The Involvement of Reactive Oxygen Species in Autophagy Regulation by Amino Acids

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ABSTRACT

THE INVOLVEMENT OF REACTIVE OXYGEN SPECIES IN AUTOPHAGY REGULATION BY AMINO ACIDS

by

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Autophagy, an intracellular bulk degradation process induced by starvation, is regulated by nutrients and different conditions. Reactive oxygen species (ROS) critically modulates autophagy in response to various cellular stresses. In eukaryotic cell, starvation prompts induction of autophagy and during this condition, intracellular ROS level increases. Hence, ROS is considered as a good signaling molecule in autophagy. Amino acid is a known autophagy regulator. However, the significance of ROS production by amino acids to their effect on autophagy has yet to be explored. In the present work, we analyzed the effects of individual amino acids on the intracellular ROS level and autophagy in H4-II-E cells. ROS measurement was performed in Spectrofluorimeter using the fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFDA). The cytosolic LC3 ratio (LC3-IIs/LC3-I) was employed as a suitable quantitative index of autophagic flux. LC3 signal was detected by Western blotting and quantified by densitometric analysis. CAA mixture showed significant

suppression of autophagy. Furthermore, all the amino acid mixtures suppressed the ROS production significantly. Most individual RegAA showed suppressive effects on ROS production and autophagy. This scenario explains the ROS production suppression observed form RegAA mixture. Interestingly, arginine, a NonRegAA showed significant autophagy suppression but its ROS production was greatly stimulated. Inhibitor study using rapamycin and wortmannin showed starvation is partially Class III PI3K dependent. Furthermore, it also suggested that ROS production involvement in the autophagy regulation by CAA and Leu (a RegAA) may also be Class III PI3K dependent. This implies the participation of ROS as signaling molecule in the autophagy regulation by CAA and Leu. Finally, ROS production by arginine exhibited partial mTORC1 pathway dependence; however, its autophagy regulation may involve another pathway.

Dedicated to

my family

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Abbreviations

Akt	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
Arg	arginine
ATG (Atg)	autophagy-related genes
CAA	complete amino acid
DCFDA	2',7'-dichlorofluorescin diacetate
DCF	Dichlorofluorescin
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
EBSS	Earle's balance saline solution
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin-3-gallate
GABARAP	γ-aminobutyric acid receptor-associated protein
GATE-16	Golgi-associated ATPase enhancer 16 kDA
HMGB	high-mobility group box protein
Leu	Leucine
MAP-LC3	Microtubule-associated protein light chain 3
Met	Methionine
mTOR	Mammalian target of rapamycin
NAC	N-acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NonRegAA	Nonregulatory amino acid
PE	Phosphatidylethanolamine
PI3K	Phosphoinositide 3-kinase
PI3(P)	Phosphatidylinositol 3-phosphate
POX	Proline oxidase

PRAS40	Proline-rich Akt substrate of 40 kDa
PUFA	Polyunsaturated fatty acid
RegAA	Regulatory amino acid
RM	Rapamycin
ROS	Reactive oxygen species
TIGAR	TP53-induced glycolysis and apoptosis regulator
TSC2	Tuberous sclerosis 2
ULK	UNC 51-like kinase

Chapter 1

General Introduction

1.1 The autophagic pathway

Cell maintains internal metabolic equilibrium by balancing biosynthesis and turnover. In eukaryotic cell, lysosome is responsible for the degradation of excess organelles and macromolecules through its large range of resident digestive enzymes. During unfavorable conditions such as nutrient deprivation, autophagy mediates a highly regulated process via lysosomes. Autophagy is a process of self-degradation of cellular components in which double-membrane autophagosome sequesters organelles or portions of cytosol and fuse with lysosomes or vacuoles for breakdown by resident hydrolases (Klionsky *et al.*, 2009). The resulting macromolecules can be recycled back to the cytosol for reuse during starvation. In general, autophagy is divided into three main types: chaperone-mediated autophagy (CMA), microautopahgy and macroautophagy (Klionsky, 2005). In CMA, a chaperone protein binds to its cytosolic target substrate and then to a receptor on the lysosomal membrane where the unfolding of protein occurs. This unfolded cytosolic protein is subsequently translocated directly into the lysosome for its degradation (Massey et al., 2004). Microautophagy translocates cytoplasmic materials into the lysosome by direct intussusceptions of the lysosomal membrane and subsequent budding of vesicles into the lysosomal lumen (Mayer, 2004). In contrast, macroautophagy is the major inducible pathway for general turnover of cytoplasmic components. This process involved formation of double-membrane vesicles, called autophagosomes, around a portion of cytoplasm containing organelles, cytoplasmic proteins and other materials. These autophagosomes ultimately fuse with lysosomes, resulting in degradation of their contents (Rubinsztein, 2009).

1.2 Molecular aspects of autophagosome formation

The molecular mechanism underlying autophagy has been extensively explained on the past decade. A series of genetic screens in yeast *Saccharomyces cerevisiae* identified

autophagy-related genes (ATG) that actively participated in autophagy (Xie and Klionsky, 2007). About half of the genes have mammalian counterparts (**Table 1-1**) and most of the core processes are conserved in humans (Ohsumi, 2001; Huang and Klionsky, 2002). The core molecular machinery of autophagy includes induction of the isolation membrane, engulfment of cytoplasm, formation of the autophagic vesicles, and fusion with lysosome (**Fig 1-1**).

Table 1-1. Autophagy genes in mammals

Gene	Protein properties/function
Atg1, ULK1	A serine/threonine protein kinase; may be involved in regulation and vesicle formation
Atg3	Functions as an ubiquitin-conjugating-like enzyme that covalently attaches Atg/LC3 to phosphatidylethanolamine
Atg4	A cysteine protease that cleaves the C-terminus of Atg8/LC3 to expose a glycine residue for subsequent conjugation
Atg5	Covalently attached to ATg12 and binds Atg16 as part of a tetrameric complex of unknown function
Atg6, Beclin 1	A component of the Class III PI3K complex that is required for autophagy
Atg7	Homologue of the ubiquitin-activating enzyme; it activates both Atg8/LC3 and Atg12 before conjugation
Atg8, MAP1LC3	Has structural similarity to ubiquitin; it is conjugated to PE, and is part of the autophagosome
Atg9	A transmembrane protein that may be involved in delivering membrane to the forming autophagosome
Atg10	Functions as an ubiquitin-conjugating-like enzyme that covalently attaches Atg12 to Atg5
Atg12	Has some structural similarity to ubiquitin; conjugated to an internal lysine of Atg5 through its C-terminal glycine
Atg16	Binds Atg5 and homo-oligomerizes to form a tetrameric complex

These autophagy genes have product confirmed to play a role in autophagy in higher eukaryotes (Rubinsztein et al., 2007).

1.2.1 Induction

At normal condition, basal-level autophagy is very low, hence inducing autophagy is very important in order for organisms to survive against stress and other cellular cues. One of

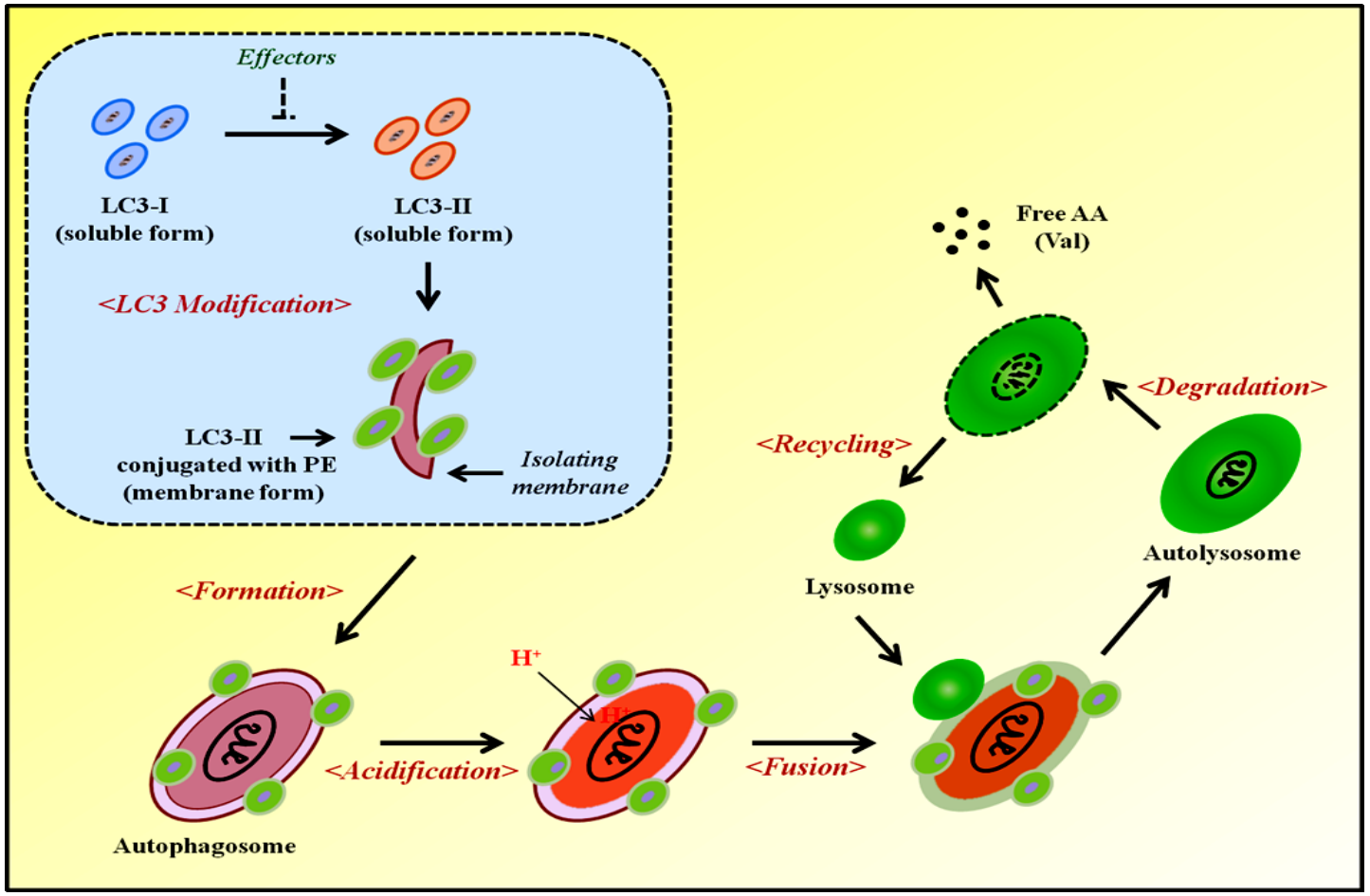

Fig. 1-1. The autophagy pathway. Autophagy involves the sequestration of cytoplasmic materials by an isolation membrane to form a double-membrane vacuole, the autophagosome. The autophagosome undergoes fusion with lysosome, to form lysosome, in which the sequestered material is degraded. See text for details.

the important ATG complex involved in autophagy induction is the Atg 1 complex. In yeast, this complex is composed of Atg1, Atg13 and the Atg17- Atg31- Atg9 subcomplex. On the other hand, the mammalian Atg 1 complex, also known as ULK complex, is composed of the mammalian Atg1 homolog Unc–51–like kinases 1 or 2 (ULK1or ULK2, respectively), Atg13 homolog, Atg17, RB1CC1, and Atg101 (Chen and Klionsky, 2011). Atg 1 complex is negatively regulated by the target of rapamycin complex 1 (TORC1). In mammals, under nutrient-rich conditions, active TORC1 associates with the ULK complex (ULK-Atg13-FIP200-Atg101) resulting to phosphorylation of ULK and thus blocks autophagy regulation. On the other hand, under starved condition, TORC1 dissociates from the ULK complex thus preventing Atg13 and ULK phosphorylation leading to autophagy induction (Chang and Neufeld, 2010; Mizushima, 2010).

1.2.2 Autophagosome formation

One of the hallmark events in the study of autophagic process is the discovery of the two novel ubiquitin-like (UBL) conjugation systems Atg12-Atg5 conjugate and the Atg8 protein. Both of these systems showed similarity to those catalyzing the ubiquitylation of the cytoplasmic proteins for their subsequent degradation by the proteasome. The systems are composed of two important enzymes, a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2).

1.2.2.1 Atg12-Atg5 conjugation system

The first of this conjugation system calls for the involvement of Atg12-Atg5 **(Fig. 1-2)**, a protein conjugate necessary for autophagosome formation. In this system, Atg12 is activated by Atg7 (E1-like activating enzyme), leading to thioester bond formation between

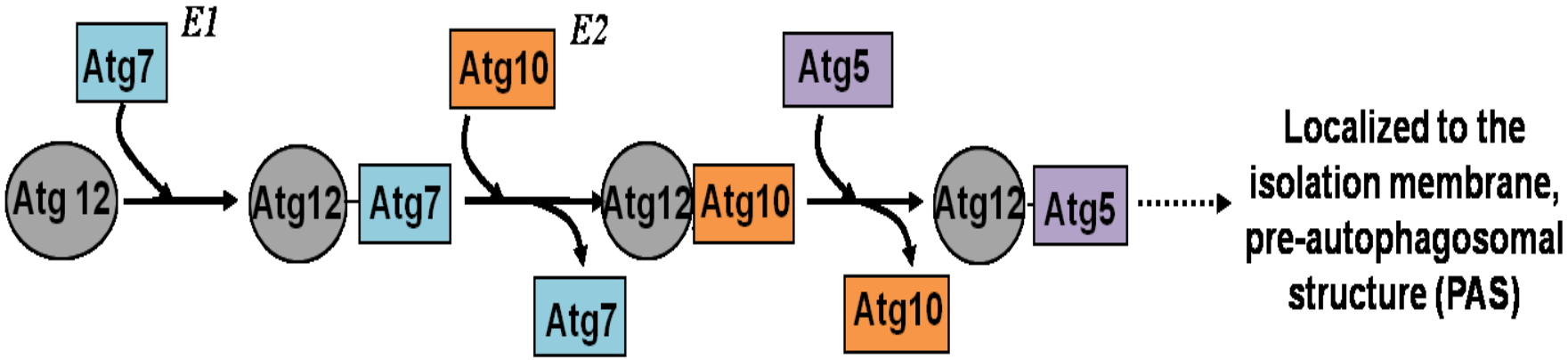

Fig. 1-2. Model for Atg12-Atg5 conjugation system. The Atg7 (E1-like) and Atg10 (E2-like) proteins from thioester intermediates through a COOH-terminal glycine of Atg12. Atg12 is ultimately conjugated to Atg5 through an internal lysine residue in Atg5 in a process that is similar to ubiquitination.

the C-terminal glycine of Atg12 and a cysteine rsidue of Atg7 (Komatsu *et al.*, 2001). Afterwards, Atg12 is transferred to Atg10 (E2-like conjugating enzyme) resulting to formation of a new thioester bond between the C-terminal glycine of Atg12 and a cysteine of Atg10 (Mizushima et al., 1998). In contrast to ubiquitination, the Atg12-Atg5 conjugation is constitutive and irreversible. In this conjugation system, it is likely that Atg10 directly catalyzes the Atg12-Atg5 formation. It is constitutively active and the formation of the Atg12-Atg5 conjugate further interacts with coiled-coil protein Atg16, which facilitates homo-oligomerization (Mizushima *et al.*, 1999). The process results into the formation of the four subunits of Atg12-Atg5-Atg16 conjugates. This complex is necessary for the elongation of the isolation membranes and during autophagosome formation, it asymmetrically localized on the outer sides of the isolation membrane. However, it starts to dissociate from the membrane before the completion of the autophagosome formation (George *et al.*, 2000).

1.2.2.2 Atg8-phosphatidylethanolamine conjugation system

The second conjugation system involves Atg8 protein elongation with phosphatidylethanolamine (PE) **(Fig. 1-3)**. This system is dependent upon the activity of Atg7 and Atg3, which have functions similar to the E1 and E2 enzymes in the ubiquitin pathway respectively (Ichimura *et al.*, 2000; Tanida *et al.*, 1999). However, Atg8 must be processed by the cysteine proteinase Atg4 (Kim *et al.*, 2001). This proteolytic modification results in the disappearance of the C-terminal region, leaving glycine at the C-terminus. The activation of Atg8 by Atg7 leads to the formation of the thioester bond between glycine terminal of Atg8 and cysteine residue of Atg7 (Kirisako *et al.*, 2000; Komatsu *et al.*, 2001). The activated Atg8 transfers to Atg3 (E2 enzyme) forming a new thioester bond (Ichimura *et al.*, 2000). The final step involves covalent conjugation of Atg8 to PE through an amide bond of glycine in the C-

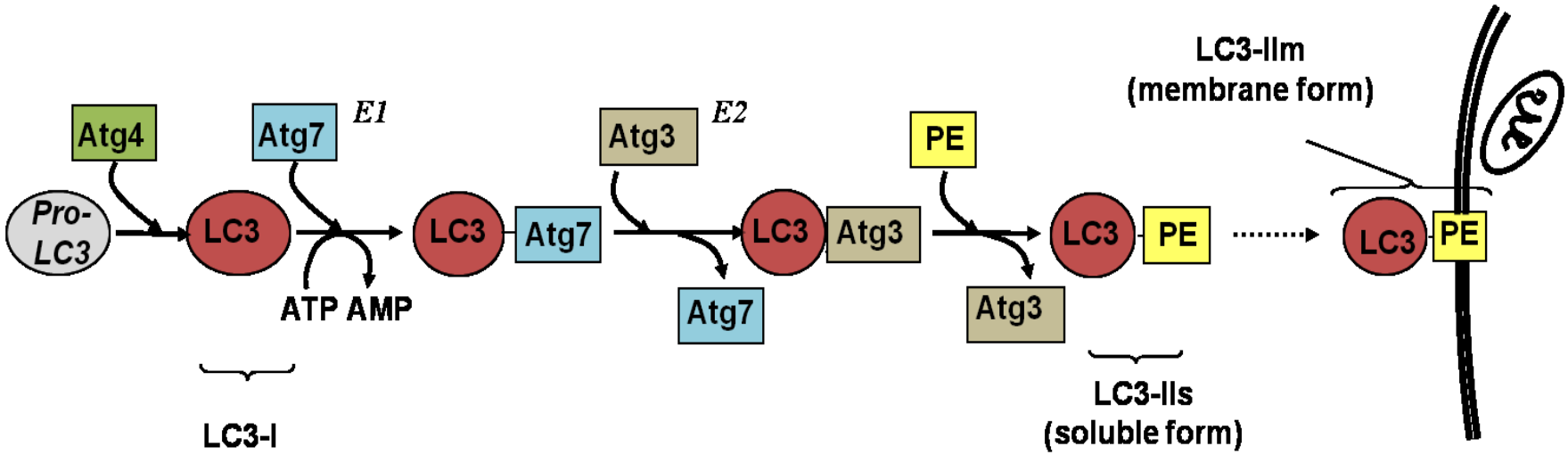

Fig. 1-3. Model for Atg8-phosphatidylethanolamine conjugation system. Atg4 modify the C-terminal of pro-LC3 enzymatically to form LC3-1 localizing in the cytosol. Atg7 (E1-like) activates LC3-1 an transferred to Atg3 (E2-like) through the formation of thioester intermediates, in a chain of reactions of similar to ubiquitination. Finally, LC3 conjugates with phosphatidylethanolamine (PE) and becomes membrane attached.

terminus of Atg8 and the amino group of PE. The presence of the conjugate Atg8-PE is fundamental for autophagosome formation. It has been suggested that a crosstalk between the Atg8-PE and Atg12-Atg5 conjugation systems is possible. Atg12-Atg5 complex may act as a ubiquitin ligase that catalyzes the conjugation of Atg8 to PE (Klionsky, 2008). Furthermore, Atg4 is required to cleave Atg8 from PE on the autophagosome membrane (referred to as deconjugation) after autophagosome formation (Chen and Klionsky, 2011). The conjugation and deconjugation processes are essential for the continuation of autophagy.

Several Atg8 mammalian homologs are involved in ATg3-dependent conjugating processes. One of the homologs, the microtubule-associated protein light chain 3 (MAP-LC3) is involved in the formation of autophagosome after a processing similar to that of yeast Atg8 (Kabeya *et al.*, 2000). MAP-LC3, which is commonly referred to as LC3, have three distinct forms: proLC3, encoding the fill-length molecule; LC3-1 (18kDa), corresponding to the proteolytically processed form; and LC3-II (16kDa), the membrane-bound form of LC3 attached to the isolation membranes or autophagosomes (Kabeya *et al.*, 2000).

The other mammalian orthologues of Atg8 that were originally identified are the GABARAP (γ-aminobutyric acid receptor-associated protein) and GATE-16 (Golgi-associated ATPase enhancer of 16kDa). GABARAP is associated with GABA receptor and with cytoskeleton and is thought to mediate endocytosis while GATE-16 is involved in intra-Golgi protein transport in vitro (Hemelaar *et al.*, 2003).

1.3 Autophagy effectors

Autophagy is a highly controlled process that can be modulated by a wide range of stimuli and conditions. Stimuli such as amino acids, hormones, and vitamins or conditions of stress such as starvation or oxidative stress may cause varied effects on autophagy.

1.3.1 Amino acids

Amino acids have been known to have regulatory activities on gene expression, mRNA translation, and proteolysis (Fafournoux *et al.*, 2000). However, not all amino acids have regulatory activities. Its regulatory property may depend on the type of cell and tissue as well as the environmental condition. In perfused liver, the extracellular concentrations of phenyalanine and leucine individually controlled autophagic proteolysis (Kadowaki *et al.*, 1992; Miotto *et al.*, 1992) while in FAO hepatocytes, leucine in particular, enhanced the phosphorylation of p70S6k (Patti *et al.*, 1998). Additionally, tyrosine does not show p70S6k phosphorylation except in pancreatic β-cells (Xu *et al.*, 1998). Based on previous studies, eight of the twenty standard amino acids showed direct suppression of autophagic proteolysis in the rat hepatocytes (Seglen *et al.*, 1980; Pösö *et al.*, 1982). The remaining 12 amino acids showed no regulatory activity even at as high as 10 times the normal plasma levels (Pösö *et al.*, 1982). However, alanine in particular, showed coregulatory effect.

1.3.2 Vitamins and antioxidants

Vitamins directly scavenge reactive oxygen species (ROS) and upregulate the activities of antioxidant enzymes. Among them, vitamin E has been recognized as one of the most important antioxidants. In human lymphocytes, vitamin E was found to inhibit ROS-induced generation of lipid peroxyl radicals thereby protecting cells from peroxidation of PUFA in membrane phospholipids, from oxidative damage of plasma very low density lipoprotein, cellular proteins, DNA, and from membrane degeneration (Topinka *et al.*, 1989). Another study demonstrated increase in brain lipid peroxidation and neurodegeneration in mice with a deficiency of α-tocopherol transfer protein (Yokata *et al.*, 2001). A more recent study demonstrated vitamin E as a novel enhancer of autophagy targeting the LC3 conversion step, an early part of autophagosome formation (Karim *et al.*, 2010). However, despite being

a typical antioxidant, vitamin E seemed to have a novel and unknown pathway different from the one utilized during antioxidative functions.

On the other hand, the antioxidant role of physiologic concentrations of vitamin C has been well established in the literature. Vitamin C, in the form of ascorbic acid, showed to stimulate autophagy in human astrocyte gilial cells, without affecting protein synthesis (Martin *et al.*, 2002). Ascorbate was also reported to inhibit non-small-cell lung cancer growth by caspase-independent autophagy and to synergistically enhance adenoviral vector-mediated transfer of the tumor suppressor gene 101F6 (Ohtani *et al.*, 2007). Furthermore, the antioxidant epigallocatechin-3-gallate (EGCG), a major ingredient of green tea, was internalized into HMGB1-containing LC3-positive cytoplasmic vesicles in macroautophages, and induced HMGB1 aggregation in a time-dependent manner; LC3-II production and autophagosome formation was also stimulated (Li *et al.*, 2011).

1.4 Signaling pathways regulating autophagy

As a great variety of stimuli are able to modulate autophagy, numerous signaling pathways have shown to control the process. However, clear effects of these stimuli on the signaling pathway controlling autophagy regulation are still largely obscure and require further identification of downstream targets. There is a great possibility that most of these signaling pathways converge at a single target to stimulate the autophagic pathway (Ogier-Denis and Codogno, 2003).

1.4.1 mTOR pathway

The classical pathway regulating mammalian autophagy involves the serine/threonine kinase mammalian target of rapamycin (mTOR) (Rubinsztein *et al.*, 2007). In the budding *Saccharomyces cerevisiae*, TOR functions as a nutrient-dependent mediator of cell

autonomous growth while in metazoans, TOR participates in both nutrient- and hormone-dependent signaling pathways (Hay and Sonenberg, 2004). mTOR partitions between two scaffold proteins, Raptor and Rictor. The Raptor/mTOR complex, called mTORC1, is rapamycin-sensitive and regulates growth via S6K1 and 4EBP1/PHAS (Hara *et al.*, 2001). On the other hand, Rictor/mTOR complex, known as mTORC2, is rapamycin-insensitive and it regulates cellular proliferation via Akt pathway (Sarbassov *et al.*, 2005) and cytoskeleton organization via protein kinase Cα (Sarbassov *et al.*, 2004).

Autophagy is found to be regulated via the mTOR complex 1 (mTORC1), which consists of raptor, GβL, and PRAS40 (Guertin and Sabatini, 2009). mTORC1 can regulate autophagy in two ways; by direct phosphorylation of Atg13 and Atg1 and by stimulating signal transduction cascade involving other proteins that regulate autophagy (He and Klionsky, 2009). These signaling cascades include adenosine monophosphate-activated protein kinase (AMPK), which activates autophagy, and Akt (protein kinase B) that downregulates autophagy (Esclatine *et al.*, 2009; He and Klionsky, 2009). It became apparent that in mammalian cells, AMPK negatively regulates mTORC1 by either direct inhibition (Yang and Klionsky, 2010) or by activating tuberous sclerosis 2 (TSC2), which is an upstream effector of mTORC (Inoki *et al.*, 2003). Additionally, recent evidence has shown that mTORC1 regulates autophagy by acting on a complex comprising mammalian Atg13, ULK1 and FIP200 (Mizushima, 2010). The activity of mTORC1 can be inhibited by rapamycin and starvation, which are established inducers of autophagy (Noda and Ohsumi, 1998). Many diverse signals such as growth factors, amino acids, and energy stress regulate autophagy by the mTORC1 pathway (Meijer and Codogno, 2006).

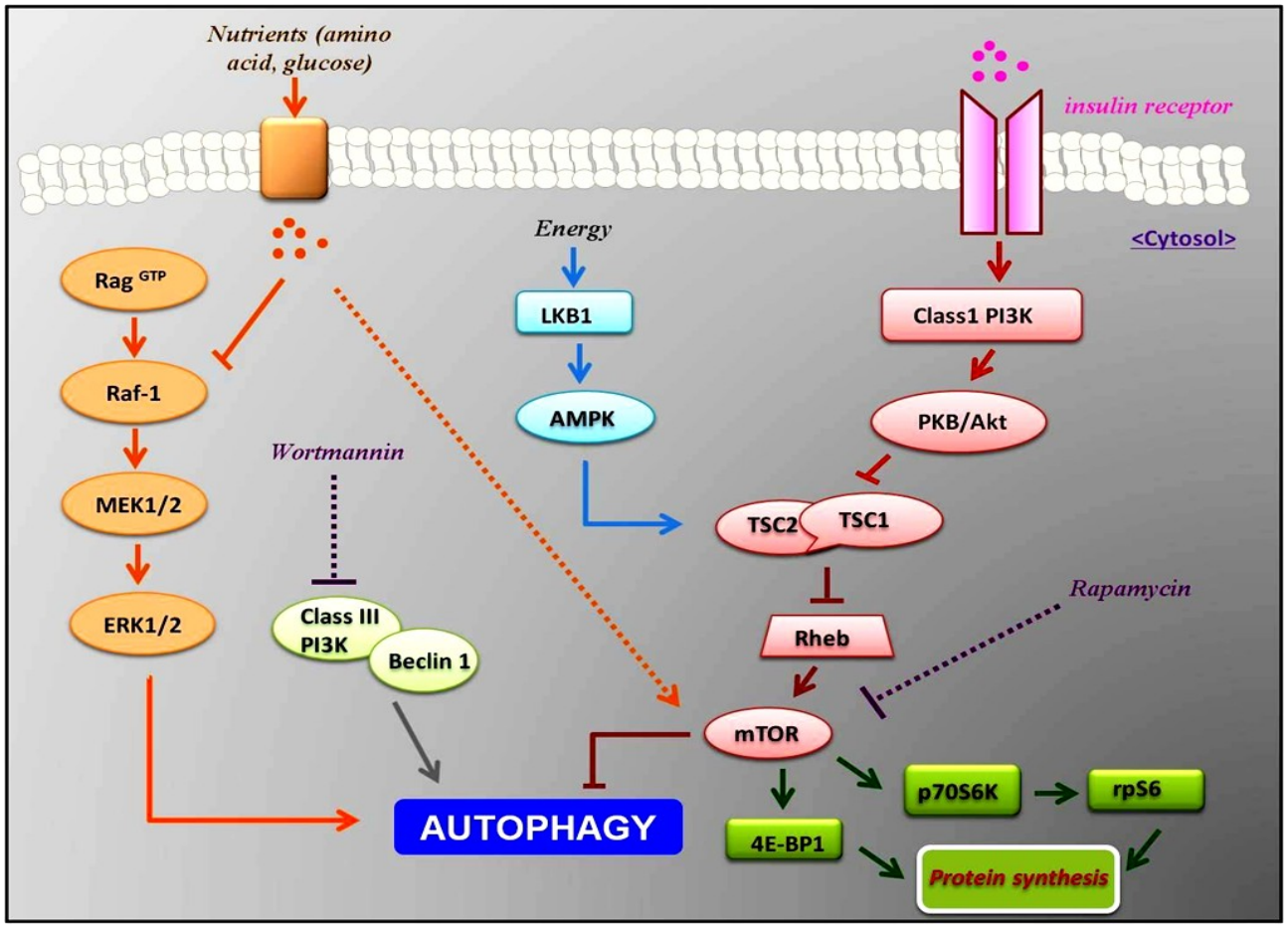

Fig. 1-4. Signaling pathway for autophagy regulation by different effectors. Amino acids modulate a number of signaling pathway as reported by several studies. However, possible convergence of signals at mTOR is still obscure. It has been noted that AMPK-activated protein kinase (AMPK; an energy sensor) may signal through TSC2 to down-regulate the activity of mTOR as well as its downstream effectors.

1.4.2 ClassIII PI3K/Beclin1 pathway

Vps34 is the Class III phosphoinositide 3-kinase (PI3K) that phosphorylates phosphatidylinositol to generate phosphatidylinositol 3-phosphate PI3(P), a phospholipid central for membrane trafficking processes (Lindmo and Stenmark, 2006). Class III PI3K plays a critical role in the early stages of autophagosome formation in mammals through formation of essential complex with Beclin1 resulting to inhibition of its activity and leads to autophagic process inhibition (Petiot *et al.*, 2000; Tassa *et al.*, 2003). The essential role of Class III PI3K in autophagy has been established largely through the use of the pharmacological inhibitors Wortmannin and 3-methyladenine, which are known to inhibit the formation of autophagosome precursors in mammalian cells. Furthermore, studies have shown that activation of the autophagic pathway in response to amino acid depletion is associated with an increase of Class III PI3K activity in marine myotubes (Tassa *et al.*, 2003).

1.5 Oxidative stress

A common intracellular stress that effectively leads to induction of autophagy is the formation of reactive oxygen species (ROS). Mitochondria are the major source generating ROS, which may in turn damage organelles. ROS-generating agents (e.g. peroxides and superoxides), or chemicals inhibiting mitochondrial electron transport chain induce ROS production and autophagic cell death in transformed and cancer cell lines (Chen *et al.*, 2008).

1.5.1 Autophagy regulation by ROS

In most studies mitochondria are the major source of ROS for autophagy induction. Recent studies demonstrate the contribution of NADPH oxidase-generated ROS in autophagy

induction (Huang *et al.*, 2009; Mitroulis *et al.*, 2008). Some studies show that ROS activate autophagy by regulating mTOR activity. In malignant glioma, ROS disrupt mitochondrial membrane potential and induce autophagy thorugh inhibiting Akt/mTOR pathway signaling (Zhang *et al.*, 2009). A novel compound, 1, 3-dibutyl-2-thioxo-imidazolidine-4,5-dione was recently found to induce autophagic cell death in tumor cells through stimulating ROS production and , in turn, activation of ERK and JNK (Wong *et al.*, 2010). Additionally, one p53-target gene encodes TIGAR (TP53-induced glycolysis and apoptosis regulator) indirectly modulates ROS level. Inhibition of TIGAR expression increases ROS production and enhances ROS-dependent autophagy (Bensaad *et al.*, 2009). ROS also regulate autophagy under amino acid and serum starvation conditions, where superoxide and peroxides has been suggested to be the ROS species involved in ROS-mediated autophagy (Chen *et al.*, 2009; Scherz-Shouval *et al.*, 2007). One mechanism by which autophagy appears to be regulated by ROS is the redox regulation of the essential autophagy protein Atg4 (Scherz-Shouval *et al.*, 2007). This protein is required for the conjugation of Atg8 to PE, a reaction that is in turn essential for the induction of autophagy.

Overall, still many unknowns exist regarding the involvement of ROS signaling in autophagy regulation. It is likely that ROS act through different mechanisms to regulate autophagy under different cellular contexts and in different cell types. Examining ROS signaling that overlap with autophagy regulation pathways may help to determine the mechanism of ROS-induced autophagy.

1.6 Aim of the present study

The main goal of this study is to investigate the involvement of ROS in autophagy regulation. Several studies have established the role of mTORC1 pathway in autophagy inhibition by amino acid and the effect of ROS production in autophagy stimulation. However, no clear evidences has been presented regarding the possible involvement of ROS as signaling molecule in amino acid regulated autophagy. In Chapter 2, I tested and validated a method that will be used in measuring intracellular ROS production in H4-II-E cells. The effect of nutrient starvation and enrichment on ROS generation was described. The antioxidant NAC and the oxidant H2O2 was used to ascertain the suitability and sensitivity of the method on any changes in ROS population.

In Chapter 3, I showed the effect of various nutrient conditions on autophagy in H4-II-E cells. I used the method of LC3 ratio measurement previously developed in our laboratory to illustrate autophagy. The second part of the chapter geared towards the application of the validated ROS measurement method on amino acids. Here, I examined the effect of amino acids (mixture and individual) on ROS production and described the similar and contrasting effects of different groups of amino acids on autophagy regulation and ROS production.

Lastly, earlier evidences indicate mTORC1 as the major signaling pathway for autophagy. Amino acids, usually in mixture form, have been shown to follow the same pathway. In Chapter 4, I described the occurrence of ROS signaling in the autophagy regulation pathway of selected amino acids by inhibitor study using rapamycin and wortmannin. I focused on the involvement of the two pathways, mTOR and Class III PI3K, both played an important role in nutrient signaling during autophagy.

Chapter 2

ROS formation assessment in H4-II-E cells

2.1 Abstract

Reactive oxygen species (ROS) are formed by the incomplete reduction of oxygen and are produced at low levels under normal physiological conditions as a result of mitochondrial respiration and a number of other processes. In this study we tested the method of measuring ROS generation in our H4-II-E cells using DCFDA as fluorescence-based probe, and spectrofluorimeter. We show that starvation stimulates formation of ROS, specifically H_2O_2 in a dose-dependent manner. The method was validated by treatment with antioxidant NAC which results to significant decrease of ROS level in the starved condition of the cell.

2.2 Introduction

Oxidative stress occurs in cells when an imbalance exists to favor the production of reactive oxygen species (ROS). Mitochondria are the major generators of these species in cells and tissues. ROS include, predominantly, the hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\bullet-}$), and the hydroxyl radical ($^{\bullet}OH$) (Finkel and Holbrook, 2000). These three major ROS are mainly produced through a chain reaction as follows: $O_2^{\bullet-}$, which is mainly produced from mETC complexes I and III, is converted to H_2O_2 by the superoxide dismutase (SOD) family of enzymes; H_2O_2 can then be converted to $^{\bullet}OH$ by ferrous iron or copper iron , or it can be catalyzed to H_2O (Chen and Gibson, 2008). Thus, the levels of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ are constantly in flux until an equilibrium is established.ROS is recognized as a central mediator in deciding the fate of a cell. Under physiological conditions, low levels of ROS activate various cellular signaling molecules, such as tyrosine kinase and small Ras proteins (Azad *et al.*, 2009). However at high levels, ROS are deleterious to cells, leading to programmed cell death (PCD) (Jabs, 1999; Lee *et al.*, 2003). 2, 7'-dichlorofluorescin diacetate (DCFH-DA) is an intracellular fluorescent probe used to measure intracellular oxidants like H_2O_2. DCFH-DA is freely permeable across cell membrane and is incorporated into hydrophobic lipid regions of the cell. The acetate moiety is deacetylated by esterases to nonfluorescent 2', 7-dichlorofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2', 7'-dichlorofluorescin (DCF) in the presence of hydrogen peroxide and peroxidase (Bass et al., 1983) **(Fig. 2-1)**. In general, DCFH-DA measurements are more than 80% sensitive to H_2O_2 and partially sensitive to other hydroxyl radicals, hence, the contribution of other ROS, including organic hydroperoxides, to DCFDA oxidation is generally minimal (McLennan and Esposti, 2000).

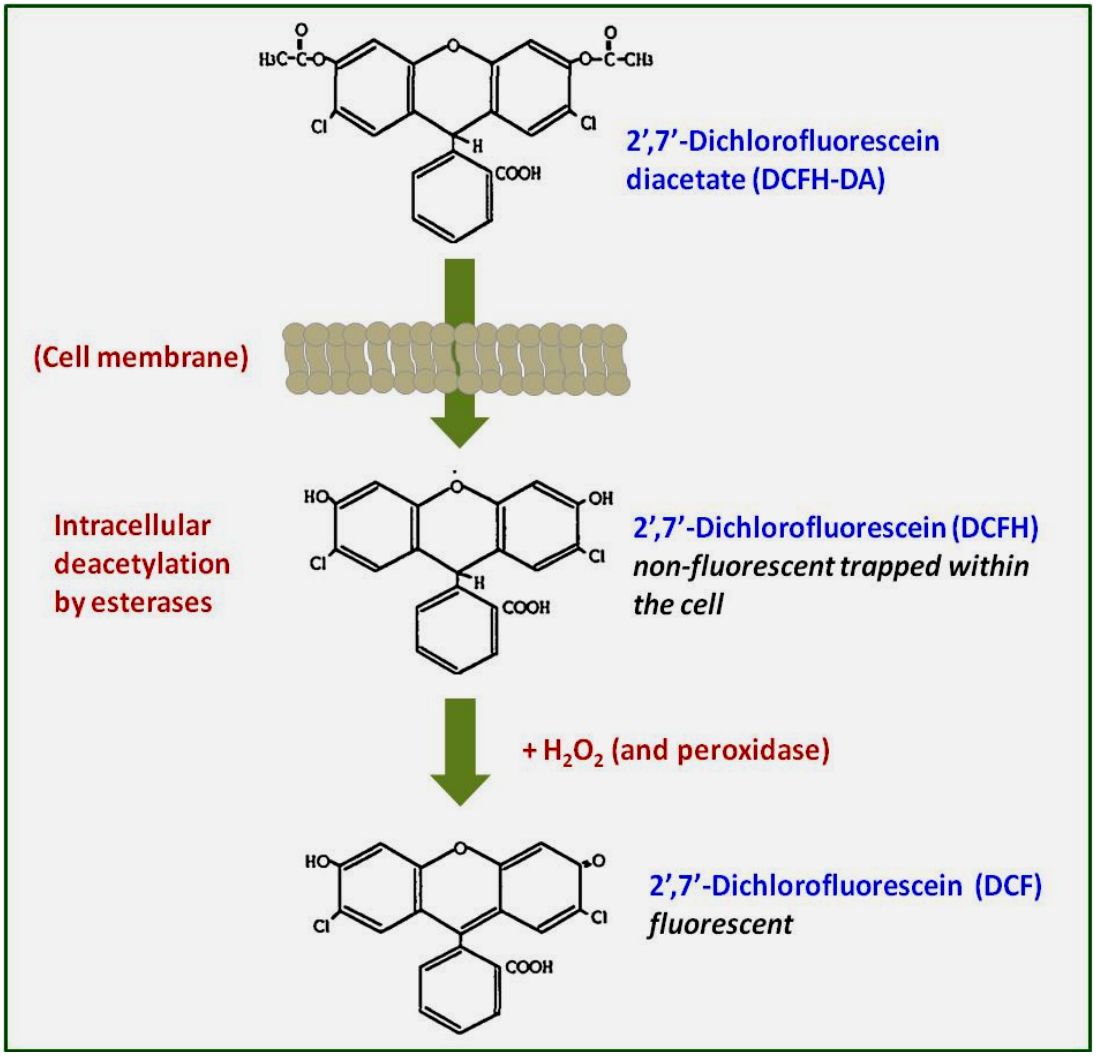

Fig 2-1. Hypothetical mechanism of the DCFDA assay. 2',7'-Dichlorofluorescin diacetate (DCFH-DA) is diffused into the cell, is trapped by deacetylation, and is available for oxidation in the presence of H_2O_2 and peroxidase.

2.3 Materials and Methods

2.3.1 Reagents

Dulbecco's Modifies Eagle Medium/Ham's F-12 (DMEM) and Earle's Balanced Salt Solution (EBSS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The fluorescence probe 2',7'-dichlorofluorescin diacetate (DCFDA) was obtained from, Molecular Probes and dissolved in DMSO. Stock solution of 10 mg/188 µl was stored in -20^{o}C and freshly diluted with medium to the appropriate concentration before using in the experiment.

2.3.2 Cell culture

Rat hepatoma H4-II-E cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). Cells were grown in DMEM/F-12 medium supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-Life Technologies), 1% Antibiotic-antimycotic (Invitrogen), and 2.5 mM L-glutamine. The cells were maintained in humidified conditions with 5% CO_2 and 37^{o}C. Cells were plated in 60 mm plates (Iwaki, Japan). Cell condition was monitored carefully until the desired confluence was reached. Prior to experiments, confluent cells were washed twice with PBS and maintained in a nutrient-rich condition (DMEM).

2.3.3 ROS measurements

H4-II-E cells were seeded in a 6-well plate (Iwaki, Japan), incubated and allowed to adhere for at least 24 hrs before being treated with 30 µM DCFDA for 15 minutes at 37^{o}C in an incubator. Cells were washed with PBS followed by starvation or amino acid treatment. Cells' DCF signal were measured using a SpectroMax Gemini Spectrolfuorometer (Molecular Devices; CA, USA) at 485 nm excitation and 535 nm emission and kept at 37^{o}C for 60-90 min. The result of the starved cells was set to 100%, and all other

treatments were normalized accordingly. The results presented for all fluorimetric measurements are the means ± standard error of the mean (SEM) of at least 3 experiments in duplicates or triplicates.

2.3.4 Statistical analysis

All the data are expressed as mean ± SEM. Student's *t*-tests were used to evaluate statistical significance. *P* values more than 0.05 were considered not significant.

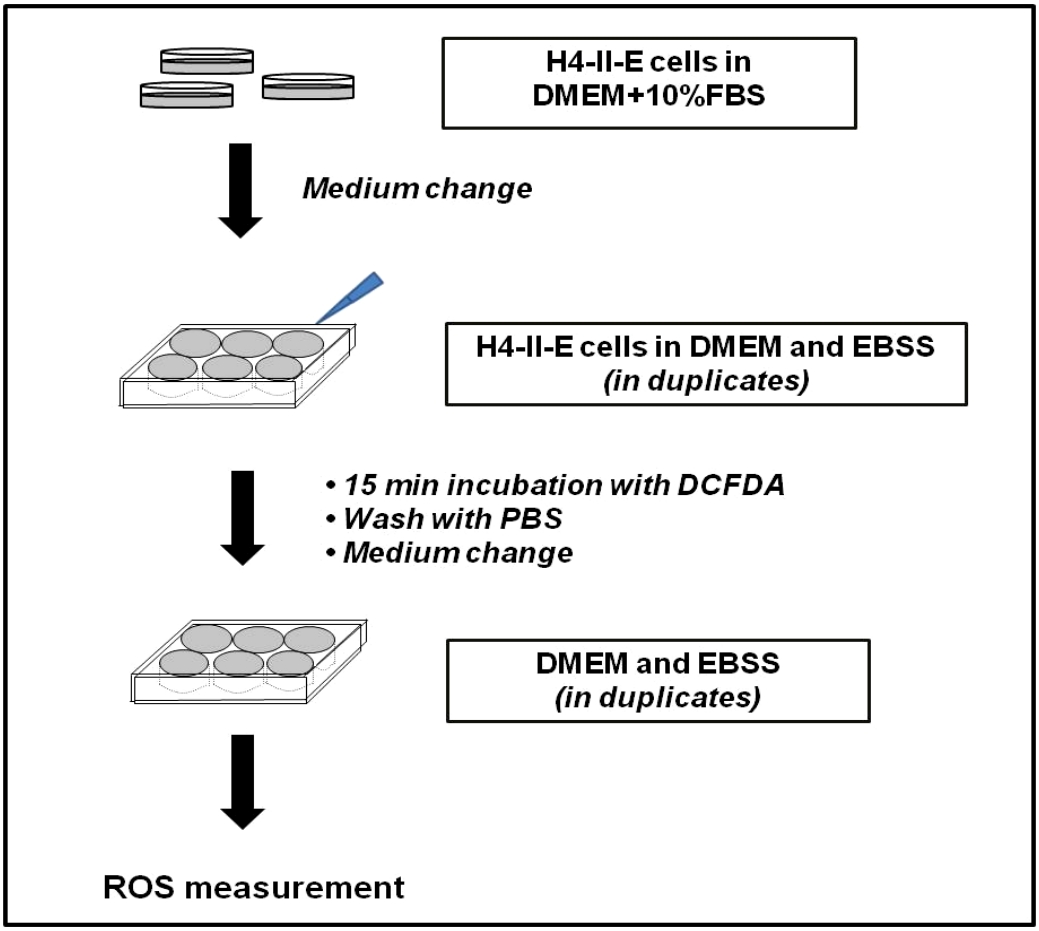

Fig 2-2. Schematic representation of ROS measurement experiment.

2.4 Results and Discussion

2.4.1 Starvation induces formation of ROS

Reactive oxygen species (ROS) serve as a signaling molecules in a variety of cellular processes. To test whether the ROS response of the cell at various nutrient conditions, we measured ROS production under nutrient-rich and starvation condition using a fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA). This probe reacts mainly with H_2O_2 to form a fluorescent compound (DCF) at the point of interaction. Since H_2O_2 is major peroxide in cells, it is generally accepted that the DCF level is proportional to the ROS level (Takanashi *et al.*, 1997). The pattern of DCF fluorescence of H4-II-E cells in nutrient-rich (EBSS) and starved (DMEM) conditions is shown in **Fig. 2-3**. In this experiment, we assessed the rise of ROS by measuring the increase in DCF fluorescence using a spectrofluorometer. In as early as 15 min, complete starvation of the cells resulted in a dramatic increase in the fluorescence signal over time as compared with nutrient-rich DMEM medium. In this study, we showed that the stress caused by starvation triggered ROS generation from mitochondria. This oxidative signal suggested that ROS, mostly as H_2O_2, served as a good signaling molecule in H4-II-E cells. Furthermore, the absence of abnormal changes in fluorescent signals indicated the stability of the reaction throughout the indicated time period.

These results were further validated by testing the effect of H_2O_2 and the antioxidant N-acetyl-L-cysteine (NAC) on starvation-induced ROS production. The addition of increasing concentration of H_2O_2 increases ROS generation **(Fig. 2-4)**.

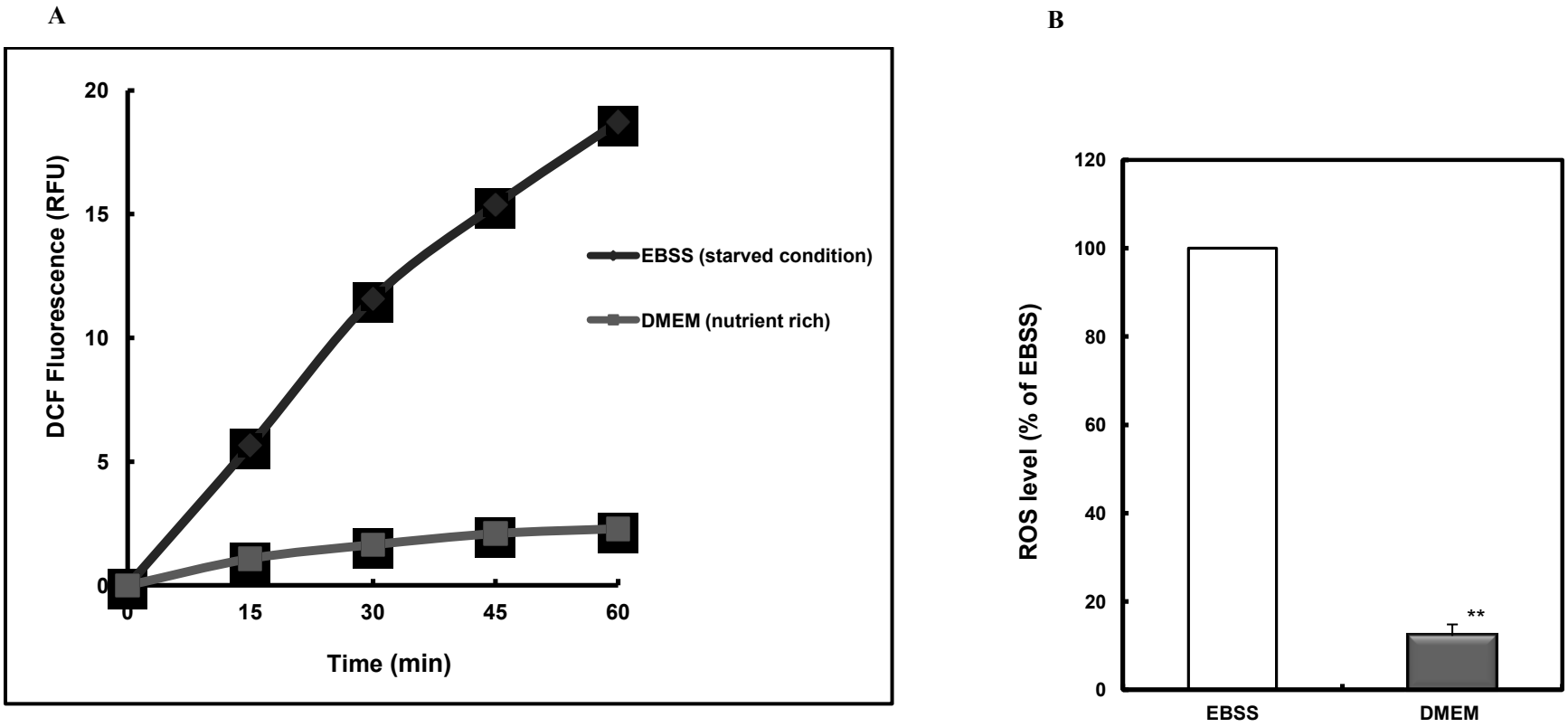

Fig 2-3. Effect of starvation on ROS production in H4-II-E cells. (A) H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates and treated with 30 μM DCFDA at 37°C for 15 min. Medium was changed in EBSS (completely starved) or DMEM (nutrient-rich). Cells' DCF fluorescence was measured with a fluorimeter for 60 min. (B) Data collected from the fluorimetric measurements were analyzed as detailed in Materials and methods. Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

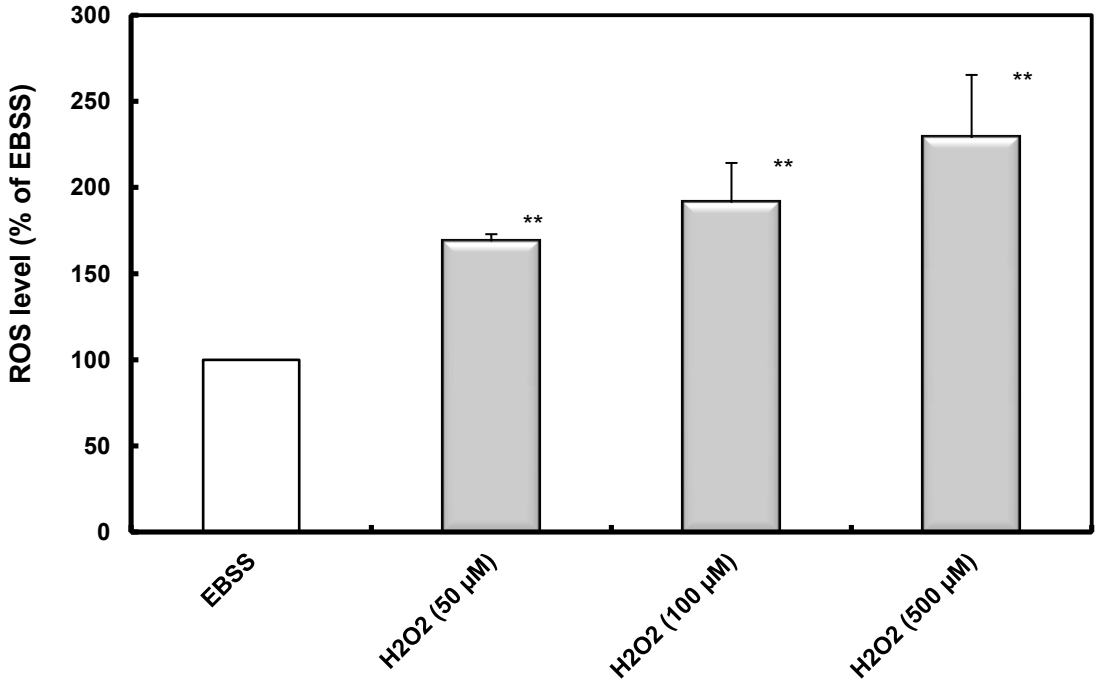

Fig 2-4. Effect of exogenous H_2O_2 addition on ROS production in H4-II-E cells. (A) H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates and treated with 30 µM DCFDA at 37^{o}C for 15 min. Medium was changed to EBSS (completely starved) or treated with different concentrations of H_2O_2. Cells' DCF fluorescence was measured with a fluorimeter for 60 min. Data collected from the fluorimetric measurements were analyzed as detailed in Materials and methods. Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

In the entirety of this study we focus specifically on H_2O_2 as our main ROS. There are several possible reasons why H_2O_2 is the more commonly investigated ROS especially during the event of starvation. First, H_2O_2 is more stable than other ROS (e.g. $O_2^{\bullet-}$) and is found to be a very good signaling molecule in many biological pathways. Second, H_2O_2 scavengers such as NAC are readily available. Our results showed that NAC can significantly inhibit ROS production to almost 45% **(Fig. 2-5)**. Moreover, exogenous ROS are also readily available and it can easily mimic the effect of intracellularly produced H_2O_2. In this study we have demonstrated direct correlation of DCF fluorescence to the concentration of H_2O_2 in the cell.

In addition, the fluorescence intensity of our cell is slightly less if compared to the fluorescence intensity of other cell types from other studies. Our cell sample, H4-II-E, is an endothelial adherent cell and as pointed out by Carter *et al.* (1994), the less intensity observed from adherent cells suggests that fluorescence intensity may be related to differences in cellular dye incorporation between adherent and nonadherent cells. Adherent cells need to be washed after treatment of the probe and before fluorescence reading, while cells in suspension need no washing. This action may cause slight disturbance in the equilibrium of the cell and may lead to some loss in fluorescence intensity. Nonetheless, this should not affect our result since we are using the same cell type and method of analysis throughout the course of our study. The fluorescence results that we obtained during starvation, exogenous H_2O_2 addition, and NAC treatment demonstrated that our cell sample is sensitive to the changes in H_2O_2 population in its environment and our method is sensitive enough to detect the changes. Taken together, this method of ROS production assessment is appropriate to be applied to amino acids.

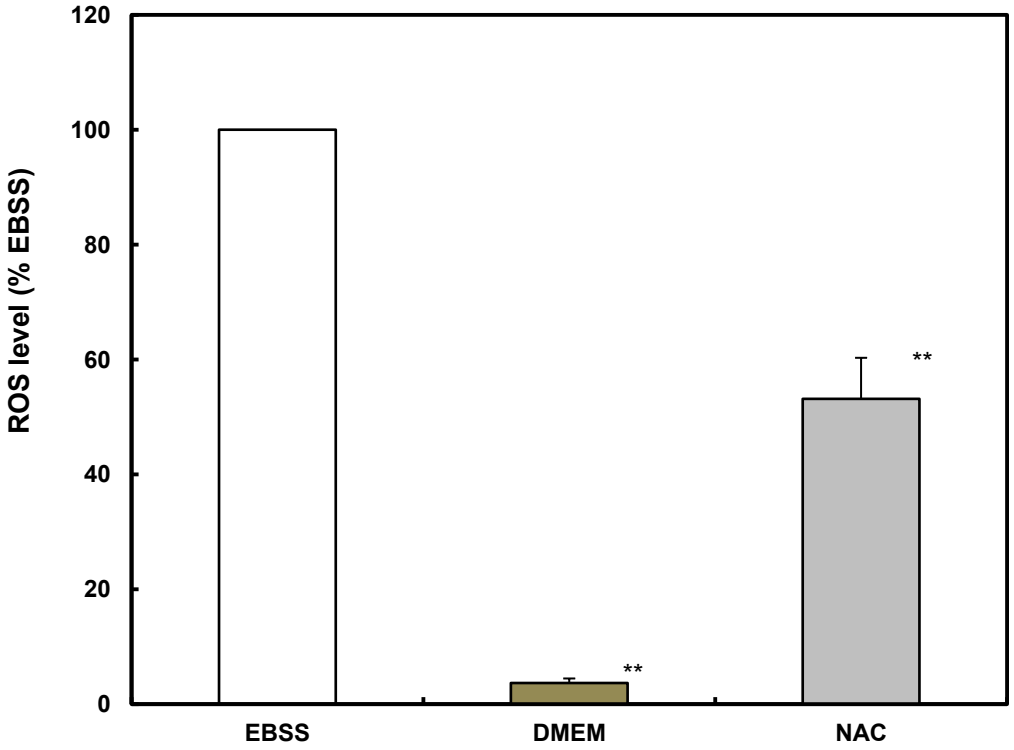

Fig 2-5. Effect of NAC addition on ROS production in H4-II-E cells. (A) H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates and treated with 30 μM DCFDA at 37^{o}C for 15 min. Medium was changed in EBSS (completely starved) or DMEM or treated with 10 mM NAC. Cells' DCF fluorescence was measured with a fluorimeter for 60 min. Data collected from the fluorimetric measurements were analyzed as detailed in Materials and methods. Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

2.4.2 Concluding remarks

In conclusion, our present study has demonstrated the utility of the fluorescent probe DCFDA in the evaluation of H4-II-E cell oxidation reaction. We have assessed the rise of ROS in a more accurate manner through the increase in DCF fluorescence. The sensitivity of our method made it feasible for amino acid ROS production profiling.

Chapter 3

Effect of amino acids on ROS production and autophagy regulation

3.1 Abstract

Autophagy is a lysosomal degradation process by which eukaryotic cells degrade and recycle macromolecules and organelles. This process is regulated by various stress conditions or nutrient availability. In this study we investigated the involvement of reactive oxygen species (ROS) production in the regulation of autophagy by amino acids in rat hepatoma H4-II-E cells. Mixture and individual amino acids were subjected in the study. Treatment with complete amino acid (CAA) mixture and individual amino acids Leu, Met, and Arg showed suppression of autophagy. Furthermore, CAA, regulatory amino acid (RegAA), and nonregulatory amino acid (NonRegAA) mixtures exhibited suppression of ROS production. We also extended our study on ROS production of individual amino acids. Leucine, from the group of RegAAs, showed decrease in ROS production and suppression of autophagy. Furthermore, methionine and proline from the group of RegAAs and arginine, from NonRegAA group, suppressed autophagy with stimulation of ROS production. Other amino acids from the NonRegAA group showed stimulating effects on ROS production but no autophagic response.

3.2 Introduction

Autophagy is the cellular pathway of self-digestion, a regulated lysosomal pathway for the degradation and recycling of long-lived proteins and organelles (Levine, 2005). Autophagy initiates with the formation of double-membraned structure called phagophores, which elongate and engulf portions of cytoplasm to form autophagosomes. These vesicles ultimately fuse with lysosomes to form the degradative autolysosomes, where the engulfed contents are degraded by the acidic lysosomal hydrolases (Xie and Klionsky, 2004). The only known mammalian protein that specifically associates with autophagosome membrane is LC3 (Kabeya *et al.*, 2000). In cells, LC3 is present in two forms. The cytosolic LC3-I form conjugates with phosphatidylethanolamine (PE) upon induction of autophagy, resulting in the autophagosome-associated LC3-II form (Kabeya *et al.*, 2000). Since autophagosome is a transient structure, the life-time of LC3-II is relatively short. Therefore, the level of LC3-II represents the autophagic activity at the moment. Majority of the recent studies assaying mammalian autophagy have relied on this specific marker to assess the extent of autophagy by measuring the levels of LC3-II protein (Klionsky *et al.*, 2008). But, previous studies have shown that detection of steady state levels of LC3-II is not exactly accurate for estimating cellular autophagic flux (Tanida et al., 2005). However, a recent study (Karim *et al.*, 2007) conducted in our laboratory on fresh rat hepatocytes and H4-II-E cell line identified the existence of a hydrophilic and nonlipidated cytosolic LC3-IIs. This study showed that the cytosolic LC3 ratio derived from the LC3-IIs is a reliable index of autophagy formation and much more sensitive and proportionate to the change in proteolytic rates than the ratio from total homogenate. This method will be used in our study to assess autophagy in H4-II-E cells.

Among a number of proteolytic systems in the body, autophagic proteolysis is the only mechanism known to be subjected to nutritional regulation. Earlier studies on this field noted the occurrence of autophagy in HeLa cells deprived of fetal calf serum and amino acids

(Mitchener *et al.*, 1976). It has also been seen in rat liver after glucagon administration during nutritional deprivation and in diabetes (Ashford and Porter, 1976; Swift and Hruban, 1964; Pain *et al.*, 1974). Thus, autophagy has shown to have somewhat physiological role in the maintenance of amino acid pools. Development of this field has eventually shown amino acid, together with hormones such as insulin and glucagon, as principal regulators of mammalian autophagy.

Recently, the themes of oxidative stress and autophagy have been brought together. Most of these studies have implicated ROS in autophagy induction by using nonspecific ROS scavengers or exogenous H_2O_2 (Bridges, 1987; Kirkland *et al.*, 2002). Several independent studies reported that ROS induce Beclin-1 expression in cancer cells (Chen *et al.*, 2008; Djavaheri-Mergny *et al.*, 2006), although the mechanism for this upregulation remains unknown. Without pinpointing specific molecular mechanisms, these studies nevertheless strongly support the involvement of ROS in autophagy regulation. One mechanism by which autophagy appears to be regulated by ROS is the redox regulation of the essential autophagy protein Atg4 (Scherz-Shouval *et al.*, 2007). This protein is required for the conjugation of Atg8 to phosphatidylethanolamine, a reaction that is in turn essential for autophagy stimulation. Multiple studies have implicated amino acids as one of the prime regulators of autophagy. A pioneering study on autophagy showed that amino acids were able to inhibit autophagic flux and the presence of insulin promotes inhibition by low concentrations of amino acids, whereas glucagon reduces it (Mortimore *et al.*, 1989). Throughout the evidences of ROS involvement in autophagy regulation and the amino acid regulation on autophagy, little is known about the effect of amino acid on ROS production in the event of autophagy regulation. For this reason, here in Chapter 3, we examined the ROS production of mixtures and individual amino acid and correlated the result to autophagy regulation in H4-II-E cells using the method (Karim *et al.*, 2007) developed previously in our research group.

3.3 Materials and Methods

3.3.1 Reagents

All individual amino acids were obtained from Wako Pure Chemical Inductries (Osaka, Japan). Polyclonal rabbit anti-LC3 antibody was from ThermoFisher Scientific (Waltham, MA, USA) and peroxidase-conjugated goat anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Trypsin-EDTA was from Invitrogen (Grand Island, NY, USA). The ECL Western blotting detection kit was from GE Healthcare Life Sciences (Pittsburgh, CA, USA). The other reagents were indicated in Chapter 2.3.1. All the chemicals were in analytical grade unless otherwise indicated.

3.3.2 Cell cultures

The H4-II-E rat hepatoma cells were prepared as mentioned in **Chapter 2.3.2**. Autophagy was induced by changing the medium to EBSS or suppressed by changing the medium back to DMEM or by treatment with complete amino acid (CAA), regulatory amino acid (RegAA), and nonregulatory amino acid (NonRegAA) mixtures, or individual regAA Leu or Met, and NonRegAA Arg. Following incubation, sampling was done at 1 hour of incubation at 37^{o}C. To collect the cells, the culture dishes were washed twice with ice-cold PBS and scraped out into microtube and spun at 3,000 rpm for 5 min and resuspended in a buffer of 0.25 M sucrose, 1 mM EDTA (pH 7.4), and homogenized with a tightly fitting Dounce homogenizer (7 ml) by 120 strokes on ice. The resulting cell lysate was regarded as the total homogenate. These homogenates were centrifuged (100,000 x g) for 1 hr at 4^{o}C with Hitachi CS 100 GXL, S 100 AT4-557 rotor. The resulting supernatants were collected and this fraction was designated as the cytosolic fraction containing the LC3 protein.

3.3.3 Western blotting

To assess autophagic proteolysis, SDS-PAGE and immunoblotting were done on the soluble cytosolic fraction. Electrophoresis sample was prepared by mixing the cytosolic fraction with the SDS sample buffer (1% bromophenol blue, 10 mM Tris-HCl, 12.5% glycerol, a.25% β-mercaptoethanol) at 4:1 ratio. The resulting sample mixture was subjected to heat treatment at 100^{o}C for 5 minutes. After heat treatment, the LC3 protein in the sample was separated by SDS-PAGE in 15% acrylamide gel (Laemmli, 1970) using the electrophoresis apparatus from Biorad, Inc. The separated proteins were transferred onto PVDF membrane (Immuno-Blot™ PVDF membrane, Biorad Inc.) using a semi-dry transferring unit (Atto Corp., Tokyo, Japan). The membrane was incubated with polyclonal rabbit anti-LC3 antibody for 1 hr followed by incubation with peroxidase-conjugated goat anti-rabbit IgG diluted in PBS (1:13,333) and allowed to react overnight. Bands were detected using the ECL western blotting detection reagents (GE Healthcare) and is exposed to Hyper film ECL. The bands were quantified by densitometric analysis (Scion Image 1.63.1, NIH image).

3.3.3 ROS measurements

H4-II-E cells were seeded in a 6-well plate (Iwaki, Japan), incubated and allowed to adhere before being treated with 30 μM DCFDA for 15 minutes at 37^{o}C in an incubator. Cells were washed with PBS followed by starvation or amino acid treatment. Cells' DCF signal were measured using a SpectroMax Gemini Spectrolfuorometer (Molecular Devices; CA, USA) at 485 nm excitation and 535 nm emission and kept at 37^{o}C for 60-90 min. ROS production was either stimulated or inhibited by treatment with complete amino acids (CAA, regulatory amino acids (RegAA; Ala, Gln, His, Leu, Met, Pro, Trp, Tyr), and non-regulatory amino acids (NonRegAA; Arg, Asn, Asp, Cys, Glu, Gly, Ile, Lys, Phe, Ser, Thr, Val), of which grouping was defined in the perfused liver study (Pösö et al., 1982). Amino acids were

added as multiples of normal plasma concentrations. The normal concentration (1-fold) of each amino acid was (μM): L-His, 92; L-Leu, 204; L-Met, 60; L-Pro, 437; L-Trp, 93; L-Ala, 475; L-Gln, 716; L-Tyr, 98; L-Arg, 220; L-Asn, 101; L-Asp, 53; L-Cys(tine), 34; L-Glu, 158; L-Gly, 370; L-Ile, 114; L-Lys, 408; L-Phe, 96; L-Ser, 657; L-Thr, 329; L-Val, 250. A 4-fold concentration was regarded as the physiologically maximum level.

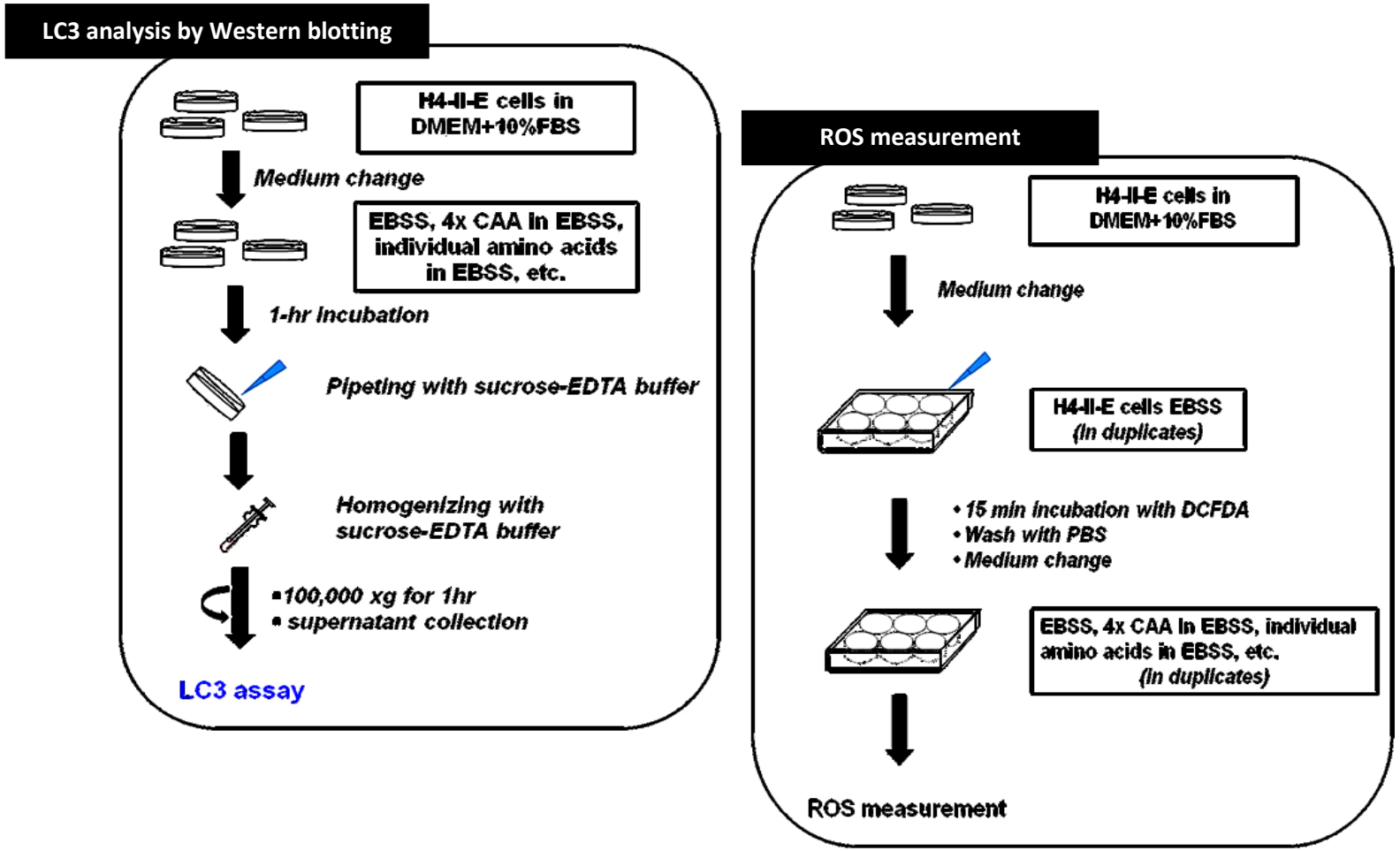

Fig. 3-1. Schematic representation of cell experiment and ROS measurement.

3.4 Results and Discussion

3.4.1 Effects of starvation and amino acid mixture on autophagy

Autophagy is stimulated by nutrient starvation when cells are not able to take up external nutrients. The first study on this chapter demonstrated the effect of starvation and amino acid on autophagy in H4-II-E cells. To evaluate autophagy, we used the method (Karim *et al.*, 2007) previously developed in our laboratory wherein a new cytosolic LC3 ratio (LC3-IIs/LC3-I) was used as a sensitive and quantitative index of autophagic flux.

The LC3 bands obtained after 1 hr incubation of our cells were quantified by densitometric analysis calculated as the ratio of LC3-IIs/LC3-I **(Fig 3-2)**. The index value was presented as percentage of control (EBSS), hence the value of LC3 ratio of the starved cells was set to 100% and the LC3 ratio values from other conditions were normalized accordingly. In these figure, stimulation and suppression of autophagy depending on the nutrient conditions were demonstrated. In a nutrient-rich condition (DMEM supplemented with FBS), minimal level of autophagy was observed. However, in the absence of amino acids and other nutrients (EBSS; starved condition), LC3-I was actively converted to LC3-II resulting to maximum autophagy stimulation. This stimulation, however, was suppressed to 49% by addition of 4-fold complete amino acid (4x CAA) mixture. Earlier studies have pointed out that this suppressive effect of amino acids is due to inhibition of the formation of autophagosome (Mortimore and Kadowaki, 1994; Seglen and Bohley, 1992). Furthermore, in the previous chapter, we pointed out that starvation significantly stimulates ROS production. All these results suggest that the increase of ROS production resulted to induction of autophagy as clearly demonstrated by the augmented LC3 ratio of EBSS. This result supported the previous study that showed induction of autophagy in H4-II-E cells upon H_2O_2 addition (Karim *et al.*, 2010)

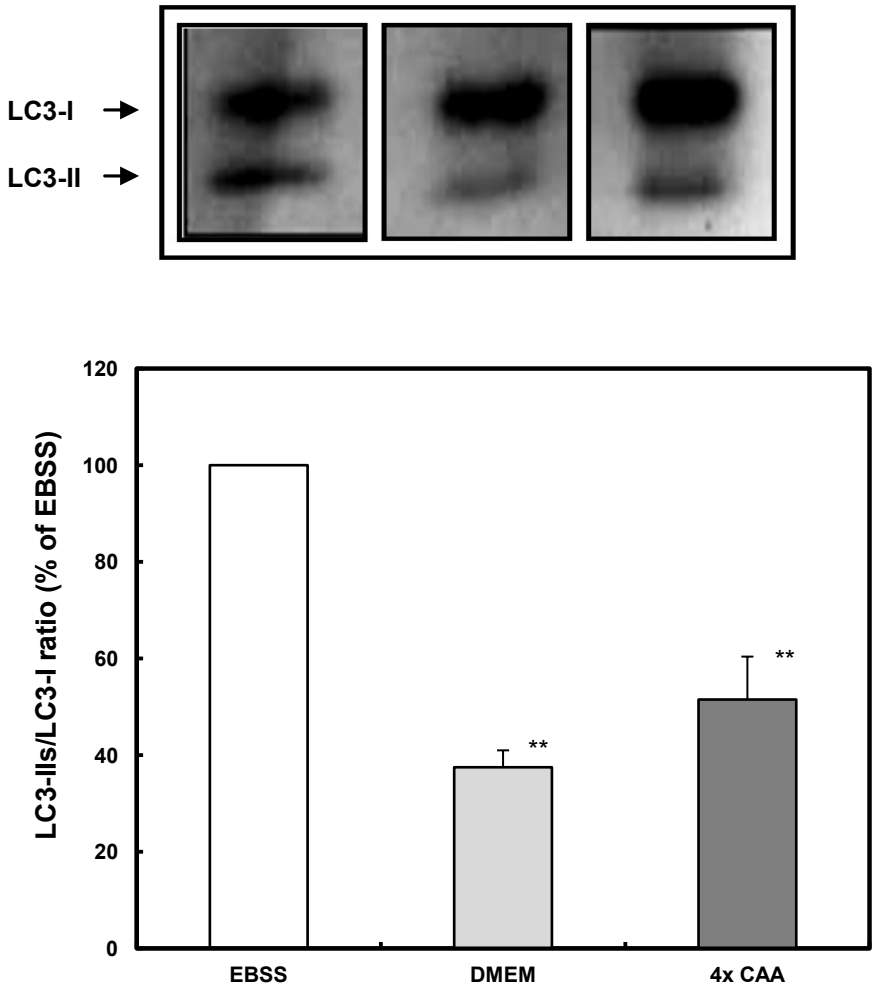

Fig. 3-2. Effects of amino acid mixture (CAA) on autophagy. H4-II-E cells were incubated in DMEM or starved in EBSS for 1 hour, then homogenized. LC3 bands in the 100,000 x g supernatant (cytosol) fraction were analyzed and visualized by SDS-PAGE and western blotting. The cytosolic LC3 ratio (LC3-IIs/I) was calculated by densitometry. Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

3.4.2 ROS production profile of amino acid mixtures

The results above clearly illustrated that starvation triggers accumulation of ROS and autophagy induction. Many studies have proven the effect of amino acid on autophagy regulation; however, no enough substantial studies have been made regarding the involvement of ROS on amino acid regulated autophagy. Since CAA clearly demonstrated suppression of autophagy, we would like to examine the effect of amino acid on autophagy regulation. We first examined the ROS production of three different mixtures of amino acids: CAA, RegAA, and NonRegAA mixtures. The amino acid composition of these mixtures is indicated in Chapter 3.3.3. The RegAA mixture is composed of amino acids that exert a direct effect on autophagic proteolysis. Our real-time DCF fluorescence result showed that in as early as 20 minutes, the three mixtures of amino acid exhibited suppression of ROS production **(Fig 3-3A)**. After extending our reading until 120 minutes, no changes were observed. This indicates that our result exhibited consistency of the trend and stability of the system. In fact, within the 60 min reading, complete suppression of ROS production was obtained in the DMEM medium and as much as 80% and 64% decrease was observed with CAA and RegAA, respectively **(Fig. 3-3B)**. Furthermore, recent study on H4-II-E cells showed that CAA and RegAA mixtures significantly suppressed autophagy by 43% and 23%, respectively, and NonRegAA mixture showed non-significant suppression, but similar to RegAA (Angcajas *et al.*, 2014). Taken together, these results supported the hypothesis that ROS production mediates the autophagy regulation by these mixtures. Even NonRegAA showed an unexpected significant suppression of ROS production. These tell us further that regardless of their autophagic regulation property, the three amino acid mixtures exhibited antioxidant properties. However, the antioxidant activity of NonRegAA may not be tightly linked to autophagy regulation.

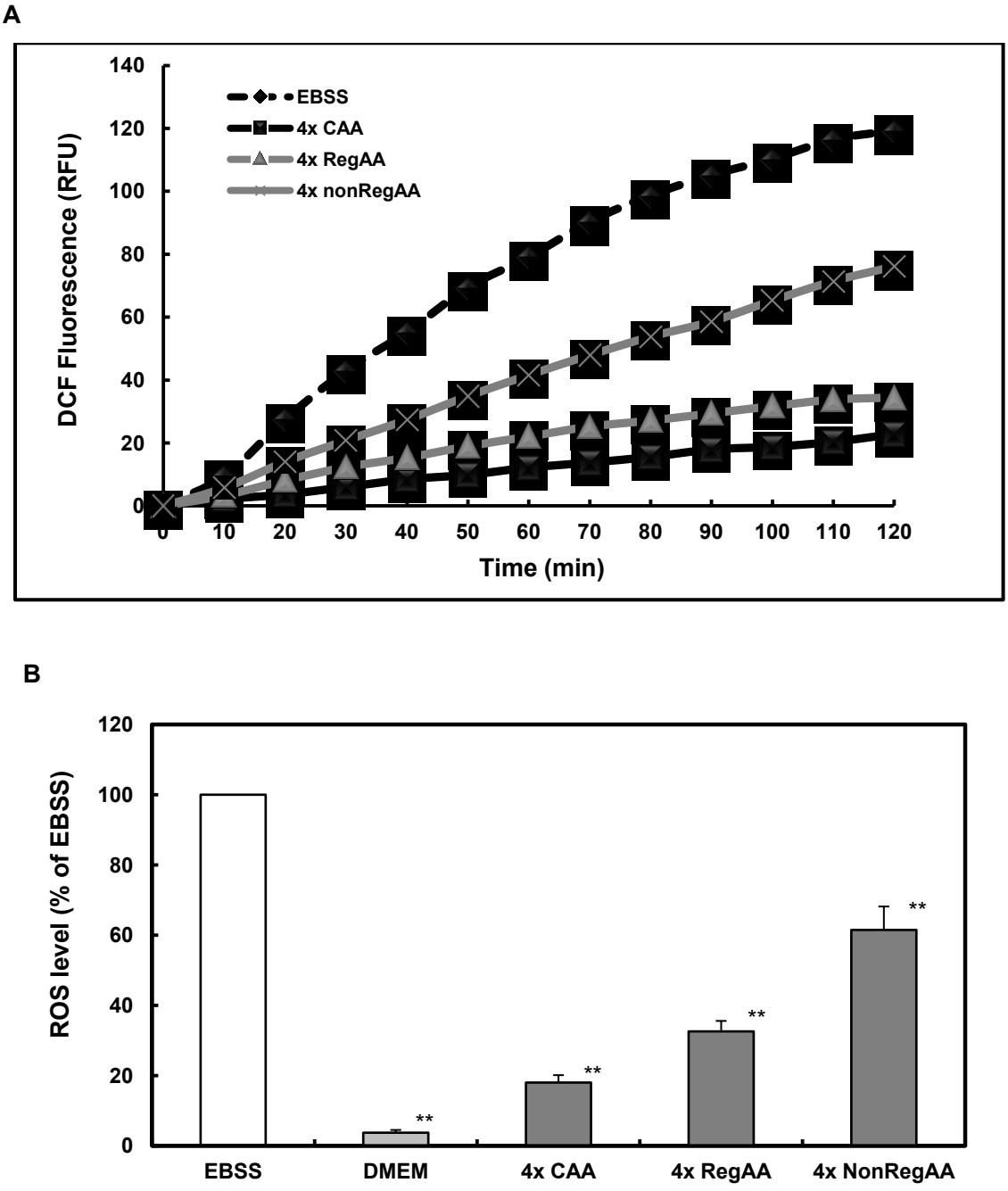

Fig. 3-3. Effects of amino acid mixtures on ROS production. (A) H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 μM DCFDA at 37^{o}C for 15 min, washed and starved by changing the medium to EBSS and treated with with 4-fold CAA, 4-fold RegAA, and 4-fold NonRegAA mixtures. Cells were subsequently analyzed with a fluorimeter for 2 hours. **(B)** Data collected from fluorometric measurements were analyzed as detailed in "Materials and Methods". Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

Some discrepancies may exist on the time of ROS production and autophagy induced by nutrient starvation between this study and other studies from literature. For example, some groups have shown that ROS and autophagy can be detected at later times (between 4 and 6 h) whereas this study showed that ROS production can be detected as early as 10-15 min and autophagy regulation can be detected in 1 h. This disparity could be due to variations of cell lines used, the relative health of the cells before starvation, or differences in detailed experimental procedures from different laboratories.

3.4.3 Effects of individual amino acids on ROS production and autophagy regulation

The unforeseen result, that NonRegAA suppressed ROS, directed us to explore the antioxidant property of individual amino acids and their capability to control autophagy. Thus, we examine the effects of individual amino acids comprehensively using the same method. Since most of the previous studies on autophagy regulation were conducted using mixtures of amino acid, we suspected that individual amino acid would mimic the ROS and autophagy response of their mixture groups. Physiologically maximum levels of amino acids (4-times their normal plasma levels) were employed (Pösö *et al.*, 1982).

In **Table 3-1**, we showed that most of individual RegAA caused a considerable decrease in ROS production, with the exception of Met and Pro. These results, suggested that the ROS suppression exhibited by RegAA mixture reflected an accumulating effect of the individual RegAA. The 55% decrease in ROS production and 31% autophagy suppression **(Fig 3-4)** demonstared by Leu supported the hypothesis that ROS plays a signaling role in autophagy regulation. Additionally, Leu exhibited an antioxidant activity that controls autophagy. On the other hand, Met and Pro showed a 28% and 63% increase in ROS production, respectively. These responses are opposite from the rest of the RegAAs. Several studies have showed that proline

oxidation can affect cellular energetics (Hagedorn and Phang, 1986; Taggart and Krakaur, 1949; Johnson and Strecker, 1961). A recent finding

Table 3-1. Effects of individual RegAA on ROS production in H4-II-E cells.

Treatment	ROS level (% of EBSS)
EBSS (control)	100
Alanine	58.5 ± 22.8*
Glutamine	47.6 ± 11.8*
Histidine	52.2 ± 10.4*
Leucine	45.4 ± 9.5*
Methionine	127.9 ± 24.3*
Proline	162.8 ± 35.8*
Tryptophan	53.9 ± 2.1*
Tyrosine	66.5 ± 8.6*

H4-II-E cells were incubated in DMEM and then starved in EBSS. Starved cells were treated with individual RegAA. ROS production was analyzed by fluorimetry, as described in "Materials and Methods". Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

proposed that p53-dependent apoptosis is preceded by the induction of proline oxidase (POX) and is accompanied by proline-mediated ROS generation (Donald *et al.*, 2001). Since POX is a mitochondrial enzyme catalyzing the conversion of proline to pyrroline-5-carboxylate with the concomitant transfer of electrons to cytochrome *c*, it is possible that proline-dependent electron transfers in the cell may contribute to ROS production. On the other hand, previous study reported that Met increases the rate of ROS generation of liver and kidney mitochondria when directly added to the organelles in vitro (Gomez *et al.*, 2011). Similarly, our result demonstrated the same ROS stimulation on H4-II-E cells; however, Met significantly suppressed autophagy to

about 35% **(Fig 3-4)**. This can be a case of indirect involvement of ROS production on autophagy regulation.

Individual NonRegAA exhibited mostly an increase or no change in ROS production **(Table 3-2)**. This result was intriguing since NonRegAA mixture significantly decreases ROS. .Arginine displayed a 20% increase in ROS production and almost 50% autophagy suppression. This is a unique result since Arg is a known nonregulatory amino acid. In fact,

Table 3-2. Effects of individual NonRegAA on ROS production in H4-II-E cells.

Treatment	ROS level (% of EBSS)
EBSS (control)	100
Arginine	120.1 ± 0.3*
Asparagine	128.4 ± 19.6
Aspartic acid	163.9 ± 21.2*
Cystine	157.5 ± 26.7*
Glutamic acid	138.5 ± 10.0*
Glycine	117.4 ± 11.4
Isoleucine	101.8 ± 18.3
Lysine	181.1 ± 11.8*
Phenylalanine	99.7 ± 10.1
Serine	115.1 ± 12.2
Threonine	128.4 ± 20.2
Valine	184.5 ± 11.7*

H4-II-E cells were incubated in DMEM and then starved in EBSS. Starved cells were treated with individual NonRegAA. ROS production was analyzed by fluorometry, as described in "Materials and Methods". Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

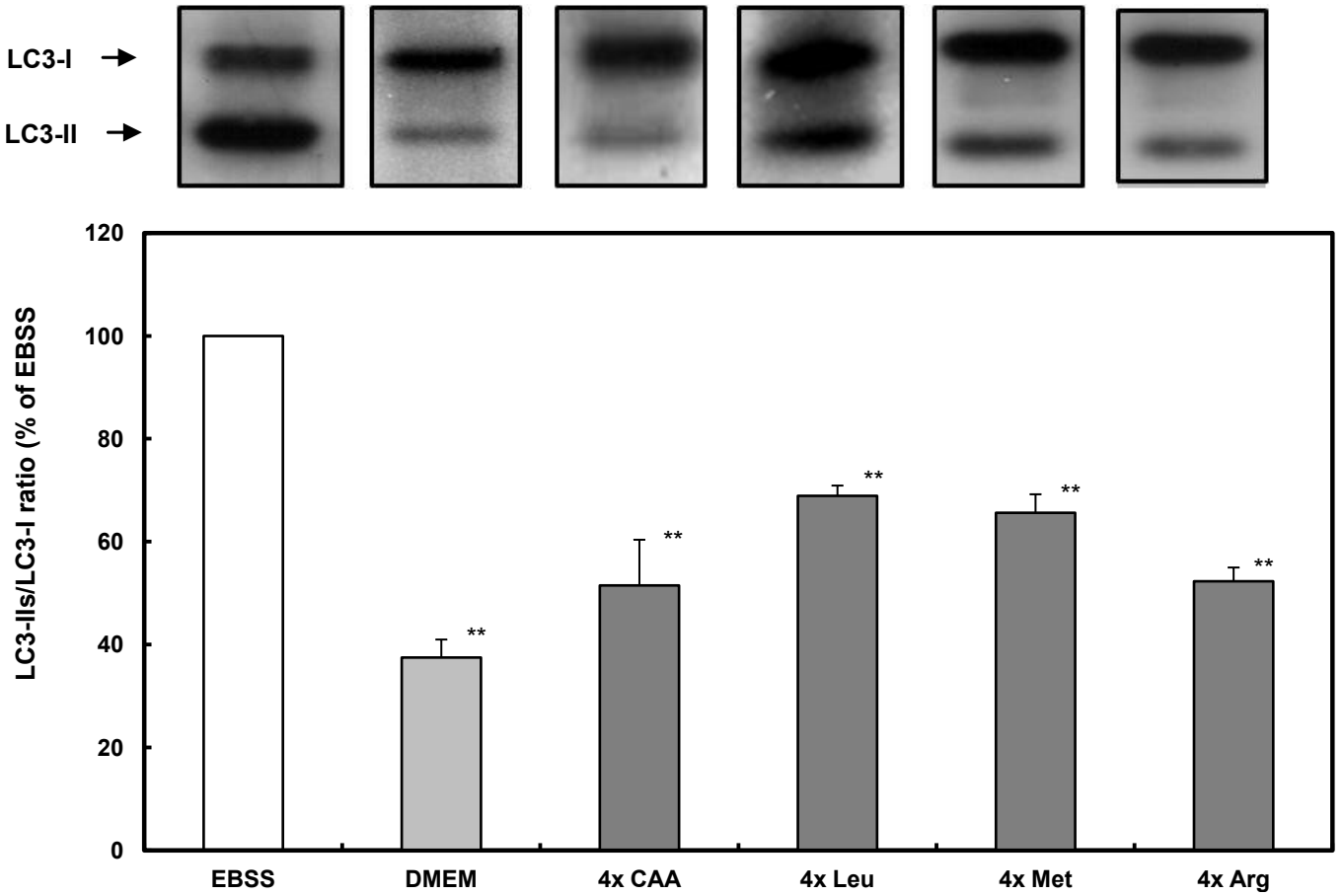

Fig. 3-4. Effects of Leu, Met, and Arg on autopahgy. H4-II-E cells were incubated in DMEM or starved in EBSS. Starved cells were treated with 4-fold CAA, 4-fold Leu, 4-fold Met, and 4-fold Arg for 1 hour, then homogenized. LC3 bands in the 100,000 x g supernatant (cytosol) fraction were analyzed and visualized by SDS-PAGE and western blotting. The cytosolic LC3 ratio (LC3-IIs/I) was calculated by densitometry. Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

preliminary ROS production study on citrulline and ornithine, two of the central substrates in urea cycle like arginine, showed the same stimulation of ROS production. Furthermore, our result also showed 58% and 39% increase in ROS production exhibited by Cys and Glu, respectively; however they significantly suppressed autophagy (Angcajas *et al.*, 2014). Taken together, the lack of correlation between ROS production and autophagy regulation were evident in some amino acids. Leu can be regarded as a regulatory amino acid in H4-II-E cells with ROS actively involved in its signaling pathway. Furthermore, it exhibited an antioxidant property that may be directly involved in autophagy regulation. In contrast, Met, Pro, Arg, Cys, and Glu can be classified as a regulatory amino acid with a signaling pathway that may not possibly involved ROS.

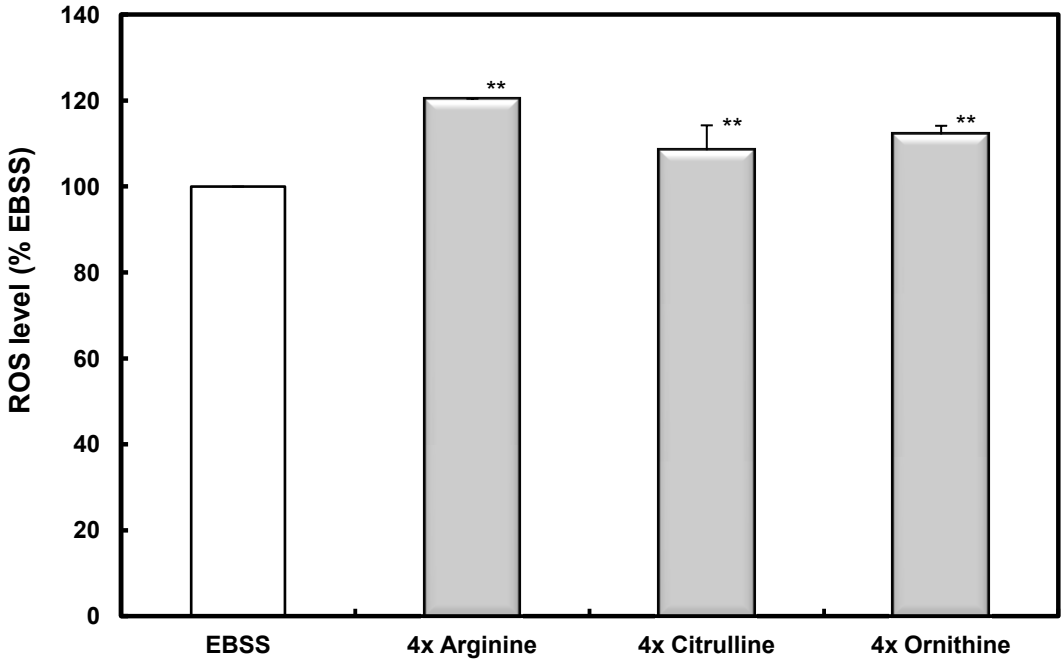

Fig. 3-5. Effects arginine, citrulline, and ornithine on ROS production. H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 µM DCFDA at 37^{o}C for 15 min, washed and starved by changing the medium to EBSS and treated with with 4-fold Arg, 4-fold citrulline, and 4-fold ornithine. Cells were subsequently analyzed with a fluorometer for 1hr. **(**Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

3.4.4 Concluding remarks

In conclusion, we have demonstrated the effect of different types of amino acids on the ROS production and autophagy regulation in H4-II-E cells. Based upon our data, it is clearly conceivable that amino acids display diverse responses on ROS production that does not necessarily coincide with its response on autophagy. Among other individual amino acids, Leu fits perfectly the scheme of ROS modulation on autophagy regulation. Additionally, our studies provide experimental evidence that ROS may have a key role in mediating autophagy regulation by some amino acid.

Chapter 4

Inhibitor study on ROS production of CAA, Leu, and Arg

4.1 Abstract

Autophagy is a ubiquitous degradation process, induced by amino acid starvation; it delivers cytoplasmic components to lysosomes. Amino acids are known as potent modulators of autophagy. In this study, the implication of reactive oxygen species (ROS) in the signaling pathway of amino acids in relation to mTOR and PI3K were investigated using rapamycin and wortmannin as inhibitors. In H4-II-E cells, rapamycin showed no inhibitory effect on starvation-induced ROS production while wortmannin blocked the ROS stimulation, suggesting involvement of PI3K pathway. Furthermore, the result obtained after addition of wortmannin suggests that PI3K pathway is involved in the ROS production by CAA and Leu. Finally, Arg exhibited a partial mTORC1 pathway dependence on its ROS production. However, it does not necessarily imply the same observation for its autophagy regulation.

4.2 Introduction

Numerous factors and signaling pathways have been shown to contribute to autophagy induction in different cellular contexts. Reactive oxygen species (ROS) are indicated to be essential signals to activate autophagy by a variety of stimulating conditions (Scherz-Shouval *et al.*, 2007). In Chapter 2 we identified ROS, probably in the form of H_2O_2, to be a good signaling molecule in H4-II-E cells.

Nutrient sensing is critical for cellular homeostasis and it is underpinned by complex signaling pathways. A key regulator of autophagy is TOR (target of rapamycin), a serine/threonine protein kinase that is inhibited by deprivation of nutrients or growth factors from extracellular milieu (Kamada *et al.*, 2004). Mammalian TOR (mTOR) directly phosphorylates ULK1 and inhibits ULK1 kinase activity, which is essential for autophagy induction (Mizushima, 2010). Thus, mTOR is a central negative regulator of autophagy. The understanding of the biological functions of mTOR was greatly facilitated by the use of rapamycin, an mTORC1 allosteric inhibitor which blocks mTORC1 activity towards some targets while sparing others (Choo *et al.*, 2008; Thoreen *et al.*, 2009). The mTORC1 components complex (containing mTOR, Raptor, PRAS40) phosphorylates ribosomal protein S6 kinase (S6K) at Thr389 and the translation repressor 4EBP1 and is rapamycin sensitive (Guertin and Sabatini, 2005; Sarbassov *et al.*, 2005; Wullschengler *et al.*, 2006). It has been shown that rapamycin inhibits mTOR by destabilizing the mTOR-Raptor complex (Kim *et al.*, 2002). Following the discovery of mTORC1, it was observed that withdrawal of amino acids from the media potently suppressed mTORC1 signaling in mammalian cells; moreover, suppressing mTORC1 by starvation strongly induced autophagy (Kroemer *et al.*, 2010). On the other hand, the autophagy specific Class III-PI3K complex appears to be essential to recruit the Atg12-Atg5 conjugates to the pre-autophagosomal structure (Suzuki *et al.*, 2001). Atg12-Atg5 conjugation is required for the elongation of the isolation membrane and for the proper localization of conjugated LC3/Atg8, the autophagosome gene that is substantially

induced under starvation conditions (Mizushima *et al.*, 2001; Ohsumi, 2001). Most importantly, Class III-PI3K functions with Beclin/Atg6 as a core complex of the autophagic system making them a central for the initiation of sequestration (Suzuki *et al.*, 2001). However, the regulation of this pathway by nutrient availability is still obscure.

In the previous chapter, we showed that mixture and individual amino acids have varied effects on ROS production that did not always fit on its autophagy regulation. In this chapter, we attempted to show the involvement of ROS in the two known signaling pathway, mTOR and Class III-PI3K/Beclin1, in autophagy regulation by amino acids. The inhibitors, rapamycin and wortmannin were used to examine the mTOR and Class III-PI3K/Beclin1 pathway, respectively.

4.3 Materials and Methods

4.3.1 Reagents

Rapamycin and Wortmannin were purchased from Wako Pure Chemical Industries (Osaka, Japan). The other reagents were specified in Materials and Methods section of Chapter 2 and 3. All the chemicals were in analytical grade unless otherwise indicated.

4.3.2 Cell cultures

The H4-II-E rat hepatoma cells were prepared as mentioned in **Chapter 2.3.2**.

4.3.3 ROS measurements

ROS measurements were performed as indicated in **Chapter 3.3.3**

4.3.3 Inhibitor study for ROS production

H4-II-E cells were maintained with DMEM and autophagy was induced by replacing the medium with EBSS, and is suppressed by adding CAA, Leu, or Arg to EBSS. Individual inhibitors, rapamycin (100 nM) and wortmannin (100 nM) were added to the medium to examine each signaling pathway.

4.3.3 Statistical analysis

All the data are expressed as mean ± SEM. Student's *t*-tests were used to evaluate statistical significance. *P* values more than 0.05 were considered not significant.

4.4 Results and Discussion

4.4.1 Starvation-induced ROS formation is modulated by Class III PI3K

In an attempt to understand where the ROS production fits in the amino acid signaling in autophagy, we monitored the effect of rapamycin and wortmannin on starvation-dependent ROS production. Starvation of cells for 1 hour in the presence of either inhibitor affected the ROS level in the cell **(Fig. 4-1)**. Our result showed that starvation promotes the increase in ROS production. Treatment with rapamycin maintains the high level of ROS, mimicking the effect of EBSS. Rapamycin did not block the effect of starvation on ROS production in the cell. In contrast, the addition of wortmannin significantly decreased the ROS level in the cell up to 30%. The contrasting effects exhibited by the two inhibitors gave us an idea on the involvement of ROS on known signaling pathways in the cell that directly affected autophagy regulation.

Rapamycin is higly selective towards mTORC1 and it blocks phosphorylation of some mTORC1 targets (Efeyan *et al.*, 2012). ULK1, one of the downstream targets of mTORC1, is dephosphorylated upon rapamycin treatment (Jung *et al.*, 2009). Dephosphorylation of ULK1 promotes autophagosome formation resulting to stimulation of autophagy. Similarly, in the event of nutrient starvation, such as amino acid depletion, mTORC1 was in inactive form inducing autophagy. In line with our result, both rapamycin and starvation showed similar stimulation in ROS production. No inhibitory effect on starvation was observed from rapamycin. In fact, addition of the same dose of rapamycin showed no effect on autophagy (Angcajas *et al.*, 2014). Collectively, these observations demonstrated the lack of involvement of mTOR pathway in both ROS production and autophagy stimulation during starvation.

In contrast, wortmannin is an inhibitor of PI3K. One type of PI3K, the Class III PI3K, plays an important role in the early stage of autophagosome formation through complex formation with Beclin1. Inhibition of this stage results to inhibition of autophagic process (Petiot *et al.*, 2000). Our result in **Fig. 4-1** showed that wortmannin significantly abolished the increase of ROS production generated by nutrient deprivation. Scherz-Shouval *et al.*, demonstrated that both wortmannin and 3-MA (another PI3K inhibitor), significantly reduced the autophagic activity of HeLa cells. Taken together, these demonstrate that ROS production is partially PI3K dependent. Proposed model based on our results is illustrated in **Fig. 4-2**.

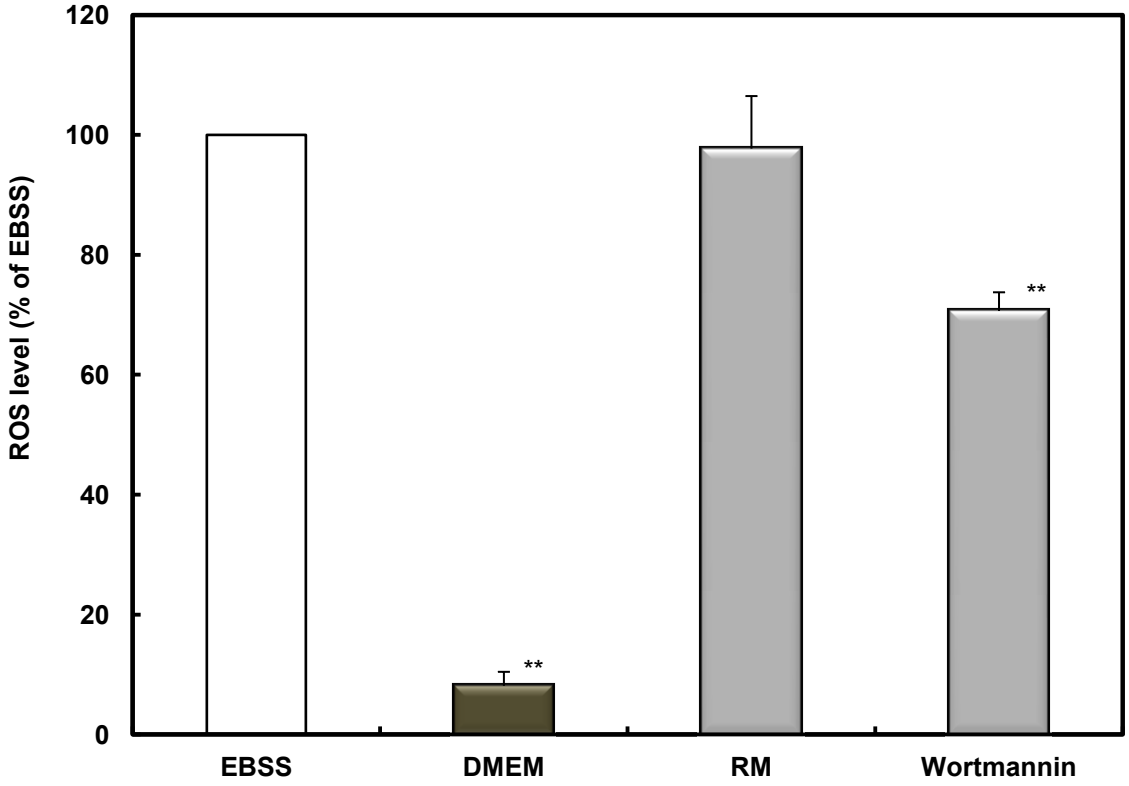

Fig. 4-1. Effect of rapamycin and wortmannin on ROS formation during starvation. (A) H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 μM DCFDA at 37^{o}C for 15 min, washed and starved by changing the medium to EBSS and treated with 100 nM rapamycin and 100 nM wortmannin. Cells were subsequently analyzed with a fluorometer for 1 hour. Data are means ± SEM (n=3). *$p < 0.05$, **$p < 0.01$ vs EBSS.

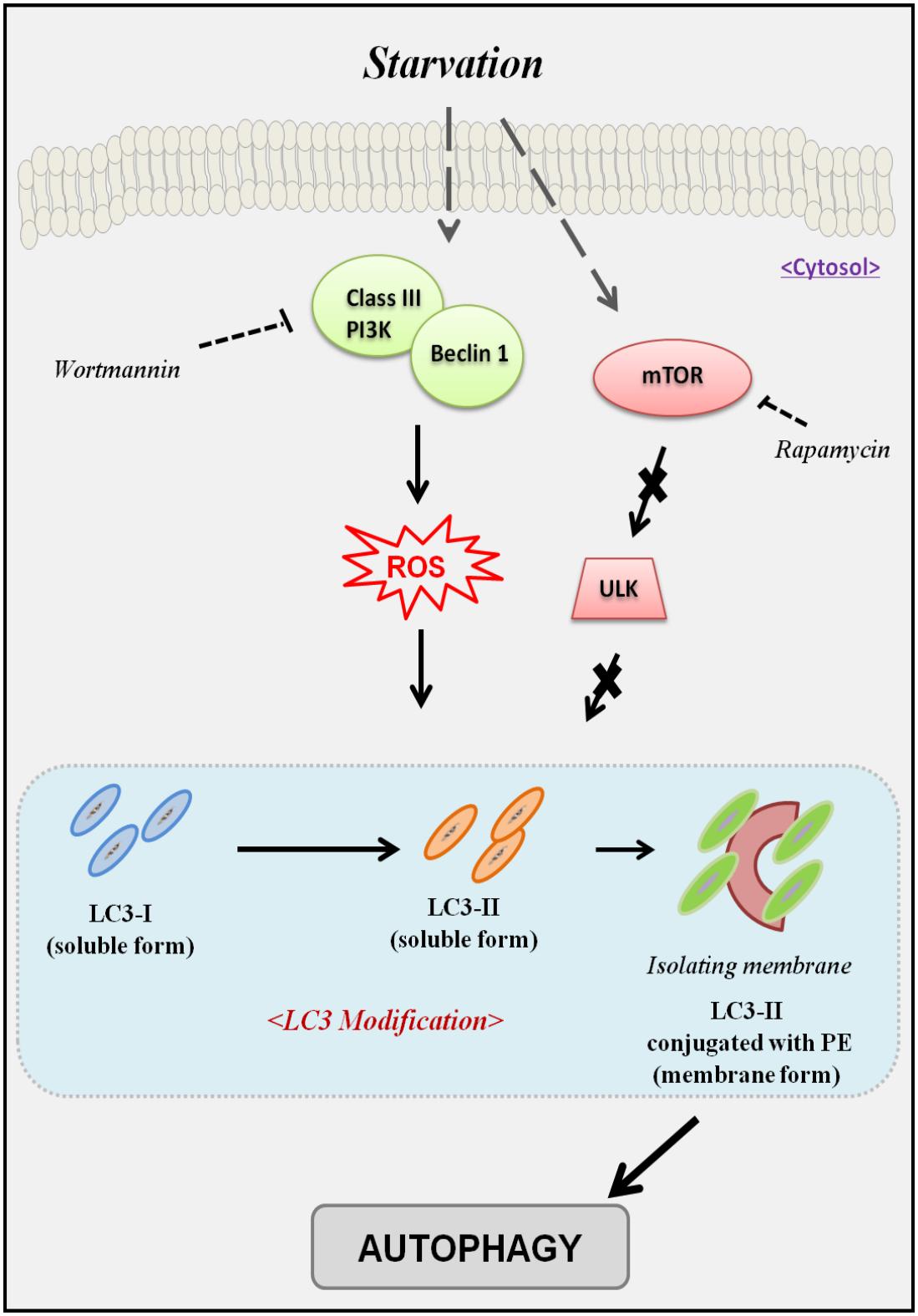

Fig. 4-2. Proposed mechanism of ROS production and autophagy stimulation by starvation.

4.4.2 ROS production does not affect downstream of mTOR in the presence of CAA and Leu

In most studies, mTOR was implicated in distinct signaling pathways for the regulation in many cellular processes. Amino acids were believed to regulate mTOR and its downstream target. However, this regulation has been subjected to intense studies, and was recently shown to be mediated by the tuberous sclerosis (TSC) complex (Gao *et al.*, 2002).

We have already illustrated from previous chapters the effect of amino acids on ROS production. However, that position of ROS in the signaling pathway of amino acids is yet to be elaborated intensively. Here we attempted to trace the possible site of ROS action in the signaling pathway of CAA and Leu. Thus, a ROS production measurement was performed on CAA and CAA treated with rapamycin (**Fig. 4-3**). We already pointed out earlier that Leu is a perfect amino acid that demonstrated suppression of autophagy and ROS. Hence, we examined the effect of Leu treated with rapamycin on ROS production (**Fig. 4-4**).

Our results showed that both CAA and Leu suppressed ROS production in H4-II-E cells. However, upon treatment with rapamycin (CAA + RM or Leu + RM), the suppressive property of the two amino acids diminished. It appears that rapamycin totally blocked the activity of CAA and Leu on ROS production. A pioneering study of Blommart *et al.*, emphasized that autophagy inhibition by amino acid is mediated by signaling pathway via phosphorylation of ribosomal protein S6 in isolated hepatocytes. This reaction directly targets mTOR (Isotani *et al.*, 1999) and this finding was supported by inhibitor study using rapamycin (Ueno *et al.*, 1999). Based on this assertion, it can be expected that ROS involvement would likely exist upstream of mTOR and will eventually lead to autophagy regulation. Additionally, the presence of amino acids activate Rag GTPases and eventually turns on the kinase activity of mTOR resulting to phosphorylation of ULK 1, a downstream target of mTOR, and eventually blocking the autophagosome formation.

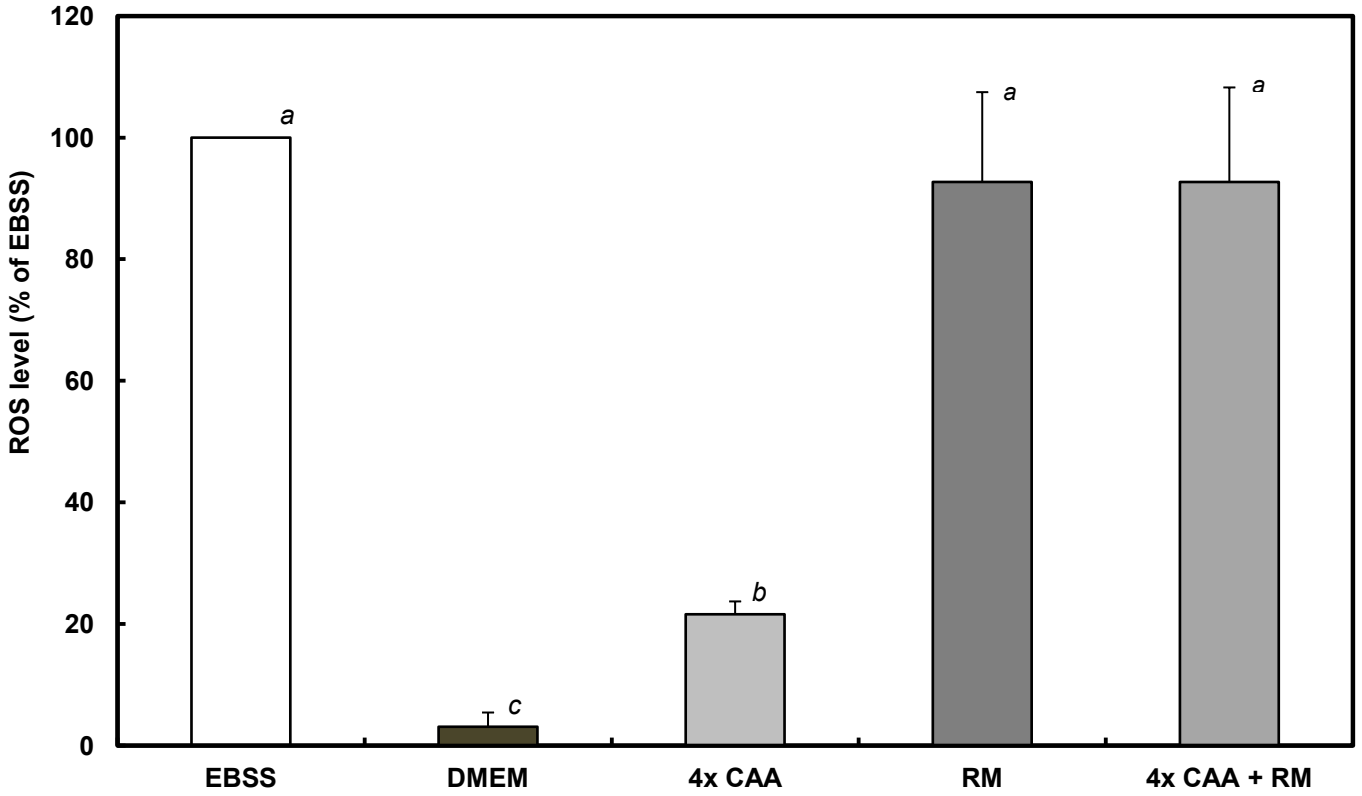

Fig. 4-3. Effect of rapamycin and CAA on ROS formation. H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 µM DCFDA at 37^{o}C for 15 min, washed and starved by changing the medium to EBSS and treated with 4-fold CAA, 100 nM rapamycin, and 4-fold CAA with rapamycin. Cells were subsequently analyzed with a fluorimeter for 1 hour. Data are means ± SEM (n=3). a, b, c significantly different $p < 0.01$.

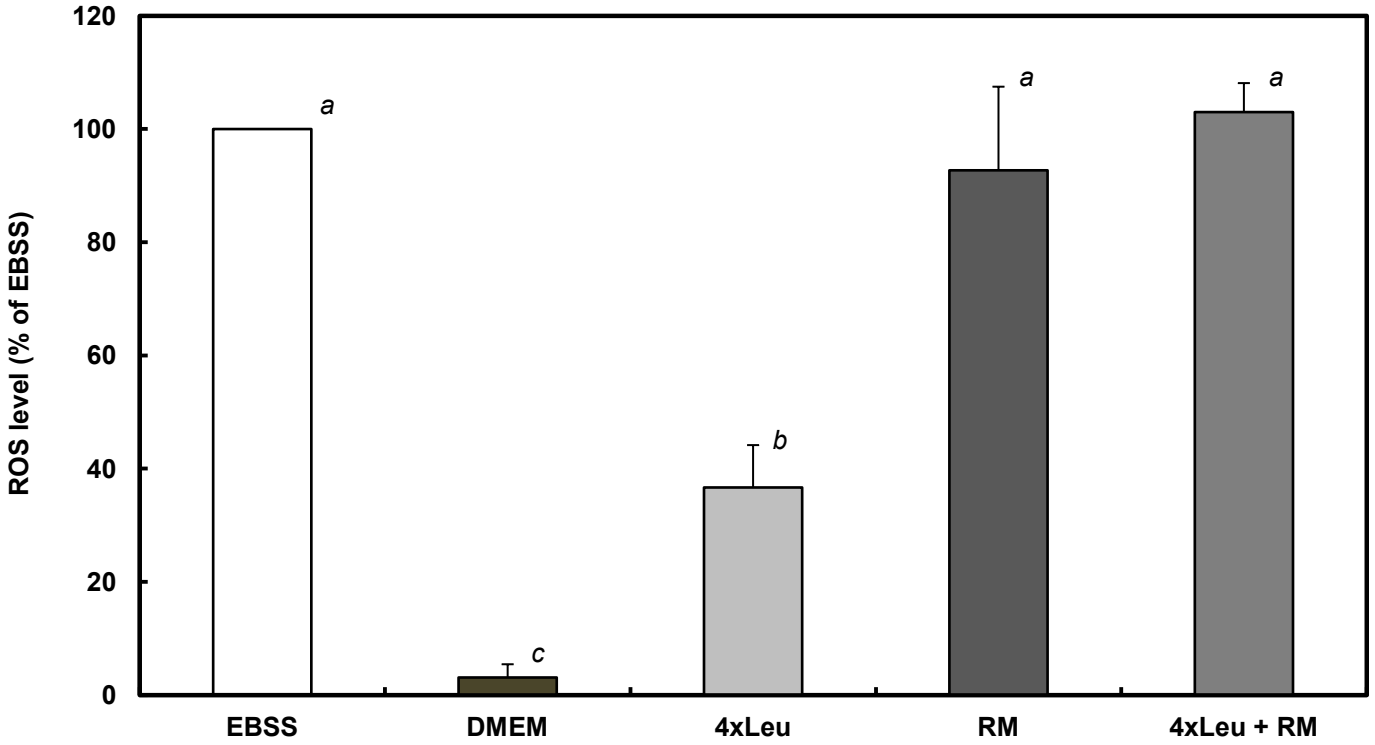

Fig. 4-4. Effect of rapamycin and Leu on ROS formation. H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 μM DCFDA at 37^{o}C for 15 min, washed and starved by changing the medium to EBSS and treated with 4-fold Leu, 100 nM rapamycin, and 4-fold Leu with rapamycin. Cells were subsequently analyzed with a fluorimeter for 1 hour. Data are means ± SEM (n=3). a, b, c significantly different $p < 0.01$.

(Efeyan *et al.*, 2012). In our findings, the disappearance of the suppressive effect of CAA and Leu upon rapamycin addition may indicate that ROS is involved downstream of mTOR. However, no previous studies have elaborated a direct ROS mediation in amino acid signaling in the downstream of mTOR. Hence, our findings here suggested the possibility of ROS to be not directly involved in the downstream of mTOR.

4.4.3 PI3K pathway affected by ROS production in the presence of CAA and Leu

The possible lack of direct involvement of ROS in the downstream of mTOR led us to investigate other pathway that is known to be regulated in the presence of nutrients like amino acids. In this experiment, we consider the effect of ROS in the PI3K pathway. We used the PI3K-specific inhibitor, wortmannin in this study.

Our result in **Fig. 4-5** showed that ROS production was suppressed upon addition of 4-fold CAA on wortmannin against EBSS. The same finding was obtained when 4-fold Leu with wortmannin was added **(Fig. 4-6).** The slight difference in ROS production between CAA and CAA combined with wortmannin as well as Leu and Leu combined with wortmannin may suggest involvement of PI3K in the ROS production by CAA and Leu.

The two PI3K that are known to be involved in autophagy are the Class I PI3K and the Class III PI3K in an insulin controlled autophagy, Class I PI3K/Akt is being activated upstream of mTOR. Activation of PI3K results in activation of PDK1, Akt, and mTOR, which is negatively regulated by phosphatase and tensin homolog (PTEN) (Chen *et al.*, 2010). Although not formally demonstrated, ROS-induced inactivation of PTEN can result to increased mTORC1 activity via PI3K/Akt pathway, leading to suppression of autophagy (Kongara *et al.*, 20102). On the other hand, Class III PI3K complex is involved in pre-autophagosomal structure formation and is outside the mTOR pathway. Additionally, in **Fig. 4-1**, we showed that ROS production is partially Class III PI3K dependent. This is an added evidence of involvement of ROS in PI3K pathway.

Based on this premise, our findings suggested that ROS production can modulate CAA and Leu signaling in autophagy by affecting the activity of both Class I and Class III PI3K. Affecting Class I PI3K would mean that CAA and Leu have the same pathway as insulin (insulin/PI3K/Akt) pathway. However, for a long time, amino acid regulation of mTORC1 remained on the factual observation that amino acids acted independently of insulin and TSC, and thus appeared to be distinct from insulin/PI3K pathway (Long *et al.*, 2005; Hara *et al.*, 1998; Wang *et al.*, 1998). Applying this assertion on our results, we can presume that Class I PI3K might not be actively involved in the ROS modulation of CAA and Leu regulated autophagy. These observations are also in agreement with previous studies performed in different cell lines, which showed that amino acids rarely affect Class I PI3K and Akt (vanSluitjers *et al.*, 2000).

We mentioned earlier that amino acid deprivation induces the formation of H_2O_2 in a Class III PI3K-dependent manner and this ROS can be essential for the induction of autophagy in response to starvation. Our result in **Fig. 4-5 and Fig. 4-6** gave us the reverse observation. The result showed that the presence of amino acid suppresses the formation of H_2O_2 through Class III PI3K pathway and this effect of ROS plays an important role in the suppression of autophagy by amino acid. The involvement of Class III PI3K in amino acid signaling has been examined by earlier studies. Corroborative evidence showed that Class III PI3K-Beclin1 complex plays a preponderant role in autophagic response of muscle cells to amino acid availability (Tassa *et al.*, 2003). Another study showed that a major pathway by which amino

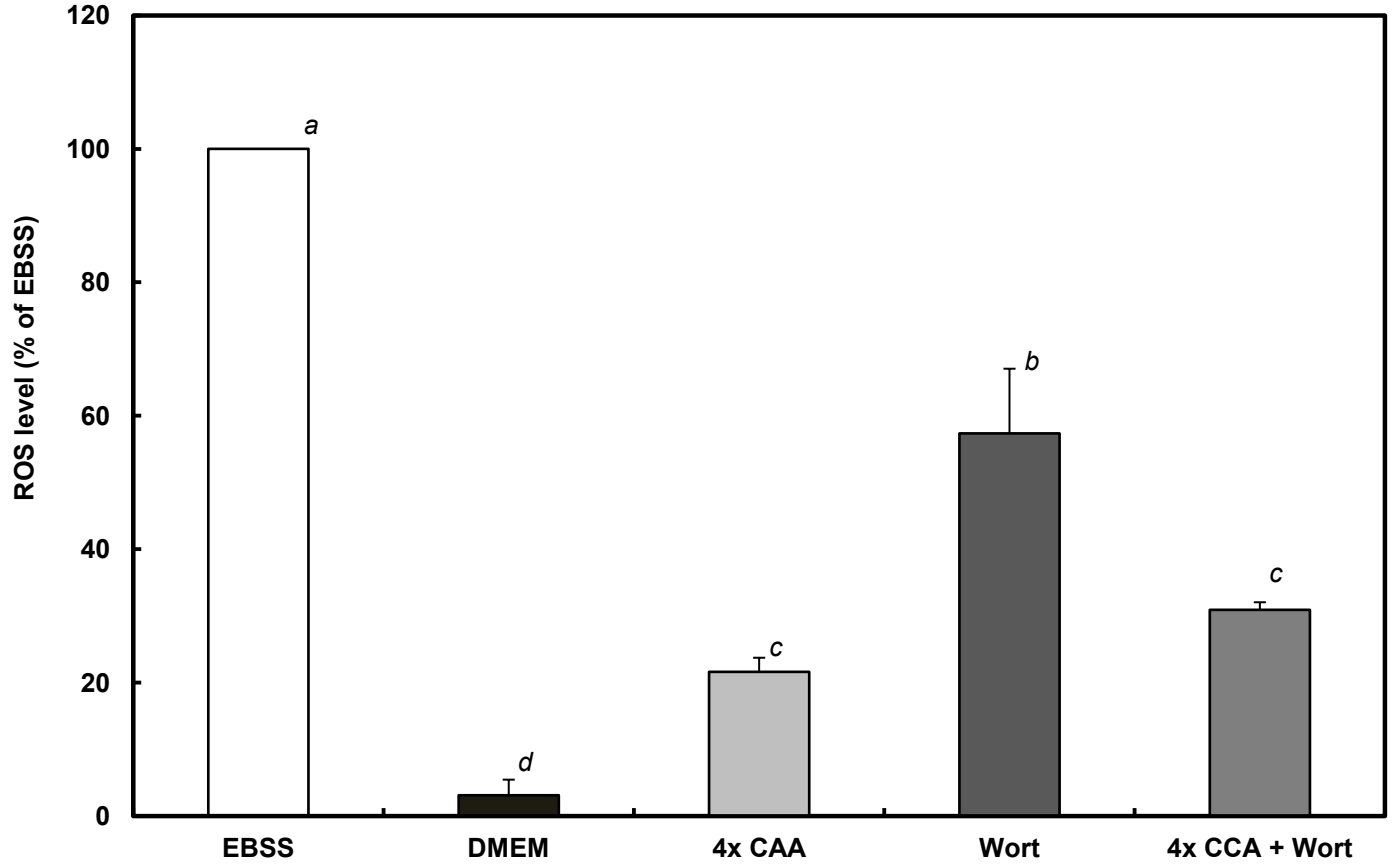

Fig. 4- 5. Effect of wortmannin and CAA on ROS formation. H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 µM DCFDA at 37°C for 15 min, washed and starved by changing the medium to EBSS and treated with 4-fold CAA, 100 nM wortmannin, and 4-fold CAA with wortmannin. Cells were subsequently analyzed with a fluorimeter for 1 hour. Data are means ± SEM (n=3). a, b, c, d significantly different $p < 0.01$.

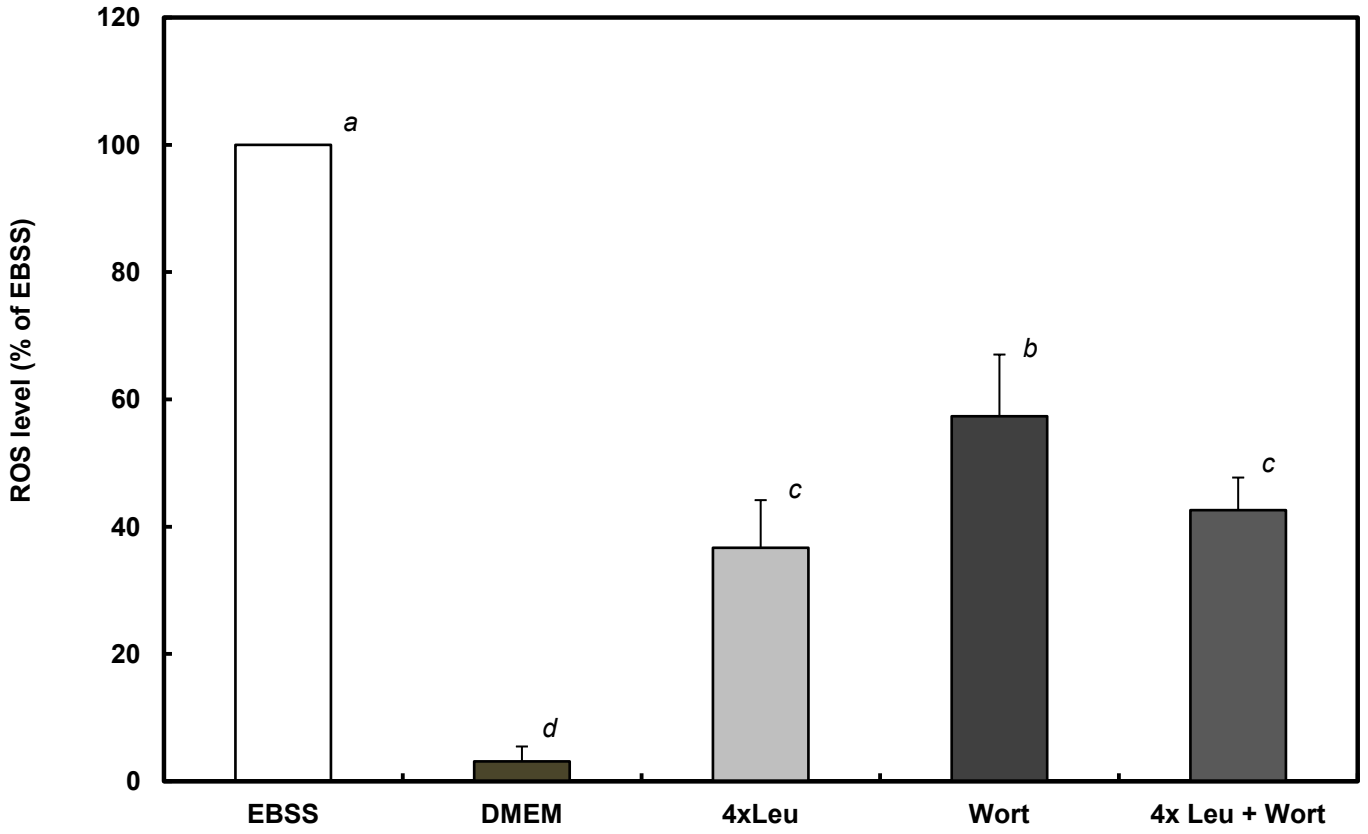

Fig. 4-6. Effect of wortmannin and Leu on ROS formation. H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 µM DCFDA at 37^{o}C for 15 min, washed and starved by changing the medium to EBSS and treated with 4-fold Leu, 100 nM wortmannin, and 4-fold Leu with wortmannin. Cells were subsequently analyzed with a fluorimeter for 1 hour. Data are means ± SEM (n=3). a, b, c, d significantly different $p < 0.01$.

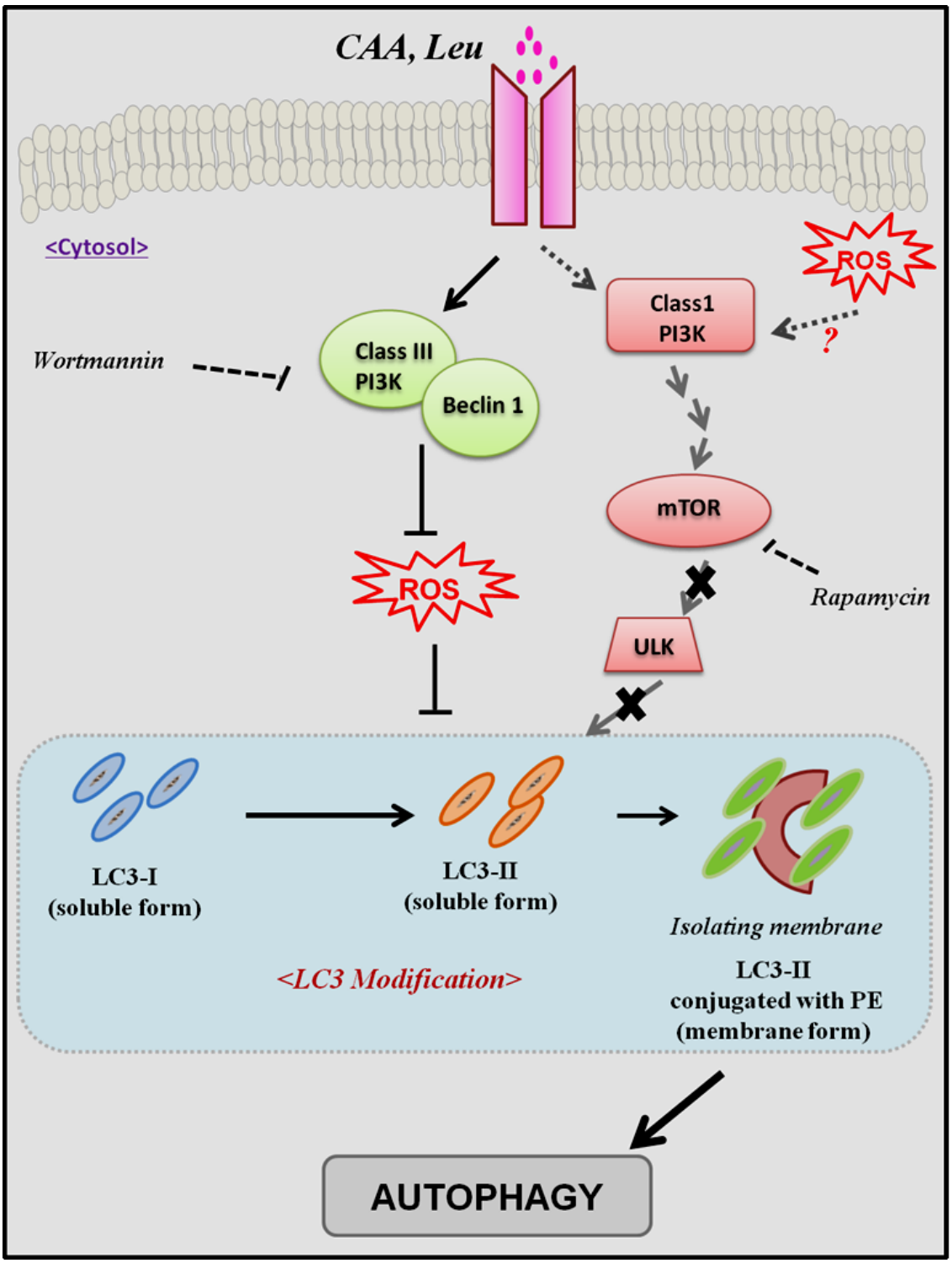

Fig 4-7. Proposed mechanism of ROS production and autophagy regulation by CAA and Leu.

acids control mTOR signaling is distinct from that of insulin and, instead of signaling through components of the insulin/Class I PI3K pathway, amino acids mediate mTOR activation by signaling through Class III PI3K, hVps34 (Nobukuni *et al.*, 2005). In line with this, we proposed that ROS mediate amino acid signaling on autophagy through the Class III PI3K.

4.4.4 Lack of involvement of PI3K in the ROS production by Arg

The ROS production of individual nonregulatory amino acids showed that arginine has distinct result among the group. Arg showed 20% increase in ROS production and 48% suppression of autophagy **(Table 3-1; Fig. 3-4)**. Because of this observation, we attempted to examine the involvement of ROS in the mTOR and PI3K pathway. We assumed that Arg regulated autophagy would follow the same possible pathway as Leu and CAA since they similarly showed autophagy suppression **(Fig. 3-4)**.

The starved H4-II-E cells were treated with 4-fold Arg or 4-fold Arg with 100 nM rapamycin or 100 nM wortmannin. In **Fig. 4-8**, we demonstrated 19% ROS level increase in the presence of Arg. Rapamycin showed almost the same ROS level as EBSS. Most importantly, the addition of Arg with 100 nM rapamycin showed no effect in ROS production with respect to EBSS, however, it slightly blocked the stimulative effect of Arg on ROS production. In contrast, the addition of Arg with 100 nM wortmannin retained the stimulatory effect of Arg on ROS production **(Fig. 4-9)**. This result implied that wortmannin was not able to block the effect of Arg in starved cell. Taken together, these findings suggested that Arg may be partially mTORC1 pathway dependent.

Upstream of mTORC1 is AMPK, a heterotrimeric enzyme. AMPK is a mediator of indirect activation of autophagy by ROS, as it is sensitive to oxidative stress induced by H_2O_2 and gets phosphorylated at Thr 172 of its α1 catalytic subunit (Choi *et al.*, 2001). Hence, ROS promotes phosphorylation of AMPK and stimulation of autophagy. Since we suggested

earlier that Arg may be mTOR pathway dependent, it may be possible that ROS modulation targets AMPK, however, our result showed autophagy suppression by Arg instead of stimulation. Another mediator of autophagy in response to ROS and oxidative stress is the c-Jun N-terminal kinase (JNK; Wu *et al.*, 2009; Wong *et al.*, 2010). ROS activates JNK1 leading to stimulation of autophagy (Kongara et al., 2012). In view of possible signaling pathway for Arg, a recent study by Sarkar *et al.*, 2011, showed that nitric oxide (NO), synthesized from L-Arg by NO synthases, negatively regulates autophagy via the JNK1-Bcl-2-Beclin1 and the IKK-AMPK-TSC2-mTOR pathways. In this relation, recent evidence, through inhibitor studies (AG and L-NMMA), showed that in H4-II-E cells, autophagy regulation by Arg is NO pathway dependent (Angcajas *et al.*, 2014). Therefore from the above arguments, we suggested that ROS production effect of Arg in our result may not be associated to its autophagy regulation. Our result hinted that the pathway for ROS production by Arg may not necessarily be the same to the signaling mechanism of its autophagy regulation. Our preliminary result also suggested that ROS may not be the main signaling molecule for its autophagy regulation. Our recent findings (Angcajas *et al.*, 2014), hinted a strong possibility of NO involvement in the autophagy regulation by arginine.

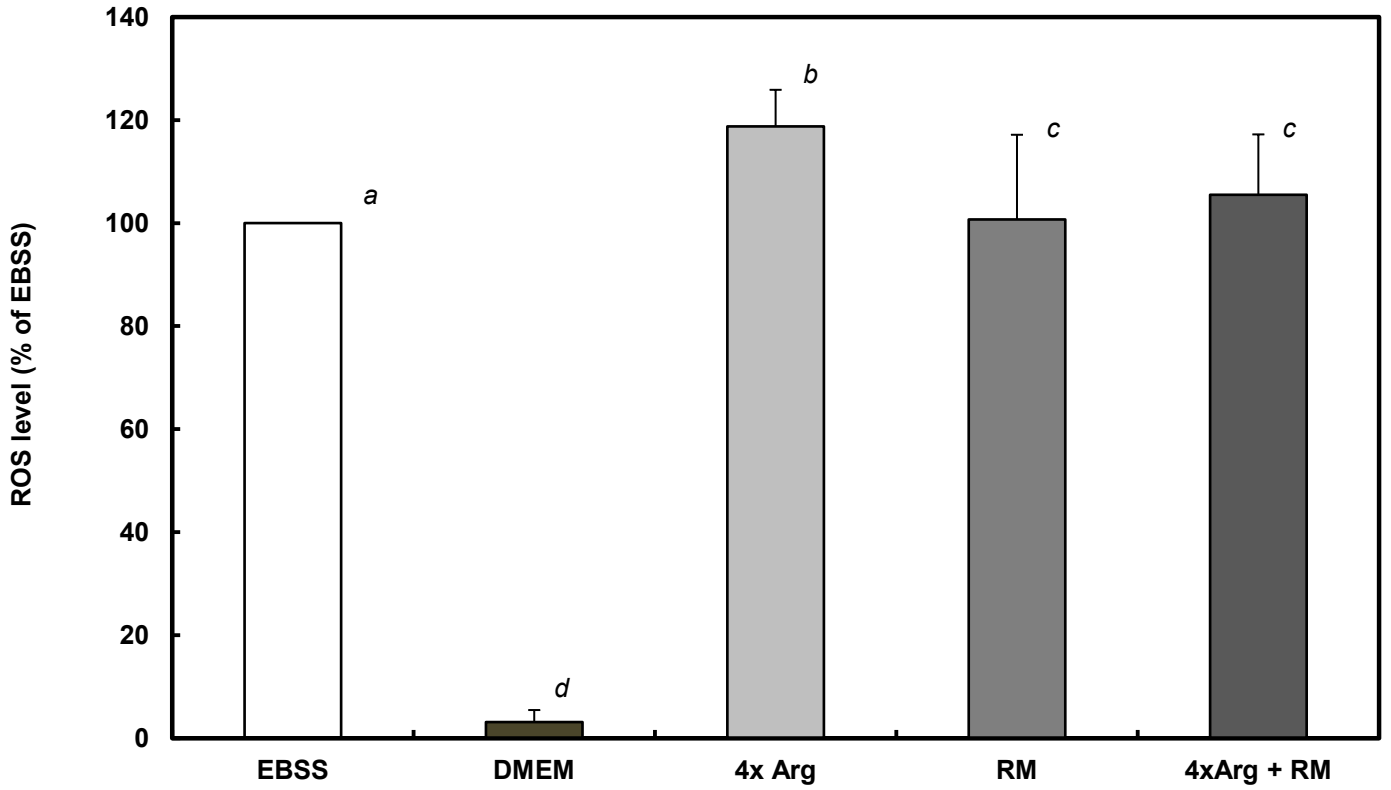

Fig. 4-8. Effect of rapamycin and Arg on ROS formation. H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 µM DCFDA at 37°C for 15 min, washed and starved by changing the medium to EBSS and treated with 4-fold Arg, 100 nM rapamycin, and 4-fold Arg with rapamycin. Cells were subsequently analyzed with a fluorometer for 1 hour. Data are means ± SEM (n=3). a, b, c, d significantly different $p < 0.01$.

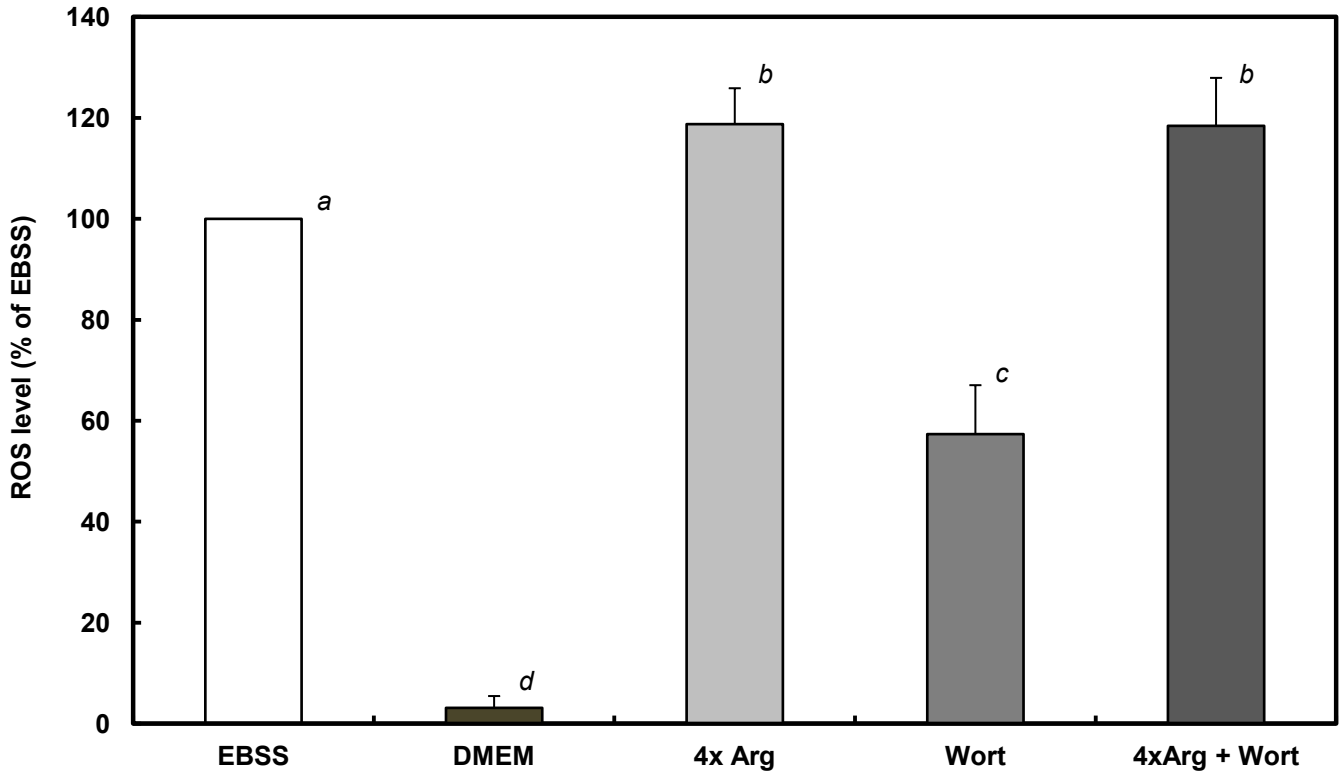

Fig. 4-9. Effect of wortmannin and Arg on ROS formation. H4-II-E cells were grown in control medium (DMEM), seeded in 6-well plates, and treated with 30 μM DCFDA at 37^{o}C for 15 min, washed and starved by changing the medium to EBSS and treated with 4-fold Arg, 100 nM wortmannin, and 4-fold Arg with wortmannin. Cells were subsequently analyzed with a fluorometer for 1 hour. Data are means ± SEM (n=3). a, b, c, d significantly different $p < 0.01$.

4.4.5 Concluding remarks

In conclusion, it has been considered for a long time that the signaling mechanism by amino acids in autophagy is mediated via mTOR pathway. Our data in this study demonstrate that ROS modulates some amino acid signaling. The effect of inhibitors on ROS production by amino acids showed that signaling pathway of amino acids may be distinct from each other. On the other hand, our result from Arg suggested that ROS may not always mediate autophagy regulation.

Chapter 5

General Discussion

In our present study, we attempted to deduce the involvement of ROS in the signaling mechanism of amino acids on autophagy regulation. To obtain results we: (A) tested and validated a method for ROS production measurement that fits to be used in our system; (B) examined the ROS profile and autophagy response of mixtures and individual amino acids and (C) performed inhibitor on ROS production by amino acids.

In **Chapter 2**, the method to be used to obtain the main results of our study was established. We used the fluorescent probe DCFDA to examine the ROS level resulting from different nutrient condition of the cell. DCFDA has high specificity to H_2O_2. Hence the DCF reading obtained in spectrofluorimeter can be considered as the level of H_2O_2 in the cell. It was shown that during starvation, the ROS level of the cell increases over time as compared to nutrient-rich condition. The addition of exogenous H_2O_2 increases the intracellular ROS level proportional to H_2O_2 concentration. We validated the method by NAC treatment. We observed that the method was sensitive to changes in H_2O_2 depending on the nutrient available. Hence, we used the same method in our study of amino acids.

In Chapter 3, we used the method validated above to examine the ROS production of different amino acids. The ROS response profile of each amino acid was used to evaluate the signaling mechanism of autophagy regulation. In addition, the autophagy regulation of selected amino acids (CAA, Leu, Met, Arg), all in 4-fold concentration, were examined using the cytosolic LC3 ratio (LC3-IIs/LC3-I), a sensitive and quantitative index of autophagic flux (Karim *et al.*, 2007). In this chapter, we showed that CAA, Leu, Met, and Arg suppressed autophagy against EBSS. The Leu and Met are both regulatory amino acid (RegAA) and their suppressive effect was expected. However, arginine is a nonregulatory amino acid (NonRegAA) but the autophagy regulation it exhibited does not fit for a NonRegAA. Next, we performed ROS study on the different amino acid mixtures CAA, RegAA, and NonRegAA mixtures individually. The result showed that all the three mixtures displayed

significant suppression of ROS production during stravation. Angcajas *et al.*, (2014) reported that CAA and RegAA mixture showed autophagy suppression. These results supported our hypothesis that autophagy regulation by these mixtures is possibly mediated by suppression of ROS production. The factual observation on ROS production by amino acid mixtures hinted that amino acid have antioxidant properties. Next, we extended our study on individual amino acids. The data showed that most individual RegAA caused a considerable decrease in ROS production, except for Met and Pro. This explained the ROS suppression exhibited by the mixture form of RegAA. Furthermore, individual NonRegAA exhibited increase or no change in ROS production as compared to EBSS. This result is unexpected since the mixture form of NonRegAA decreases the ROS production significantly. Another unanticipated result is the increase in ROS production exhibited by Arg. Altogether, the lack of correlation between ROS production and autophagy suppression was evident from our results.

Next, we checked the involvement of ROS in the signaling pathway of amino acid regulated autophagy. The mTOR pathway with ULK in the downstream is the most considered pathway in autophagy regulation. Class III PI3K /Beclin1 is another pathway known to be involve in autophagy regulation. In this study, we utilized rapamycin and wortmannin as specific inhibitor for mTORC1 and PI3K , respectively. Here in **Chapter 4**, it was showed that starvation–induced ROS production is partially Class III PI3K dependent. This was demonstrated clearly by suppression of ROS production upon addition of wortmannin in starved cell. Furthermore, inhibitor study on CAA and Leu showed that these two amino acids have the same response on ROS production upon treatment with rapamycin or wortmannin. The inhibitor study on amino acids showed that Class III PI3K/Beclin1 may be involved in the ROS modulated autophagy regulation by CAA and Leu. The slight difference in ROS production between CAA and CAA combined with wortmannin as well as Leu and Leu combined with wortmannin suggested a possible dependence in Class III

PI3K/Beclin1. The lack of inhibition from CAA or Leu added with rapamycin may indicate noninvolvement of mTORC1 in the regulatory activity of these amino acids. On the other hand, results obtained from inhibitor study on Arg, may suggest that its ROS production is partially mTORC1 dependent. This result, however, does not agree with the autophagy inhibition of Arg. If the ROS production is mTORC1 dependent this would lead to stimulation of autophagy. In contrast, Arg exhibited suppression of autophagy. This observation led us to conclude that the ROS production and autophagy regulation displayed by Arg may involved two different signaling pathways.

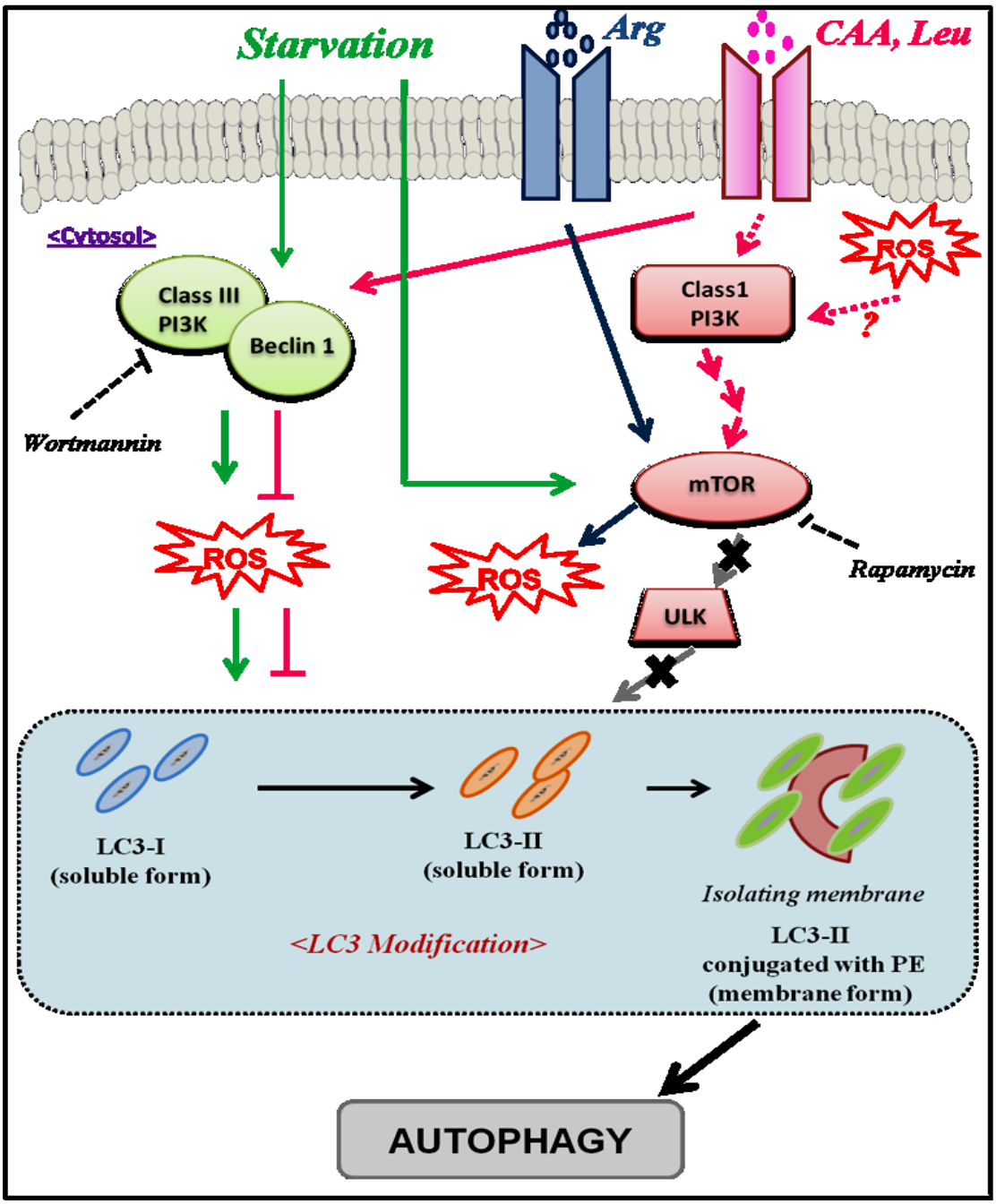

Fig. 5-1. Hypothetical illustration of ROS modulation on autophagy regulation by amino acids. Although further mechanistic study is needed, our results propose the above depiction of ROS involvement in autophagy regulation. During **starvation**, Class III PI3K-Beclin1 complex formation is induced resulting to stimulation of ROS release and induction of autophagy. Moreover, the lowering of ROS level on wortmannin with **CAA or Leu** is Class III PI3K dependent and resulted to suppression of autophagy. Lastly, ROS production of **Arg** is partially due to mTOR pathway activation, however, it did not relate to autophagy suppression displayed by Arg. Hence, autophagy regulation of Arg may have different pathway from its ROS production.

Summary

Autophagy, an intracellular bulk degradation process induced by starvation, is regulated by amino acids. However, signaling mechanism of amino acids is still obscured and many aspects are yet to be investigated. Studies showed that during starvation, intracellular reactive oxygen species (ROS) production increases. With this premise, ROS can be a good candidate signaling molecule for autophagy regulation by amino acids. Therefore, it is essential to have a method that is sensitive in measuring changes in ROS level intracellularly. For this reason, we employed the DCFDA method in measuring the intracellular ROS level of H4-II-E cell. We observed that starvation condition prompted the release of H_2O_2. Addition of exogenous H_2O_2 and antixodant NAC validated the method. Thus, it can be employed to examine ROS production of amino acids.

Next, to examine autophagy regulation, cytosolic LC3 ratio (LC3-IIs/LC3-I) was used. CAA mixture, Leu, Met, and Arg suppressed autophagy as exhibited by the decrease on their LC3 ratio. Using the validated method, the ROS generation of different amino acid mixtures, complete amino acid (CAA), regulatory amino acid (RegAA), and nonregulatory amino acid (NonRegaAA) mixtures were inspected. We observed that all the mentioned amino acid mixtures showed suppression of ROS production against EBSS. Interestingly, even NonRegAA showed significant suppression. We extended this study to individual amino acids. Most of the regulatory amino acids exhibited suppression of ROS production. Our observation showed that Leu fits well to our hypothesis on ROS signaling modulation on autophagy regulation. It suppressed both the ROS generation and autophagy. We expected this observation since Leu, being a RegAA, should have the property of inhibiting autophagy and this inhibition can be a consequence of the lowering of ROS level. Moreover, all individual NonRegAAs showed an increase in ROS production against EBSS. Another interesting finding that we observed is that Arg increases ROS production but it significantly suppressed autophagy. It is an unexpected result for a NonRegAA like Arg. However, this

could mean that Arg may have a signaling pathway in controlling autophagy that is unique from the rest.

On the basis of the results presented in Table 3-1 and Table 3-2, showing differing response on ROS production and autophagy regulation, We suspected that the signaling mechanism of individual amino acids may be diverse. Hence, we searched a possible signaling pathway that would fit and explain the ROS production and autophagy regulation exhibited by the amino acids CAA, Leu, and Arg. The mTORC1 pathway with ULK1 on its downstream is the most considered and studied conduit for autophagy regulation by nutrients, most especially amino acid. Another considered pathway is the Class III PI3K complexed with Beclin1, which played an important role in early autophagosome formation. Rapamycin, an mTOR inhibitor, and wortmannin, a PI3K inhibitor were used in the inhibitor study. We examined the effect of these inhibitors on the ROS production of the aforementioned amino acids. We observed that addition of rapamycin on starved cell resulted to rise in ROS level up to almost the same level as EBSS. This observation signifies lack of inhibitory effect of rapamycin on starvation. On the other hand, wortmannin addition suppresses ROS production, exhibiting its inhibitory effect. Furthermore, we also observed that rapamycin, when added with CAA or Leu, exhibited a ROS production almost similar to rapamycin alone. This effect may indicate that ROS production takes place downstream of mTORC1, probably affecting the ULK1. However, direct targeting of ROS to ULK1 has yet to be studied elaborately. Thus, we suggested that mTORC1 may not be directly involved in ROS modulation on autophagy regulation by CAA and Leu. Next, we examined the possibility of PI3K involvement, and found out that wortmannin with CAA or Leu showed inhibition of ROS production against EBSS. Possible partial involvement of PI3K was observed and previous researches have indicated the implication of Class III PI3K in amino acid signaling. Thus, in this study, we suggested that ROS modulation on CAA or Leu- regulated autophagy is Class III PI3K

dependent. Lastly, we observed that PI3K showed no involvement in Arg signaling. However, rapamycin addition with Arg resulted to suppression of ROS production, compared with Arg alone. This observation suggested that ROS production of Arg can be mTORC1 dependent. Choi *et al.*, (2001) earlier observed that AMPK, which is located upstream of mTORC1, indirectly activates autophagy by ROS. Hence, dependence of Arg ROS production on mTORC1 should result to stimulation of autophagy, but our result showed otherwise. Therefore, this observable fact suggested that ROS production by Arg is not directly involved on its autophagy regulation.

Concluding remarks:

Altogether, my present studies indicated that:

(1) Starvation results into increase in ROS production and stimulation of autophagy. Moreover, the inhibition study reflected that starvation is partially Class III PI3K dependent.

(2) CAA and RegAA mixtures significantly suppressed ROS production and autophagy. Likewise, individual amino acids have varied effects on ROS production and autophagy regulation.

(3) ROS modulation on CAA and Leu signaling was found to affect Class III PI3K, hinting that the ROS and autophagy regulation by CAA and Leu may actually be Class III PI3K dependent.

(4) Arginine stimulation on ROS production is mTORC1 dependent. However, its autophagy regulation is not. These effects insinuate that ROS production and autophagy suppression by arginine may involved pathways unique form each other.

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