death (PCD) are classified into three major types: apoptosis, autophagic cell death (ACD), and necrosis (Clarke, 1990). Although the most studied mode of PCD is apoptosis, recently there has been increasing interest in ACD as an alternative, non-apoptotic mode of PCD. ‘Autophagy’ means ‘self-eating’ in Greek. Double-membrane autophagosomes encircle damaged organelles or proteins for degradation. After autophagosomes turn to autolysosome through fusion with lysosome, lysosomal enzymes hydrolyze constituents for self-digestion (Shintani & Klionsky, 2004) (Figure 1). Proper level of autophagy recycles amino acid or other cellular components and provide them to the cells. Thereby, autophagy has protective effects on the cells. Accordingly, autophagy deficit results in various kinds of human diseases, such as cancers and neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Ghavami et al., 2014). However, not only decreased autophagy level is harmful, also excessive autophagy can be toxic to the cells

leading to ACD (Shintani & Klionsky, 2004).

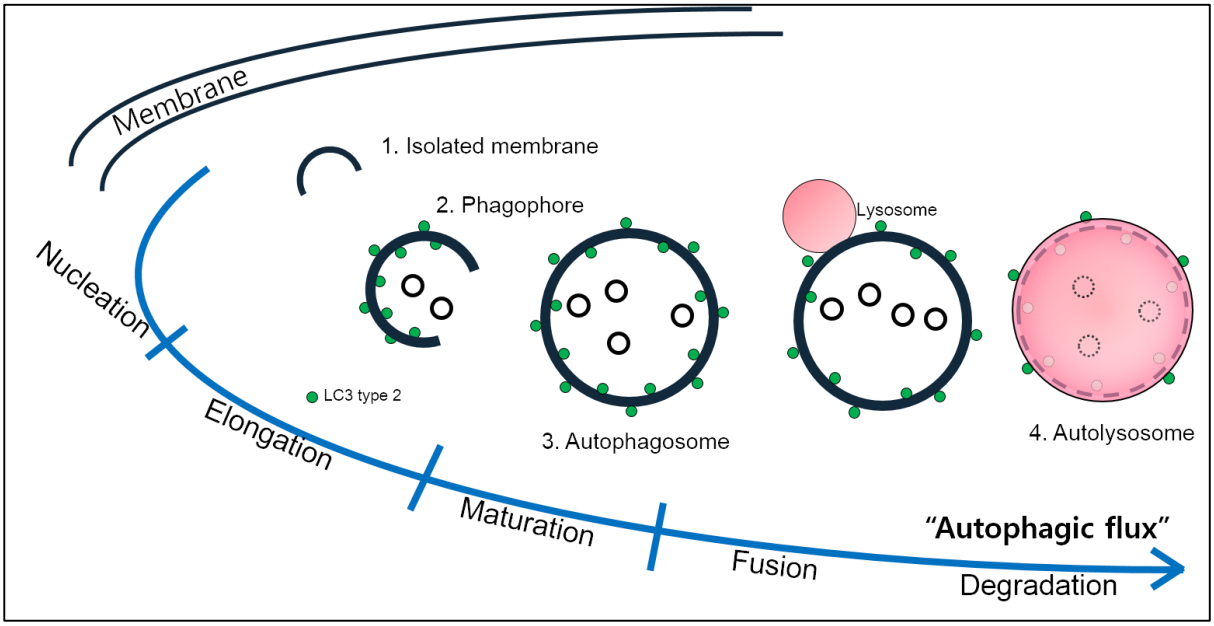

Figure 1. The process of autophagy.

Autophagy begins with the phagophore formation by sequestration (nucleation), which expands to a double-membrane autophagosome (elongation). Autophagosome surrounds damaged organelles or proteins. LC3 type 1 conjugated with phosphatidylethanolamine and converted to LC3 type 2 which is attached to the autophagosome membrane. The completed autophagosome fuses with a lysosome which provides acid hydrolases (fusion). Eventually, autolysosome degrade the cargo (degradation).

AMPK is a serine/threonine kinase mediating cellular metabolic control (D Grahame Hardie, Carling, & Carlson, 1998). It is an evolutionary conserved molecule and a key regulator for energy sensor in whole body. Also, in the brain, AMPK controls energy homeostasis and survival (Poels, Spasić, Callaerts, & Norga, 2009; Spasić, Callaerts, & Norga, 2009). AMPK regulates glucose transport GLUT3 in neurons (Amato & Man, 2011). Therefore, research efforts to understand the pathogenic mechanisms of metabolic disorders such as obesity and diabetes have been focused on AMPK. AMPK has been known to be activated by AMP/ATP ration increase and various stimuli.

When AMP/ATP level is low, AMPK is activated by allosteric mechanism (Figure 2). Upon AMPK activation, cellular metabolism is altered, and gene expression profile is changed to stimulate catabolism, inhibit anabolic processes and restore ATP (Ronnett, Ramamurthy, Kleman, Landree, & Aja, 2009). Additionally, AMPK is stimulated by drugs, hormones and stressors even though a change AMP/ATP ratio is not involved. AMPK senses both physiological and pathophysiological cellular stress stimuli (D. G. Hardie, 2007; Kahn,

Alquier, Carling, & Hardie, 2005). AMPK has several pharmacological activators such as 5-aminoimidazole-4-carboxamide riboside (AICAR), metformin, A769662. AICAR already proved to improve insulin sensitivity in aniamal model with insulin resistance (Buhl et al., 2002; Iglesias et al., 2002) . Metformin is already used anti-diabetic drug and has been known that it activates AMPK via LKB1 in liver. Another activator, A769662 is relatively recent developed activator. It is small molecule activator and confirmed to effect on ob/ob mice.

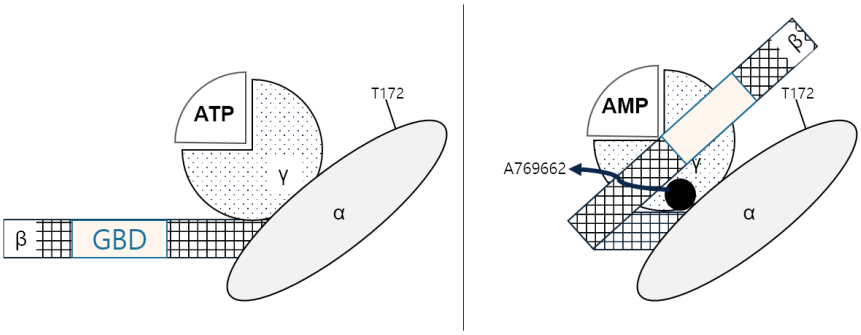

Figure 2. Model for AMPK activation.

Activation of AMPK occurs with conformational change. When AMP:ATP ratio is low AMPK T172 site is phosphorylated. If A769662 (AMPK activator) is treated, not only it induced conformational change interacting with β subunit of AMPK, it also block de-phosphorylation of T172.

As a controller of cellular metabolism, AMPK is related to mitochondrial dysfunction (D. Hardie, 2008), circadian rhythm control of cell polarity, migration and cytoskeletal dynamics (Mihaylova & Shaw, 2011). Also, in the brain, AMPK has already been reported as an important factor of energy homeostasis and cell survival in neuron (Spasić et al., 2009).

Given the role of AMPK as a cellular metabolism and stress sensor, AMPK is closely related with autophagy. Firstly, in the yeast, ortholog of AMPK (SNF1) was analyzed as a positive regulator of autophagy (Wang, Wilson, Fujino, & Roach, 2001). By addressing long-lived protein degradation in HT-29 human colon cancer and HeLa cells, the essential role of AMPK for the regulation of autophagic proteolysis in mammalian cells was confirmed (Meley et al., 2006). In addition to the role of energy sensor of AMPK, recent studies suggested another role of AMPK as an autophagy inducer by inactivating mammalian target of rapamycin (mTOR) (Klionsky & Emr, 2000) and also by directly phosphorylating Unc051 like autophagy activating kinase 1 (ULK1) (Kim, Kundu, Viollet, & Guan, 2011). Autophagy induced by activated AMPK can be triggered by starvation

through liver kinase B1 (LKB1), by cytosolic Ca^{2+}concentration increases with calcium/calmodulin-dependent protein kinase kinase 2, beta (CaMKKb) or by some cytokines with TGF-beta activated kinase 1 (TAK1) (Poels et al., 2009).

In the previous study (Yu et al., 2008), it has been shown that (HCN cells undergo ACD following insulin withdrawal. Of note, apoptosis hall markers such as caspase-3 and PARP-1 cleavage were absent in insulin-deprived HCN cells. On the other hand, when autophagy regulator gene, Atg7 gene was silenced, cell death rate decreased and autophagy marker LC3 type 2 also reduced. Since the cell death of insulin deprived HCN cell is well proportional to autophagy without apoptosis, and suppressed autophagy regulator gene prevented autophagic cell death, 'HCN cell death following insulin withdrawal' meet the standard to be considered as a genuine model of ACD in mammalian system.

Taken together, we propose that AMPK can acts as an ACD regulator in HCN cells. These study will focus on AMPK's role in autophagy and whether AMPK can regulate ACD in insulin deprived HCN cells. This study will contribute to the understanding of autophagy mechanism especially in the NSCs. Furthermore, this study will contribute to understanding autophagy mechanisms and it will support to get knowledge of the treatment of neurodegenerative diseases.

2. Materials and method

2.1 Cell culture

HCN cells were isolated following the method reported by Gage and his colleagues (Palmer, Takahashi, & Gage, 1997) and cultured as follows. Cells were plated on poly-L-ornithine (Sigma, P3055) and laminin (Corning, 354232) coated multi-well plates or tissue culture dishes. HCN cells were cultured in serum-free medium with Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with N2 components. N2 components contains apotransferrin (Prospec, pro-325), putrescine (Sigma, p5780), progesterone (Sigma, P130), and sodium selenite (Sigma, S5261). Cells were plated at a density of 1×10^5 cells /cm^2 and incubated at 37℃ in 5% CO^2. Basic FGF (Peprotech, 100-18B) was added daily. Medium was complemented with sodium bicarbonate (Sigma, S5761) 1.27 g/L and adjusted to pH 7.2. Insulin was added to control medium to separate with insulin–free medium.

2.2 Antibodies and chemicals

Antibodies directed against p-AMPK (#2535, 1:1000), t-AMPK (#2532, 1:1000), and cleaved caspase-3 (#9664, 1:1000) were obtained from Cell Signaling Technology. Antibodies directed against LC3 (NB100-2220, 1:1000) was purchased from Novus, p62 (P0067, 1:1000) were obtained from Sigma. Other antibodie, Actin-HRP was purchased from Santa Cruz.

Stock solutions of Z-VAD.fmk (BD Pharmingen, 550377), A769662 (Tocris, 3336), and staurosporine (Cell Signaling Technology, S5921) were prepared in DMSO as appropriate concentrations.

2.3 Cell death assay

HCN cells were seeded in 96-well plate with 1.0×10^5 cells per cm^2. Cells were stained with propidium iodide (PI) (Invitrogen, H1399) and Hoechst 33342 (Invitrogen, P3566) (1% volume of media in the well, final 1/1000 dilution). PI and Hoechst solutions were diluted with phosphate-buffered saline (PBS). After 20 minutes at 37℃ incubating in the dark, blue and red signal positive cells were counted under fluorescence microscope (Carl Zeiss,

Axiovert 40 CFL). The percentage of cell death was calculated as follows:

Cell death (%) = [PI (red) positive cell number/total cell number (blue)] × 100

2.4 GFP-LC3 puncta assay

The GFP-LC3-transfected HCN cells were plated on glass coverslips in 24-well plates at a cell density of 2.0×10^5 cells/mL. The HCN cells were fixed in 4% paraformaldehyde (PFA) solution for 10 min at room temperature. After removal of PFA and rinsing in PBS twice, the cells were mounted on slides with Mount solution (Dako, S3023). The counting of GFP-LC3-positive cells was carried out under a microscope (Carl Zeiss, LSM700).

2.5 Western blot

HCN cells were harvested. The lysate was lysed with lysis buffer (250 mM sucrose, 50 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, and 1 mM phenyl-methysulfonyl fluoride in 20 mM Tris-HCl, pH7.2) containing 1× protease cocktail inhibitors (Thermo Scientific, 78429), and 1×phosphatase cocktail inhibitors (Thermo Scientific, 78427) for 30 min in ice. Following centrifugation (13200 rpm, 20 min), protein concentration was determined with BCA kit (Pierce). Proper amount of protein samples (5 μg-10 μg) were loaded to SDS-polyacrylamide gel and separated. Electrotransfer was performed with a Trans-Blot SD Semi-Dry Electorphoretic Transfer Cell (Bio-rad) to polyvinylidene fluoride membrane (Millipore, IPVH00010). Transferred membrane was blocked with 5% blocking buffer which is diluted in 1×Tris-buffered saline with 0.1% of Tween 20 with non-fat dry milk in room temperature for 1 hr. Then, the membrane was incubated with primary antibody with overnight in 4℃. Horseradish peroxidase-conjugated secondary antibodies were incubated for 1 hr in room temperature.

2.6 Transfection

HCN cells were transfected with GFP-LC3, AMPK-wild type (AMPK-WT), AMPK-constitutive active form (AMPK-CA) vector using Lipofectamine 2000 (Invitrogen, 11668) according to the manufacturer's instructions. Transfection was performed in culture medium without insulin and penicillin/streptomycine. After 6 hr,

transfection media was replaced with culture medium.

2.7 Sh-RNA Lentiviral system

Lentivirus vector pLKO – sh-RNA system, pLKO.1 Sh-Atg7 vector were purchased from Sigma. Packaging vector psPAX2 (plasmid 12260), lentivirus vector pLKO.1 scramble shRNA (plasmid 1864), and enveloper vector pMD2.G (plasmid 12259) were used to produce sh-atg7 lentivirus and obtained from addgene. Lentiviruses were produced following the manufacturer's instruction. Lentiviruses were concentrated using ultracentrifuge (Beckman Coulter, Optima XPN-100) 25000 rpm for 5hr. Virus pellet was resuspend with culture medium. After virus infection 24hr, replaced virus-free media and incubated for 24hr. Infected cells were plated newly and performed selection using puromycin.

2.8 Statistical analysis

All data values were presented as mean ±standard deviation (SD), and obtained by averaging the data from at least 3 independent experiments. Statistical significance was determined by the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using GraphPad Prism (GraphPad Software).

3. Results

3.1 AMPK activator, A769662 increased cell death following insulin withdrawal.

Since AMPK is a well-known molecule to sense cell energy changes and control cellular metabolism, we checked activation of AMPK in HCN cells upon insulin deprivation. Then, we could confirm p-AMPK (T172) is gradually increased induction of LC3 type 2 conversion which is a marker of autophagosome (Figure 3A). Through the time course data, we assumed that AMPK could be an autophagy initiator and the key regulator of autophagy of HCN cell following insulin withdrawal. To confirm AMPK activation effects on autophagy, we used A769962 which is a small molecule activator of AMPK through allosteric activation and inhibition of AMPK de-phosphorylation. When we treated A769662 50uM, cell death rate was elevated dramatically compared to the cell death rate of only insulin withdrawal condition (Figure 3B). Whether A769662 is working well was confirmed by western blotting that p-ACC which is a representative downstream molecule of AMPK was increased with AMPK activator (Figure 3C). The autophagosomal lipidated microtubule-associated protein 1 light chain 3 (LC3) which are turned over from LC3-I is often used to monitor autophagy (Kabeya et al., 2000). And especially, visualized LC3 puncta usually used to show autophagy flux. Using GFP-LC3 vector expression, we tested LC3 punta with A769662 treated HCN cell. We detected LC3 puncta was increased in the HCN cells treated A769662 by confocal image (Figure 3D). Altogether, we suggested AMPK activator induced autophagy and cell death.

3.2 Ectopic expression of AMPK constructs modulated cell death.

To investigate whether AMPK functions to induce the autophagic cell death, we overexpressed AMPK Wild Type (WT) and constitutive active form (CA) in insulin withdrawal condition (Figure 4A). As a result of the over-expression of AMPK constructs, we detected cell death was increased with WT and more extent with CA (Figure 4B). We observed P-ACC was increased compared to EV and further more increased at CA overexpression (Figure 4D). Based on downstream molecules increased by transfection and GFP mouse antibody results (Figure 4C), we could confirm ectopic AMPK over-expression was successful (Figure 4D). These suggested over-expressed AMPK constructs effected on HCN cell's cell death to elevate following insulin withdrawal.

3.3 Silencing of AMPK reduced ACD in insulin-deprived HCN cells.

To investigate whether silence of AMPK also effects on autophagic cell death, we used CRISPR/cas-9 system. CRISPR/cas9 system need two components a guide RNA (gRNA) and a non-specific CRISPR-associated endonuclease (Cas9). The gRNA acts for binding to Cas-9 using short synthetic RNA which has a "scaffold" sequence and "spacer" or "targeting" sequence of gRNA defines the genomic target to be modified (Figure 5A). Using this system, we could regulated the genomic target of Cas9 by easily changing the targeted sequence displayed in the gRNA. In this study, we knock-out AMPK α2 by CRISPR/cas9 system. In the cell death assay, AMPK a2 knock-out prevented cell death in HCN cell following insulin withdrawal (Figure 5C). Also, we could confirmed LC3 type 2 of AMPK α2 knock-out cell was reduced compared to the scrambled insulin deprived cell (Figure 5D). Based on this, we propose that AMPK α2 could be an important factor for autophagic cell death in HCN cells following insulin deprivation.

3.4 Cell death mode induced by genetic and pharmacological activation of AMPK was ACD, not apoptosis.

All previous data showed AMPK induced cell death and autophagic flux. To clarify all AMPK-induced cell death in HCN cell is autophagic cell death, we checked apoptosis marker. We used Z-VAD, staurosporine (STS) and checked Caspase-3 activation to confirm. We tested whether cell death induced by A769662 show apoptosis features using Z-VAD.fmk which is a pan caspase inhibitor blocking caspase proteases. STS is commonly used as an apoptosis inducer. In accordance to cell death induced by STS successfully decreased by Z-VAD, AMPK activator-induced cell death did not effected by Z-VAD in HCN cells following insulin withdrawal (Fig.6A). Also, A769662 induced cell death increased LC3 type 2 without activation of caspase-3 (Figure 6B). These results indicated that A769662 induced non apoptotic cell death in HCN cell upon insulin withdrawal.

We also analyzed cell death rate in overexpression of AMPK WT and AMPK CA form with Z-VAD. Both cell death induced by WT and CA did not changed despite of Z-VAD treatment (Figure 6C). Furthermore, both overexpressed AMPK WT and CA did not activate caspase-3. Not only caspase-3 activation, LC3 type 2 level increased in both A769662 treated and AMPK WT and CA over-expressed cell death (Figure 6D). Taken together, the results suggested that AMPK induced non-apoptotic, autophagic cell death.

3.5 Suppression of autophagy related gene, Atg7 prevented ACD.

To verify the autophagic nature of the AMPK-induced cell death, we used ATG7 shRNA expressing lentivirus and knocked down the Atg7. Although scrambled cell increased LC3-II levels and cell death, Atg7-knocked down cell decreased both LC3-II and cell death rate (Figure 5A, B). Also, no changes observed in LC3-II levels even with A769662 treatment. Therefore, we suggested that AMPK-induced cell death is atg7-dependent. In other words, AMPK-related cell death is autophagic cell death.

4. Discussion

Stem cell therapy has been much interested as a solution for many diseases. Due to stem cell seemed realizable concept to substitute aged or hurt organ and cells, not only scientists, also the public eagle to stem cell therapy would be successful. However, stem cell transplantation was not as good as expected from ethical and technical issue. For the next solution of those diseases, endogenous stem cell regulation has been discussed. However, the knowledge of exact mechanism of stem cell survival and death is lack. Although a few studies processed to comprehend of stem cell death so far, if enough studies conducted, further steps for the stem cell control and utilization will be satisfactory.

Adult HCN cells has a multipotent ability to differentiate to neuron, oligodendrocyte, and astrocyte during whole life. Those consist of brain and acts important role for neurogenesis in the adult hippocampus. Also, many reports suggested that new neurons developed in adult period related with memory. Moreover, its dysfunction can be associated with neuro-degenerative diseases. Therefore, the knowledge of adult HCN cell death mechanisms expected to be useful for neurodegenerative disease therapy and it may be applied to developing drugs.

Because HCN cell has intact ability to dead apoptosis pathway and showed non-apoptotic, autophagic cell death features specifically in insulin deprivation condition, we thought about stimuli, especially ‘insulin’. Due to insulin depletion induced metabolic stress, we hypothesized insulin-depletion induced stress influenced AMPK which is a well-known intracellular change sensor.

AMPK is a well-studied molecule to monitor intracellular changes in early point. Therefore, it already well adjusted to develop treatment for metabolic disease such as diabetes. From the understanding of AMPK in metabolic diseases, many researches extends interest to the neuron and brain. Also, from canonical AMPK activator of AMP/ATP ratio and Ca^{2+} and etc, various stimuli got interests for AMPK activator such as drugs, hormones and stressors even though it does not change AMP/ATP ratio. It means AMPK senses both physiological and pathophysiological stimuli (D. G. Hardie, 2007; Kahn et al., 2005).

Not only as a sensor of changes of intracellular environment, also AMPK has been studied the relationship with autophagy. AMPK has a critical role in autophagy induction through inactivation of the mTOR

(Klionsky & Emr, 2000). Links between Cellular metabolic status and autophagy also reported and AMPK was pointed as an key factor of it.(He & Klionsky, 2009).

Then, we focused on AMPK and hypothesized that AMPK can be a regulator of autophagic cell death of HCN cells following insulin withdrawal. We manipulated AMPK levels using pharmacological, ectopic AMPK over-expression and silenced AMPK a with CRISPR/cas9 system. As results, elevated AMPK induced cell death and increased LC3 type without apoptosis marker but, silenced AMPK showed opposite results also without capase-3 activation. All the results concluded that AMPK induced autophagic cell death in HCN cells following insulin withdrawal. Furthermore, we genetically suppressed Atg-7 gene and no changes occurred with even AMPK activation. These results showed AMPK can be a regulator of autophagic cell death of HCN cells following insulin withdrawal.

Still, we do not study which molecules effected by AMPK and how it is activated. Among upstream of AMPK to activate autophagy molecules such as LKB1, CaMKK beta and TAK1, which molecules is related is unveiled so far. LKB1 is the essential AMPK-activating kinase which is activated when the AMP:ATP ratio increased (Woods et al., 2003). Other kinase, CaMKKb is prevailing expressed in neural tissue and linked with AMPK phosphorylation and activation (Carling, Sanders, & Woods, 2008; Fogarty et al., 2010). Last kinase, TAK1 was also confirmed as an important AMPK kinase in TRAIL-induced autophagy (Herrero-Martín et al., 2009; Xie et al., 2006). All three upstream molecules has efficient possibilities to be an effector of AMPK in HCN cells following insulin withdrawal. If specific or several upstream molecules related is proved, those knowledge will contribute to understand not only what is upstream protein, but also what component is involved to the AMPK activation among AMP:ATP ratio, Ca^{2+} level, hormones or others. Also, we speculate many molecules involved with autophagy can be candidates of AMPK downstream such as p62. Further study about upstream/downstream of AMPK is in need. Based on our research, we expect to understand mechanisms of autophagy and it attributes to develop the cure of neurodegenerative diseases.

5. Figure legends

Figure 1. The process of autophagy.

Autophagy begins with the phagophore formation by sequestration (nucleation), which expands to a double-membrane autophagosome (elongation). Autophagosome surrounds damaged organelles or proteins. LC3 type 1 conjugated with phosphatidylethanolamine and converted to LC3 type 2 which is attached to the autophagosome membrane. The completed autophagosome fuses with a lysosome which provides acid hydrolases (fusion). Eventually, autolysosome degrade the cargo (degradation).

Figure 2. Model for AMPK activation.

Activation of AMPK occurs with conformational change. When AMP:ATP ratio is low AMPK T172 site is phosphorylated. If A769662 (AMPK activator) is treated, not only it induced conformational change interacting with β subunit of AMPK, it also block de-phosphorylation of T172.

Figure 3. Increase of cell death with A769662 following insulin withdrawal

A) Time course of p-AMPK following insulin withdrawal. HCN cells were cultured with insulin (I (+)) or without it (I (+)) and incubated for the indicated times.

B) Cell death was measured by the PI staining.

C) Effects of A769662 (50 uM, 8 hr) treatment to HCN cells.

D) Increase of autophagic flux was visualized with I (+), I (-) and A769662 treated I (-).

Figure 4. Ectopic expression of AMPK constructs effected on cell death.

A) An experimental scheme for the over-expression of AMPK constructs.

B) The AMPK WT and CA forms elevated cell death with more effective increases observed for the CA

form in insulin-withdrawal HCN cells. EV, empty vector. WT, wild type vector. CA, constitutive active form.

C) The over-expression of ectopic AMPK forms was verified by western blotting analyses with GFP-mouse antibody

D) The over-expression of ectopic AMPK forms resulted P-ACC, AMPK downstream molecule increase. P-ACC, phosphorylated ACC. T-ACC, total ACC.

Figure 5. Silencing of AMPK decreased ACD in HCN cells following insulin withdrawal.

A) Cas9 system scheme

B) Target sequence we used for AMPK α2 knockdown using CRISPR/cas-9 system.

C and D) The Sg-AMPK α2 HCN cells markedly reduced ACD and autophagy (D) in insulin-deprived HCN cells. Sg- AMPK α2 was transfected HCN cells with Cas-9 and sgRNAs.

Figure 6. Cell death mode induced by genetic and pharmacological activation of AMPK was ACD, not apoptosis.

A) CDA showed no change with Z-VAD, apoptosis inhibitor. As a positive control, STS cell death decreased upon Z-VAD. C-Cas3, cleaved caspase-3. STS, staurosporine.

B) AMPK activator, A769662 increased LC3 type 2 without activation of caspase-3. STS was treated for 6hr in I (+) as a positive control for caspase-3 activation.

C) Z-VAD treated did not present cell death rate changes with the AMPK over-expression.

D) The absence of activation of C-Cas3 with over-expression of AMPK.

Figure 7. Suppression of autophagy related gene, Atg7 prevented cell death in insulin-deprived HCN cells.

A) Knockdown of Atg7 decreased cell death greater with A769662. pLKO.1- Atg7 shRNA and control scramble shRNA lentivirus infected to HCN cells. Quantitative data are presented as the mean ± SD (n = 3). *$p < 0.05$.

B) Suppressed Atg7 resulted decreased LC3 type 2 increase intensity of A769662.

Figure 8. Schematic drawing of suggested autophagy regulation by AMPK in HCN cells.

When insulin is deprived, AMPK is phosphorylated through T172 site in HCN cells. Activated AMPK induced autophagic cell death.

6. Figures

Figure 1. The process of autophagy.

Figure 2. Model for AMPK activation.

Figure 3. Increase of cell death with A769662 following insulin withdrawal.

Figure 4. Ectopic expression of AMPK constructs effected on cell death.

Figure 5. Silencing of AMPK decreased ACD in HCN cells following insulin withdrawal.

Figure 6. Cell death mode induced by genetic and pharmacological activation of AMPK was ACD, not apoptosis.

Figure 7. Suppression of autophagy related gene, Atg7 prevented cell death in insulin-deprived HCN cells.

Figure 8. Schematic drawing of suggested autophagy regulation by AMPK in HCN cells.

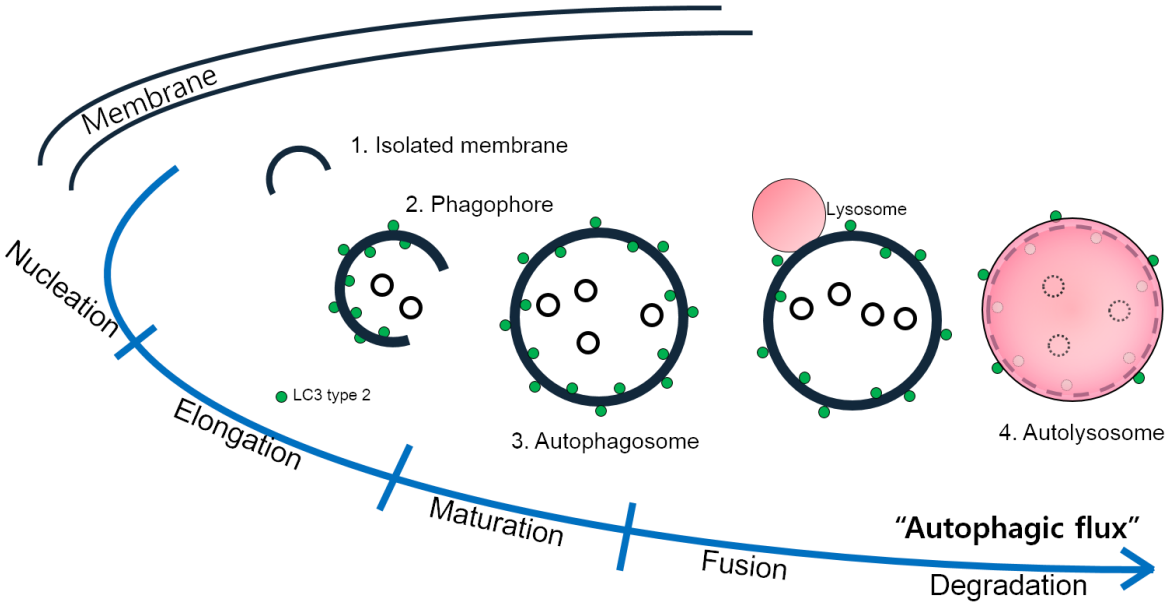

Figure 1. The process of autophagy.

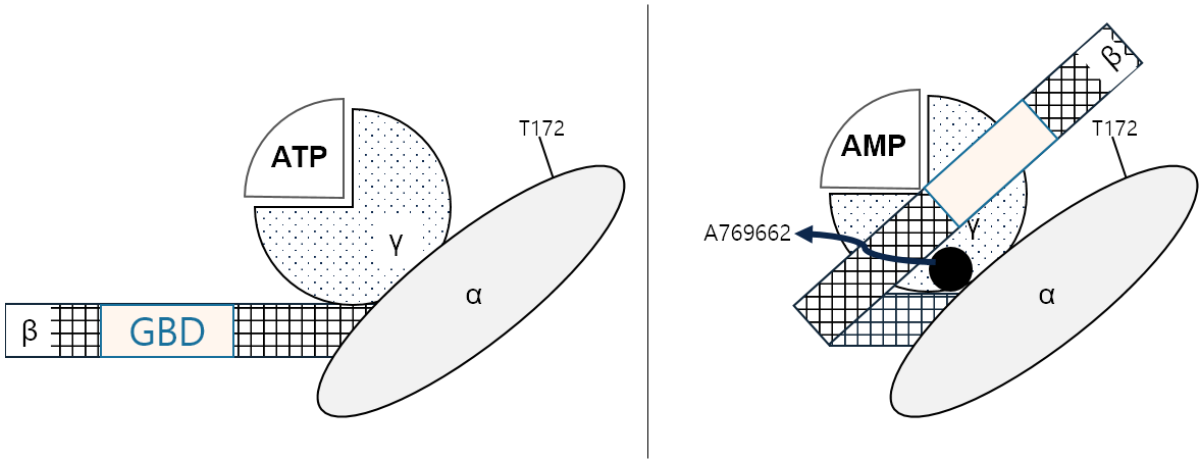

Figure 2. Model for AMPK activation

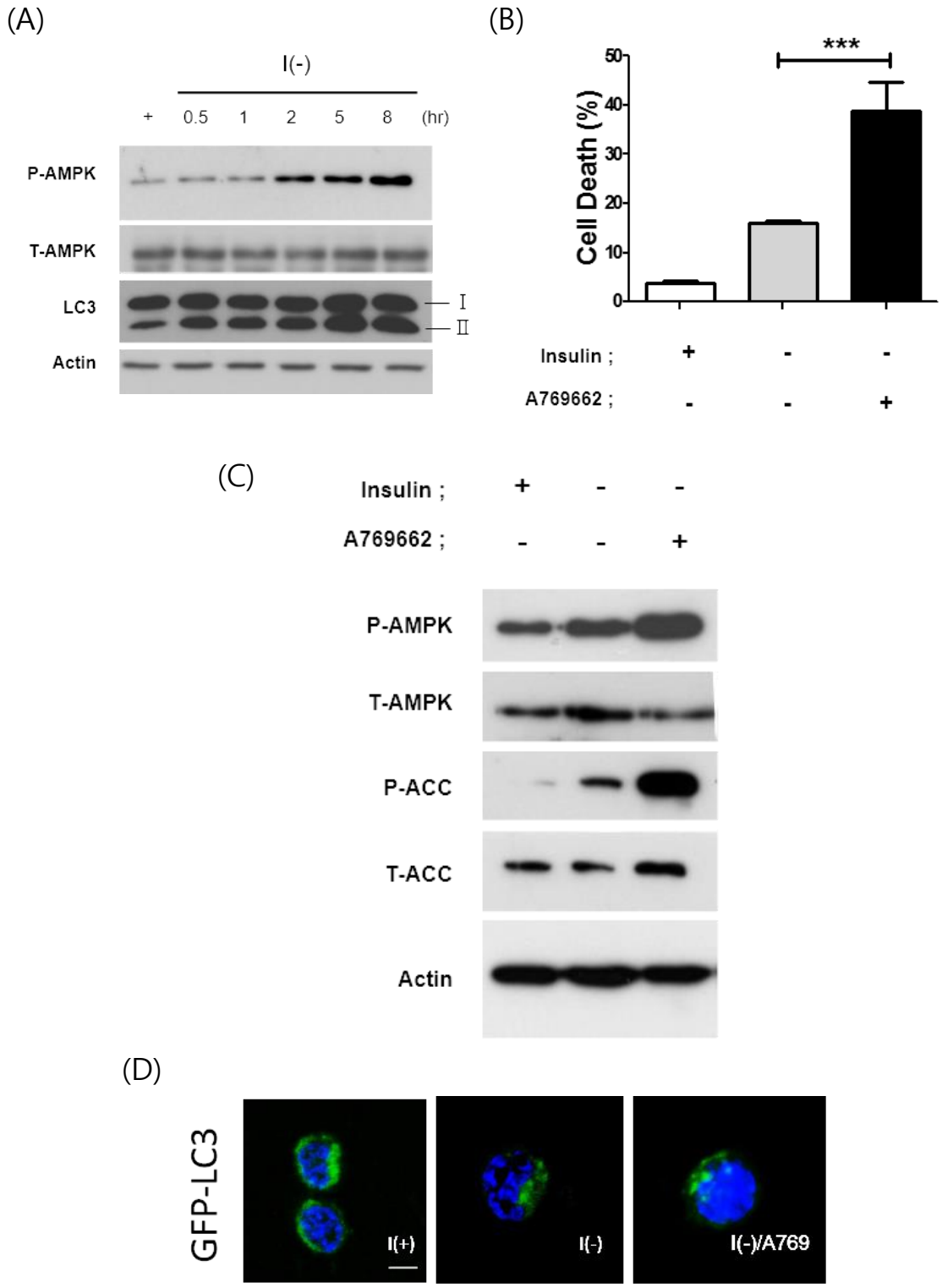

Figure 3. Increase of cell death with A769662 following insulin withdrawal.

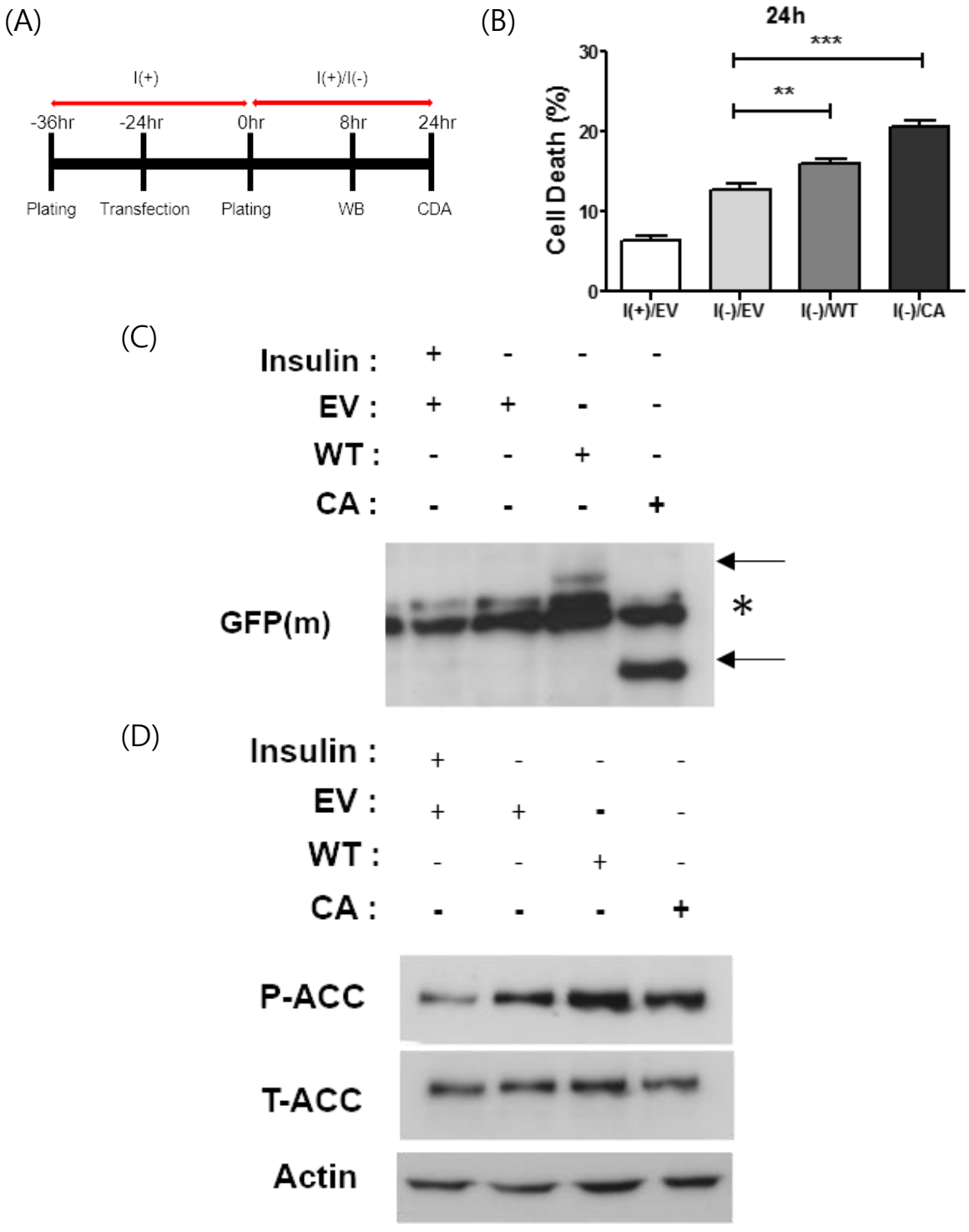

Figure 4. Ectopic expression of AMPK constructs effected on cell death.

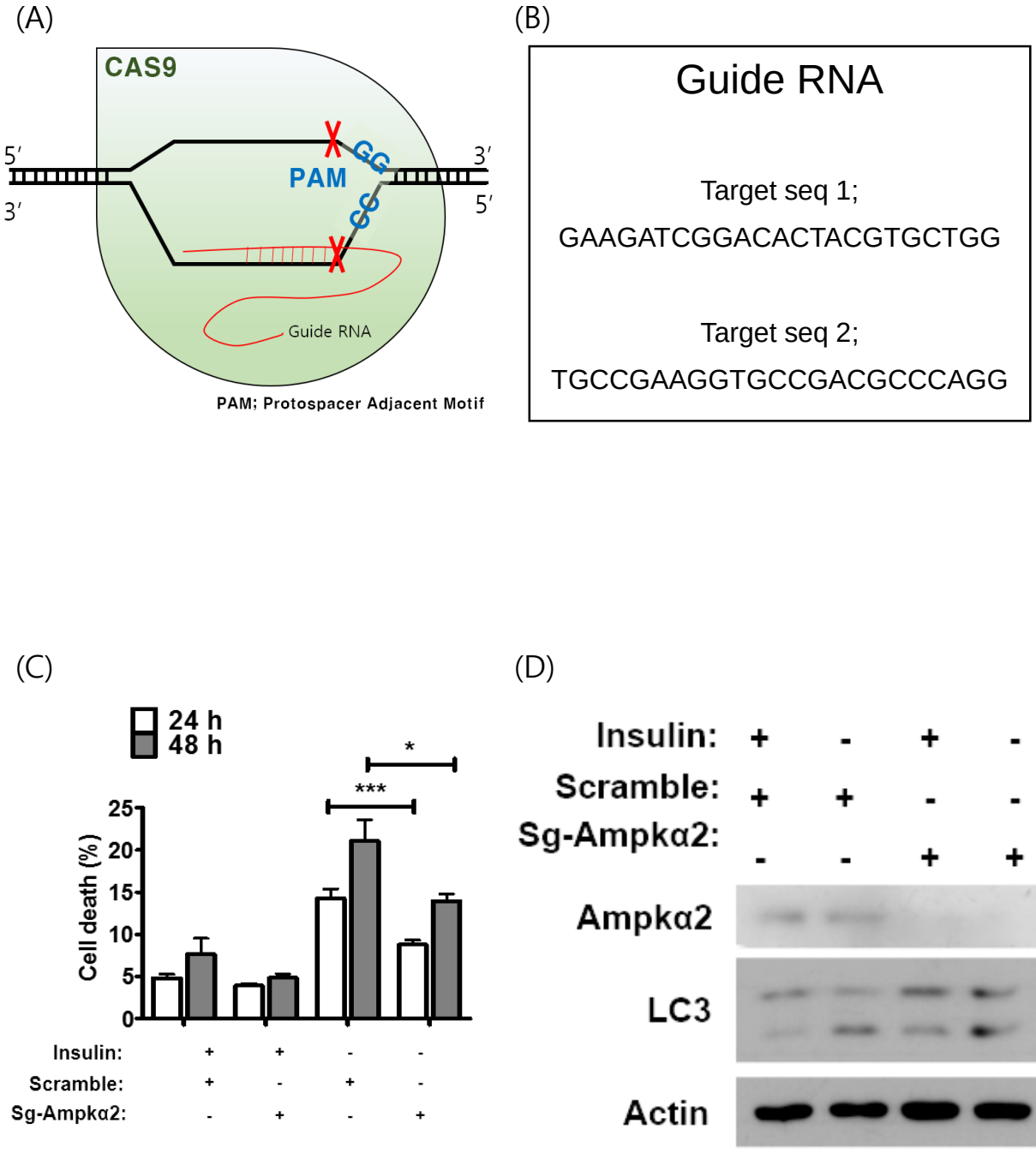

Figure 5. Silencing of AMPK decreased ACD in HCN cells following insulin withdrawal

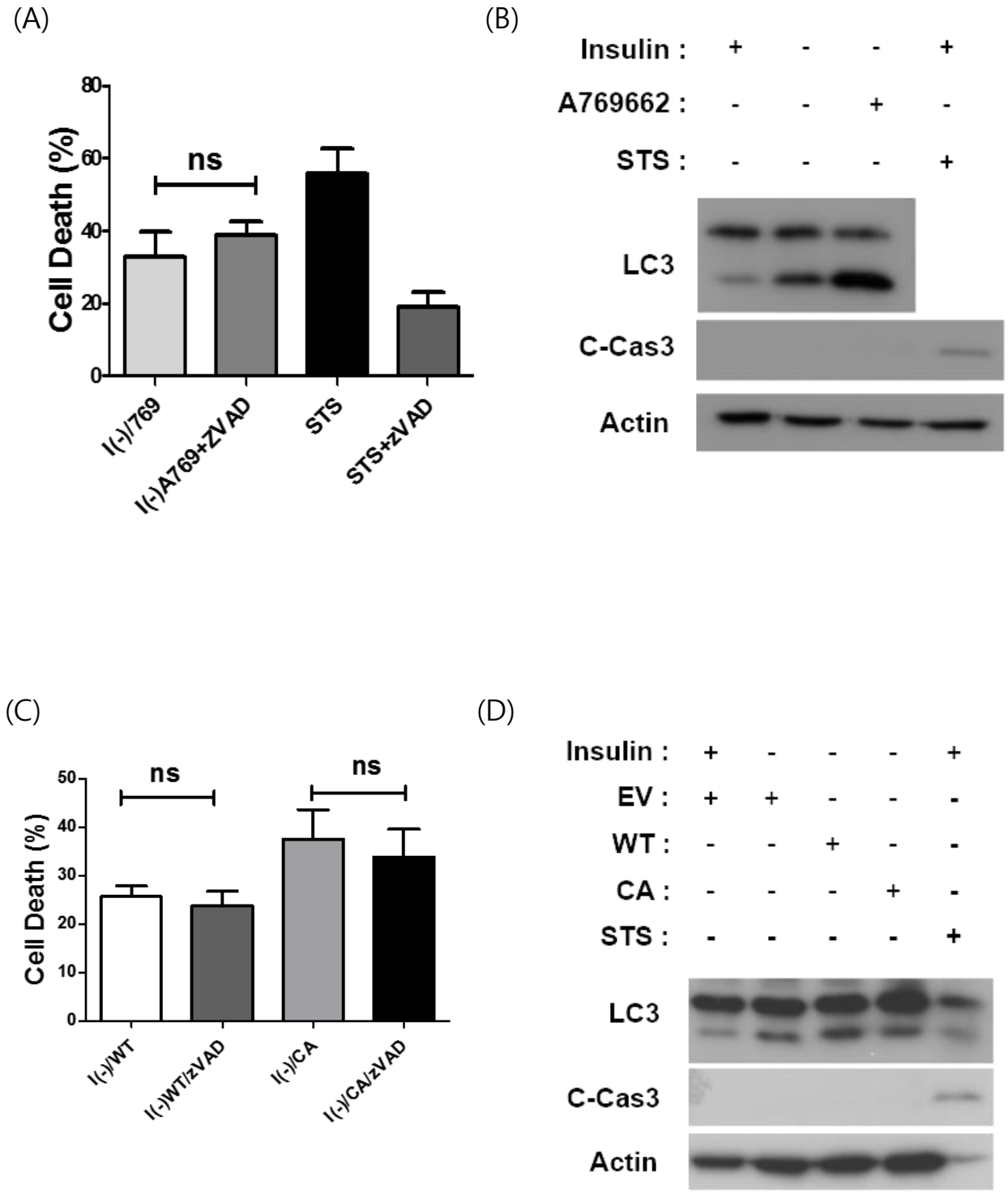

Figure 6. Cell death mode induced by genetic and pharmacological activation of AMPK was ACD, not apoptosis.

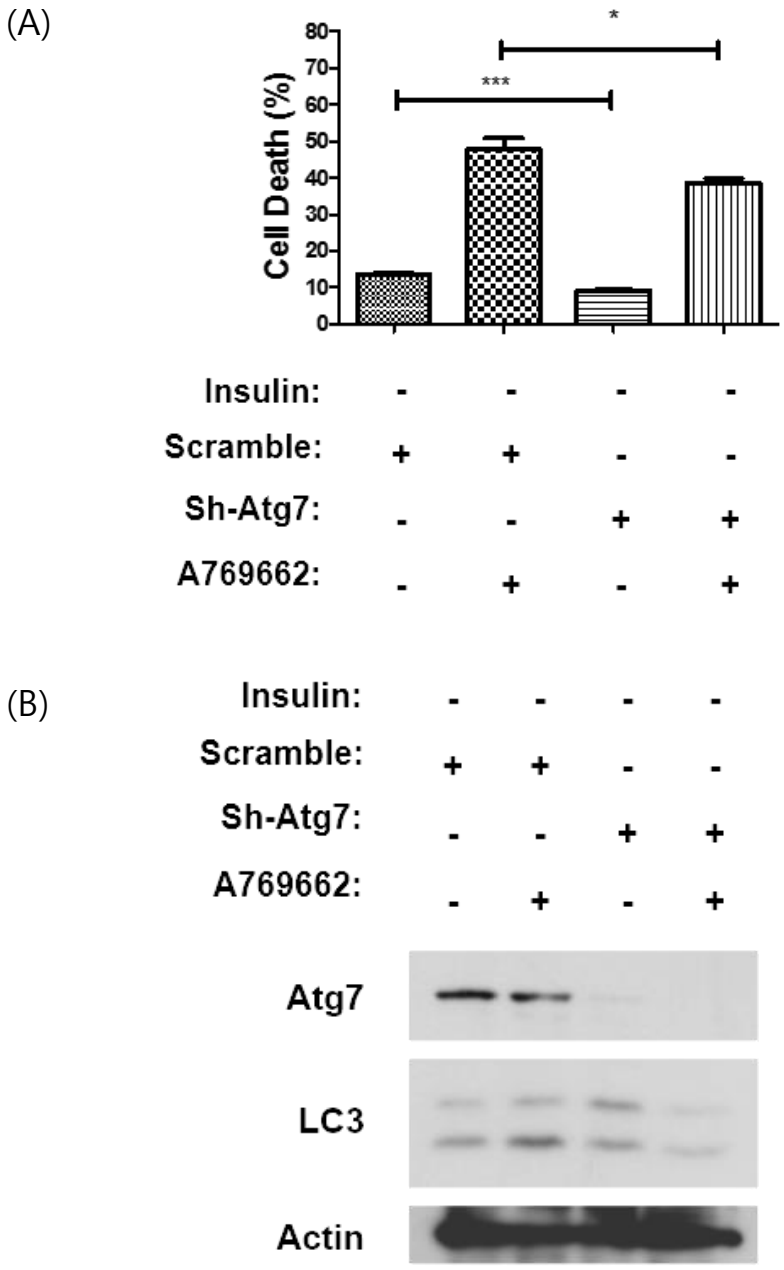

Figure 7. Suppression of autophagy related gene, Atg7 prevented cell death in insulin-deprived HCN cells.

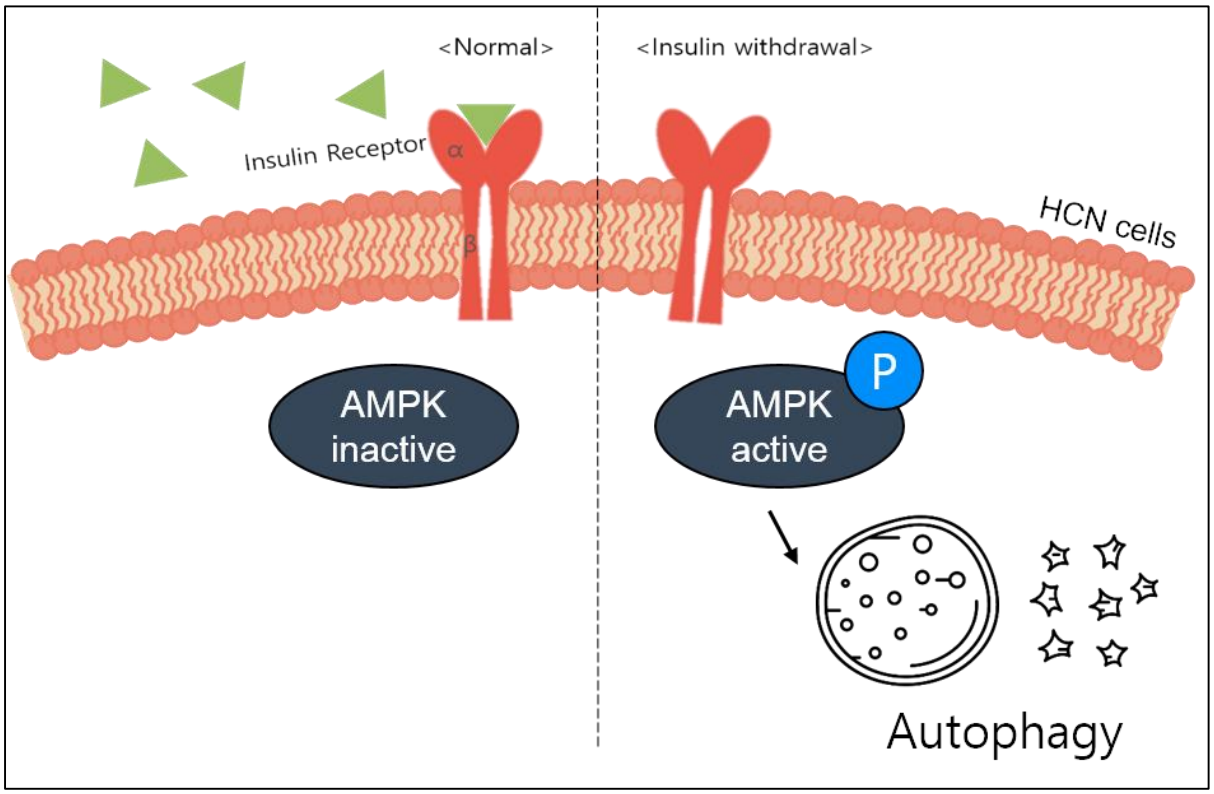

Figure 8. Schematic drawing of suggested autophagy regulation by AMPK in HCN cells.

Reference

Amato, S., & Man, H. Y. (2011). Bioenergy sensing in the brain: the role of AMP-activated protein kinase in neuronal metabolism, development and neurological diseases. *Cell Cycle, 10*(20), 3452-3460. doi:10.4161/cc.10.20.17953

Buhl, E. S., Jessen, N., Pold, R., Ledet, T., Flyvbjerg, A., Pedersen, S. B., . . . Lund, S. (2002). Long-term AICAR administration reduces metabolic disturbances and lowers blood pressure in rats displaying features of the insulin resistance syndrome. *Diabetes, 51*(7), 2199-2206.

Carling, D., Sanders, M., & Woods, A. (2008). The regulation of AMP-activated protein kinase by upstream kinases. *International journal of obesity, 32*, S55-S59.

Clarke, P. G. (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol (Berl), 181*(3), 195-213.

Fogarty, S., Hawley, S., Green, K., Saner, N., Mustard, K., & Hardie, D. (2010). Calmodulin-dependent protein kinase kinase-beta activates AMPK without forming a stable complex: synergistic effects of Ca2+ and AMP. *Biochem. J, 426*, 109-118.

Ghavami, S., Shojaei, S., Yeganeh, B., Ande, S. R., Jangamreddy, J. R., Mehrpour, M., . . . Łos, M. J. (2014). Autophagy and apoptosis dysfunction in neurodegenerative disorders. *Progress in Neurobiology, 112*(0), 24-49. doi:http://dx.doi.org/10.1016/j.pneurobio.2013.10.004

Hardie, D. (2008). AMPK: a key regulator of energy balance in the single cell and the whole organism. *International journal of obesity, 32*, S7-S12.

Hardie, D. G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nature Rev. Mol. Cell Biol., 8*, 774-785. Retrieved from http://dx.doi.org/10.1038/nrm2249

Hardie, D. G., Carling, D., & Carlson, M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annual review of biochemistry, 67*(1), 821-855.

He, C., & Klionsky, D. J. (2009). Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet, 43*, 67-93. doi:10.1146/annurev-genet-102808-114910

Herrero-Martín, G., Høyer-Hansen, M., García-García, C., Fumarola, C., Farkas, T., López-Rivas, A., & Jäättelä, M. (2009). *TAK1 activates AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells* (Vol. 28).

Iglesias, M. A., Ye, J.-M., Frangioudakis, G., Saha, A. K., Tomas, E., Ruderman, N. B., . . . Kraegen, E. W. (2002). AICAR administration causes an apparent enhancement of muscle and liver insulin action in insulin-resistant high-fat-fed rats. *Diabetes, 51*(10), 2886-2894.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., . . . Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO journal, 19*(21), 5720-5728.

Kahn, B. B., Alquier, T., Carling, D., & Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell metabolism, 1*(1), 15-25.

Kim, J., Kundu, M., Viollet, B., & Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol, 13*(2), 132-141. doi:10.1038/ncb2152

Klionsky, D. J., & Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science, 290*(5497), 1717-1721.

Meley, D., Bauvy, C., Houben-Weerts, J. H., Dubbelhuis, P. F., Helmond, M. T., Codogno, P., & Meijer, A. J. (2006). AMP-activated protein kinase and the regulation of autophagic proteolysis. *Journal of Biological Chemistry, 281*(46), 34870-34879.

Mihaylova, M. M., & Shaw, R. J. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nature cell biology, 13*(9), 1016-1023.

Palmer, T. D., Takahashi, J., & Gage, F. H. (1997). The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci, 8*(6), 389-404. doi:10.1006/mcne.1996.0595

Poels, J., Spasić, M. R., Callaerts, P., & Norga, K. K. (2009). Expanding roles for AMP-activated protein kinase in neuronal survival and autophagy. *Bioessays, 31*(9), 944-952.

Ronnett, G. V., Ramamurthy, S., Kleman, A. M., Landree, L. E., & Aja, S. (2009). AMPK in the brain: its roles in energy balance and neuroprotection. *Journal of neurochemistry, 109*(s1), 17-23.

Shintani, T., & Klionsky, D. J. (2004). Autophagy in health and disease: a double-edged sword. *Science, 306*(5698), 990-995. doi:10.1126/science.1099993

Spasić, M. R., Callaerts, P., & Norga, K. K. (2009). AMP-Activated Protein Kinase (AMPK) Molecular Crossroad for Metabolic Control and Survival of Neurons. *The Neuroscientist, 15*(4), 309-316. doi:10.1177/1073858408327805

Wang, Z., Wilson, W. A., Fujino, M. A., & Roach, P. J. (2001). Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase, and the cyclin-dependent kinase Pho85p. *Molecular and cellular biology, 21*(17), 5742-5752.

Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G., Neumann, D., . . . Carling, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Current biology, 13*(22), 2004-2008.

Xie, M., Zhang, D., Dyck, J. R., Li, Y., Zhang, H., Morishima, M., . . . Schneider, M. D. (2006). A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proc Natl Acad Sci U S A, 103*(46), 17378-17383. doi:10.1073/pnas.0604708103

Yu, S. W., Baek, S. H., Brennan, R. T., Bradley, C. J., Park, S. K., Lee, Y. S., . . . Kim, S. W. (2008). Autophagic death of adult hippocampal neural stem cells following insulin withdrawal. *Stem Cells, 26*(10), 2602-2610. doi:10.1634/stemcells.2008-0153

요 약 문

성체해마신경줄기세포의 자가포식현상에서 AMPK의 자가포식현상 조절자로의 역할

장수 100세 시대에 이르며 퇴행성 뇌질환 환자가 증가하고 있으며, 뚜렷한 치료방법이 없어서 증가하는 환자에 대한 해결방안이 제대로 마련되지 않는 것이 사회적 문제로 대두되고 있다. 대표적 퇴행성 뇌질환인 알쯔하이머 치매나 파킨슨병과 같은 질병들은 신경세포들이 죽어서 수가 절대적으로 줄어드는 증상을 보인다. 자가포식현상은 포유류의 몸 속에서 대사산물로 발생한 몸에 필요치 않는 물질들을 없애기 위한 방법으로 세포 내 자기항상성에 기여를 하는 아주 중요한 과정이다. 그러나 어떤 이유로 인해 체내 자가포식현상이 정상적으로 일어나지 않으면, 많은 질병 특히나 퇴행성 뇌질환을 야기한다는 연구들이 많이 진행되었다. 하지만 중요도에 비해 정확한 자가포식현상, 궁극적으로 세포사멸 기전에 대한 이해가 부족해 더 많은 연구가 이루어져야 한다고 보고 있다.

본 논문은 성체해마신경줄기세포의 세포사 기전 중에서도 세포 내 항상성을 유지하는 역할을 하는, 또한 비교적 이른 시간에 반응하는 AMPK라는 단백질의 자가포식현상에 미치는 영향에 대해 다루었다. 성체해마신경줄기세포에서 인슐린 결핍 시 자가포식현상만이 일어나므로, 성체해마신경줄기세포를 이용하였다. 이 세포에서 AMPK가 인슐린 결핍상황에서 일어나는 자가포식현상을 증가시키는 것을 확인하였다. 단순히 현상뿐 아니라 유전적인 방법과 약물처리 등 여러 방법을 이용하여, 증가된 자가포식현상이 AMPK에 의해 조절된 것임을 증명하였다. 더 나아가 이 연구는 충분하지 못한 자가포식현상으로 인해 일어나는 퇴행성 뇌질환 또는 불필요하게 과활성화 된 뇌세포에서의 자가포식현상을 조절하기 위해, AMPK 촉진제 또는 억제제를 사용한다면 치료제로도 충분히 사용할 수 있을 것이라는 가능성을 보여주고 있다.

핵심어: 성체해마신경줄기세포, 인슐린 결핍, 자가포식현상, AMPK

Acknowledgements

I would like to thank Professor Seong-Woon Yu for his expert advice and encouragement as well as Shinwon Ha for extraordinary support in this thesis process.

I would also like to thank my lab members for their assistance and encourage to approve my acknowledgment.