Thesis for the Degree of Doctor of Philosophy

Induction of Endoplasmic Reticulum Stress, Autophagy and Apoptosis in Human Lung Adenocarcinoma A549 Cells by Anacardic Acid

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(A549 폐암 세포에서 Anacardic Acid에 의한 ER stress, Autophagy, Apoptosis 유도)

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List of Abbreviation

AA	Anacardic acid
AIF	Apoptosis-inducing factor
ATF6	Activating transcription factor-6
Atg	Autophagy-related genes
BiP/GRP78	Immunoglobulinheavy-chain-bindingproteinB/Glucose RegulatedProtein78)
CHOP	CCAAT/enhancer binding proteins (C/EBPs) homologous protein
DAPI	4',6-diamidino-2-phenylindole
DAPK	Death-associated protein kinase
ER	Endoplasmic reticulum
ERO-1	ER oxidoreductin-1
IRE1α	Inositol-requiring enzyme 1α
LC3	Microtubule-associated protein1 light chain 3
PARP	Poly (ADP-ribose) polymerase
PDI	Protein disulfide isomerase
PERK	Protein kinase RNA (PKR)-like ER kinase
TEM	Transmission electron microscopy
UPR	Unfolded protein response
Z-VAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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Abstract

Anacardic acid (2-hydroxy-6-pentadecylbenzoic acid, AA) is a constituent of the cashew-nut shell as well as several other plants including *Ginkgo biloba*. AA displays a variety of beneficial properties including its anti-microbial effects and capacity to treat cancer. In addition, AA has been used as a traditional medicine for treatment of gastric ulcers, gastritis, and stomach cancers in many localized regions of the world including Mexico. Although it is known that AA suppresses the expression of nuclear factor-κB-regulated genes, the molecular mechanism of cell death in AA-treated cancer cells is not clear.

This research was conducted to investigate AA's anti-cancer effect and to reveal the possible pathway by which it induces cell death. While AA exhibited cytotoxic activity in all cancer cells used in this research, the degree of cytotoxicity was different depending on the cell lines. Considering our interest in lung cancer, we used A549 cells (lung

adenocarcinoma derived from human alveolar basal epithelial cells) to reveal the molecular mechanism of cell death. Currently, the collective evidence indicates that AA induces UPR (unfolded protein response), endoplasmic reticulum stress, autophagy and apoptotic cell death. The results of Ca^{2+} mobility, transmission electron microscopy (TEM), Western blot, gene transfection, fluorescence activated cell sorting (FACS) and staining with 4', 6-diamidino-2-phenylindole (DAPI) showed clear evidence of the induction of the unfolded protein response, autophagy and apoptosis in AA-treated cells.

The efflux of Ca^{2+} from the ER to the cytosol, the increase of misfolded or unfolded proteins in the ER lumen by reduced p-PERK, the activation of ATF6 and the increase of IRE1α may activate the UPR. In addition to the increased expression of autophagy-related proteins to protect from ER stress, the gradual increase of Beclin-1 and DAPK3 by AA may contribute to the cause of death by inducing overactivated autophagy.

Especially, our results showed the possibility that apoptosis is a caspase-independent apoptotic pathway with no inhibition of cytotoxicity by pan-caspase inhibitor, Z-VAD-fmk and induced by two different pathways. One is a GADD153/CHOP-related pathway via UPR and another is a mitochondial-mediated apoptosis through the activation of an apoptosis-inducing factor (AIF) and intrinsic pathway executioner such as cytochrome c.

For the first time, this research showed that AA induces ER stress and autophagy in lung cancer cells. Additionally we suggest the

potential cell death signaling pathway in AA-treated A549 cells. This study will be helpful in revealing cell death mechanisms and in developing potential drugs for lung cancer using AA.

PART Ⅰ

General Introduction of Anacardic Acid and Cell Death Pathways

Chapter 1.

General Introduction

Cancer afflicts many people globally. Lung cancer particularly is one of the three most common cancers and one of the three leading causes of cancer death in men and in women and men in the United States, respectively. According to the 2009 annual report of the National Cancer Institute (NCI), the rates of new diagnoses and death from all cancers combined have declined significantly for men and women, but the incidence rate for lung cancer in women has increased (http://progressreport.cancer.gov).

Lung cancer is usually formed in the cells lining air passages. Cancers spread and grow differently depending on their type. Treatment is also different according to the stage and progression of cancer. Treatment may include surgery, chemotherapy, radiation therapy, immunotherapy and vaccine therapy. Traditional cancer therapies are mainly directed at enhancing cell apoptosis. However, it is well-established that many cancer cells are chemo-resistant and defective in apoptosis induction. Understanding the signaling pathways involved in carcinogenesis, tumor growth and metastasis may lead to the discovery or design of potential new molecules for targeted therapy.

This research was performed in order to investigate the anti-cancer effect and to reveal the possible cell death pathway induced by anacardic acid. Investigation of the cell death-signaling pathway could be helpful to treat cancer and to develop a useful drug for lung cancer.

1.1. Anacardic acid (AA)

Anacardic acid (2-hydroxy-6-pentadecylbenzoic acid, 6-pennta decyl -salysilic acid, AA, Figure 1.1) is a bioactive constituent of the cashew-nut shell (*Anacardium occidentale*, anacardiaceae) and is the product of the hydrogenation of naturally occurring unsaturated anacardic acids which are the chief constituents of cashew nut shell liquid [Kubo *et al*., 1994; Shobha and Ravindranath, 1991]. The same compound has also been identified in several plants such as *Ozoroa insignis* (an African medicinal plant), zonal geranium (*Pelargonium hortorum*; Geraniaceae family), *Knema elegans* (Myristicaceae family), *Philodendron scandens* (Araceae family) and *Ginkgo biloba* (Ginkgoaceae; an Asian medicine), and has been widely used in Mexico as a traditional medicine for the treatment of gastric ulcers, gastritis, and stomach cancers [Rea *et al*., 2004; Gerhold *et al*., 1984; Spencer *et al*., 1980; Reffstrup *et al*., 1982; Itokawa *et al*., 1987; Olivera, *et al*., 1999; Acevedo *et al*., 2006].

AA has antimicrobial, insecticidal and acaricidal properties as well as potent molluscicidal effects [Himejima and Kubo, 1991; Muroi and Kubo, 1993; Schultz *et al*., 2006; Castillo-Juárez *et al*., 2007; Pan *et al*., 2006; Kubo *et al*., 1986; Sullivan *et al*., 1982]. Furthermore, this compound has been shown to exhibit antitumor, anti- inflammatory and anti-oxidant characteristics [Kubo *et al*., 1993; Yang *et al*., 2004; Oliveira *et al*., 2011; Trevisan *et al*., 2006; Kubo *et al*., 2006; Sung *et al*., 2008]. AA plays a role in reducing obesity in rats as well as inhibiting in the lipid synthesis of bacteria, yeast and animal cells [Toyomizo *et al*., 2003; Murata *et al*., 1997]. However, this compound showed no cytotoxic or genotoxic effects on CD1 male mice [Acevedo

et al., 2006]. AA acts as a mitochondrial uncoupler of oxidative phosphorylation and sensitizes human tumor cells to ionizing radiation by inhibitng histone acetyltransferase activity [Toyomizu *et al*., 2000; Sun *et al*., 2006]. In addition, it has been shown to inhibit the activity of various enzymes such as tyrosinase, xanthine oxidase, lipoxygenase, urease, glycerol-3-phosphate dehydrogenase(GAPDH), α-lactamase, R-Glucosidase, aldose reductase, prostaglandin endoperoxide synthase, prostaglandin synthase, matrix metalloproteinase-2 and matrix metalloproteinase-9 [Kubo *et al*., 1994; Masuoka *et al*., 2004; Grazzini *et al*., 1991; Ha and Kubo, 2005; Kubo *et al*., 1999; Murata *et al*., 1997; Hird and Milner *et al*., 1994; Toyomizu *et al*., 1993; Grazzini *et al*., 1991; Kubo *et al*., 1987; Omanakuttan *et al*., 2012].

Furthermore, AA inhibits the activation of both inducible and constitutive expression of nuclear factor-kappa B (NF-κB) activated by carcinogens and growth factors, and potentiates apoptosis in tumor cells [Sung *et al*., 2008]. AA inhibits protein SUMOylation both in vitro and in vivo, induces caspase-independent apoptosis and radiosensitizes pituitary adenoma cells [Fukuda *et al*., 2009; Sukumari-Ramesh *et al*., 2011].

The pharmacological roles of anacardic acids are summarized in Table1.

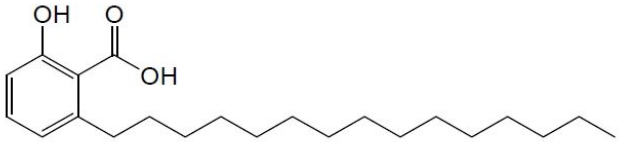

Figure 1.1. The structure of anacardic acid (AA): MW; 348.8, $C_{22}H_{36}O_3$

Table 1. Pharmacological roles of anacardic acids [Hemshekhar *et al*., 2011].

Compounds	Pharmacological role	Model used to test	Dose
AA (C15:0)	ROS generation inhibition		0.053 ± 0.005 mM
	Xanthine oxidase inhibition		0.0043 ± 0.0005 mM
	Anti-bacterial	*Propionibacterium acnes*	0.0015 mg/mL
		Corynebacterium xerosis	0.0062 mg/mL
		S. aureus	0.025 mg/mL
		MSSA ATCC12598	0.0031–0.0062 mg/mL
		MRSA, B. subtilis	
	Zoosporicidal activity	*Aphanomyces cochlioides*	
	Anti-parasitic activity	Colorado potato beetle larvae	
	HATs inhibition p300		0.0085 mM
	PCAF		0.005 mM
	Cytotoxicity		0.008 mM
	PfGCN5 HATs inhibition		
	NFκB inhibition		
	Anti-cancer activity	Pituitary adenoma cells, melanoma cells	
	Aurora kinase A activation		
	Anti-obase activity	Wistar rats	
	Tyrosinase inhibition	Mashroom tyrosinase	0.18 mg/mL
	Urease inhibition		0.12 mg/mL
	Selective metal ion chelation		
AA (C15:1)	LOX-15 inhibition	Soybean lipoxygenase	0.05 mM
	Cytotoxicity	HeLa cells	0.01 mM
AA (C15:2)	Anti-bacterial	*Helicobacter pylori*	0.2 mg/mL
AA (C15:3)	Anti-bacterial, Molluscidal activity	*H. pylori,* Snails	0.2 mg/mL
AA (C24:1)	Anti-proliferation of ERα	MCF-10A, MCF-7, MDA-MB-231 breast cancer cells	

1.2. The Endoplasmic Reticulum (ER) Stress

The endoplasmic reticulum (ER) is a subcellular organelle involved in the synthesis of secretory and membrane proteins and lipids. ER plays a role in the regulation of protein synthesis, folding and targeting, and in maintaining cellular calcium homeostasis. As a protein-folding compartment the ER is exquisitely sensitive to alterations in homeostasis and to the accumulation of unfolded proteins in the ER lumen, a condition referred to as ER stress. ER stress induces unfolded protein response (UPR) [Kaufman, 1999]. Various physiological and pathological conditions such as hypoxia, ER-Ca^{2+} depletion, oxidative injury and viral infections may break the balance between ER protein folding load and capacity. ER stress induces three major cellular responses: unfolded protein response (UPR), ER-associated degradation (ERAD) and apoptosis [Kruse *et al*., 2006].

The activation of UPR in mammalians is mediated by three distinct ER stress sensors, IRE1 (inositol-requiring protein-1), PERK (protein kinase RNA (PKR)-like ER kinase) and ATF6 (activating transcription factor 6). These are integral membrane proteins that reside in the ER lumen, which senses ER stress [Rasheva and Domingos, 2009]

1.2.1. PERK

PERK is a transmembrane kinase that phosphorylates translation initiation factor eIF2α (eukaryotic translation initiation factor 2α). Upon ER stress, activated PERK (p-PERK) phosphorylates the eIF2α, which transiently blocks most protein translation and reduces the influx of nascent proteins into the ER lumen except for the transcriptional factor ATF4 that activates the expression of several UPR target genes.

Therefore, cellular protein synthesis and the load of proteins entering into the ER are reduced [Shi *et al*., 1998; Harding *et al*., 1999].

1.2.2. IRE1

IRE1 is a type I transmembrane protein with both kinase and endoribonuclease (RNase) domains. In cells undergoing ER stress, autophosphorylation of IRE1 results in the production of XBP1s, active leucinezipper (bZIP) transcription factor, which regulates the transcription of several genes involved in ER quality control mechanisms [Yoshida *et al*., 1998; Lee *et al*., 2003]. In addition, another role of IRE1's kinase is to activate the c-Jun N-terminal kinase (JNK) signaling pathway through the MAP3K cascade [Urano *et al*., 2000].

1.2.3. ATF6

ATF6 is a type II transmembrane protein with a cytosolic domain containing a basic-leucine zipper motif (bZIP), transcription activation domain (TAD) and an ER luminal domain that binds BiP [Rasheva and Domingos, 2009].

The Releasing of Bip allows 90 kDa full-length ATF6 to translocate from the ER to the Golgi complex, where it is cleaved by the proteases SP1 and SP2. Cleaved ATF6 (50 kDa) translocates to the nucleus and plays a role in an active transcription factor for the UPR target genes, including XBP-1, Bip and CHOP/GADD153 [Haze *et al*., 1999; Yoshida *et al*., 2000].

1.2.4. Grp78/Bip

ER stressed cells trigger a set of intracellular signaling pathways to

the UPR to stop new protein synthesis and protect the cells [Ron and Walter, 2007]. Several chaperone proteins are involved in this pathway. A well-known regulator of the UPR response is Grp78 (glucose-regulated protein). GRP78 is also referred to as BiP, the immunoglobulin heavy chain-binding protein, which was originally found to bind to the immunoglobulin heavy chains of pre-B cells [Haas and Wabl, 1983]. Grp78/Bip, which was enhanced in tissue culture cells grown in medium deprived of glucose, was first discovered as a 78,000 Da protein [Shiu *et al.*, 1997]. Upon ER stress, Bip is released from three ER transmembrane signal transducers, PERK, IRE1 and ATF6. The misfolded/unfolded proteins bind with free Bip and the reduction of free Bip triggers the UPR pathway [Lee, 2005].

Furthermore, recent research revealed that knockdown of the upstream UPR regulator Grp78/BiP by siRNA inhibits autophagosome formation induced by both ER stress and nutrient deprivation, but does not affect the conversion of LC3-I to LC3-II in mammalian cells. This research suggests that Grp78/BiP is an obligatory factor for autophagy and may function at the phagophore expansion step rather than the induction step [Li *et al.* 2008].

1.2.5. CHOP

CHOP is the transcription factor C/EBP homologous protein and one of the most commonly used indicator of ER stress [Wang *et al.*, 1996; Wang and Ron, 1996]). CHOP, a 29 kDa protein, was first identified as a member of the CCAAT/enhancer binding proteins (C/EBPs) [Ron and Habener, 1992]. CHOP, also known as DNA damage inducible gene 153 (GADDI53), is a group of leucine zipper proteins that share extensive amino acid homology with the DNA binding domain of

C/EBP DNA-damage-inducible transcript 3 (DDIT3) and C/EBPz [Fornace *et al*., 1988; Luethy *et al*., 1990]. The CHOP gene is highly inducible by genotoxic and mutagenic agents [Fornace *et al*., 1988; Luethy *et al*., 1990], as well as by agents that produce stress in the endoplasmic reticulum and directly induce apoptosis [Wang *et al*., 1996]. CHOP works as a transcriptional factor that regulates genes involved in either survival or death [Zinszner *et al*., 1998]. As an important pro-apoptotic transcription factor, the expression of CHOP/GADD153 is under the control of PERK, ATF6 and IRE1α signaling [Li *et al*., 2008; Yoshida *et al*., 2000; Wang *et al*., 1998].

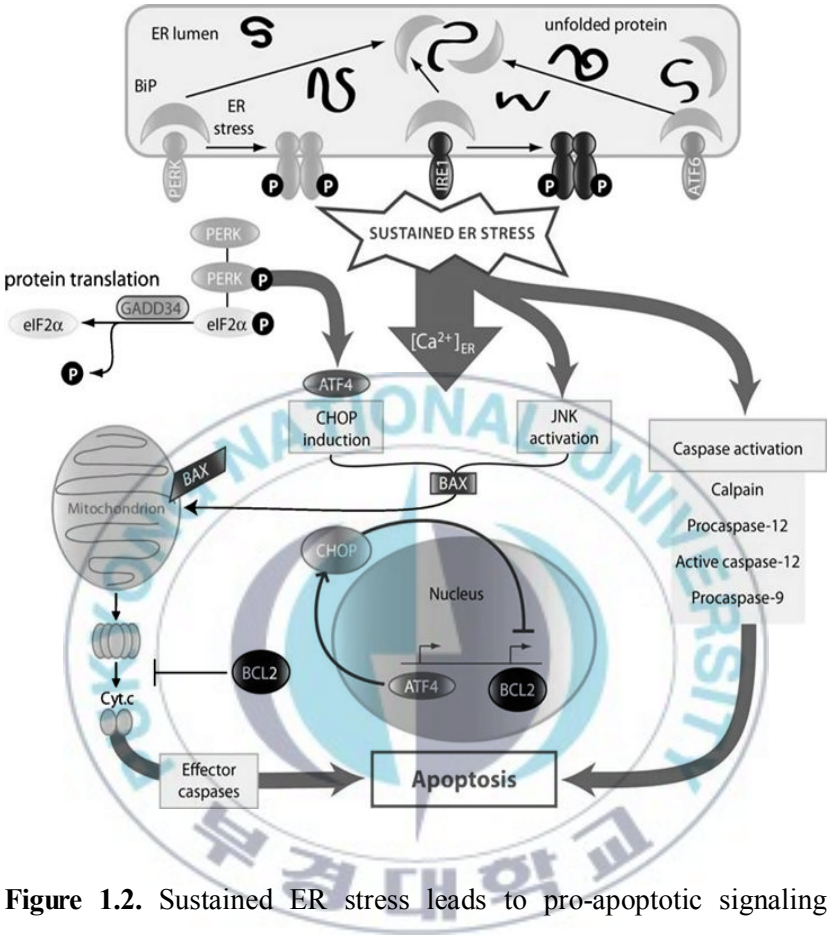

Figure 1.2. Sustained ER stress leads to pro-apoptotic signaling [Naidoo, 2009].

1.3. Autophagy

Autophagy (presently synonymous with macroautophagy) is an alternative, non-apoptotic route of programmed cell death, in which cytoplasmic components including proteins and damaged organelles are delivered to the lysosome for bulk degradation. Morphological changes involving double-membrane autophagic vacuoles, called autophagosomes, are observed in this type of cell death [Reggiori and Klionsky, 2005]. Autophagosomes degrade and recycle long-lived organelles and proteins by merging with lysosomes to form autolysosomes [Levine and Klionsky, 2004; Eskelinen, 2005]. Autophagy may function as either a cellular survival mechanism during starvation or a cell death mechanism when other cell death pathways such as apoptosis are deficient [Gozuacik and Kimchi, 2004].

The autophagic process is formally divided into several steps; initiation of autophagy at the preautophagosomal site, the formation of the autophagosome, the fusion of the autophagosome and the lysosome, the digestion of the autophagosome content, and the release of the digested components back into the cytosol [Verfaillie *et al*., 2010]. There are 31 autophagy-related (Atg) genes [Huang and Klionsky, 2007; Klionsky *et al*., 2003]

1.3.1. Atg5 and Atg8/LC3

Atg12 and Atg8/LC3, the ubiquitin-like proteins, are two protein conjugation systems that participate in autophagy and both conjugation systems are evolutionarily conserved from yeast to humans [Ohsumi, 2001; Yang and Klionsky, 2009]. Upon the stimulation of autophagy, Atg12-Atg5 conjugates are formed. This reaction is catalyzed by two

additional proteins; Atg7 and Atg10 [Mizushima *et al*. 1998]. Atg7 is homologous to the E1 ubiquitin-activating enzyme and Atg10 functions as an E2 ubiquitin conjugating enzyme [Tanida *et al*. 1999; Shintani *et al*. 1999]. Atg5 is further bound Atg16 to form an Atg12-Atg5-Atg16 complex that is functionally essential for autophagy [Mizushima *et al*., 1999; Kuma, 2002].

Another ubiquitin-like protein, Atg8/LC3, is conjugated to a membrane lipid, phosphatidylethanolamine (PE) [Ichimura *et al*., 2000]. Atg8 is initially proteolytically cleaved by a cysteine protease, Atg4 [Kirisako *et al*., 2000] and then Atg7 activates Atg8, which is transferred to the E2-like protein Atg3. As a last step in Atg8/LC3 modification this protein conjugates to phosphatidylethanolamine (PE) [Verfaillie *et al*., 2010]. Upon autophagy induction, after lipidation, Atg8/LC3 associates with the autophagosome. It is most abundant in autophagosomal membranes and is used as a marker to monitor the autophagosome and autophagic activity [Yang and Klionsky, 2009].

1.3.2. Beclin-1

Beclin-1 is one of the Vps34-interacting proteins required in the initiation process of autophagosome formation. Beclin-1 has a BH3-only domain that permits the interaction of this protein with the antiapoptotic proteins Bcl-2 and Bcl-XL. The interaction of antiapoptotic proteins abrogates Beclin-1's ability to induce autophagy [Levine and Kroemer, 2008; Liang *et al*., 1999; Pattingre *et al*., 2005].

1.3.3. DAPK

DAPK, death-associated protein kinase, is a calcium/calmodulin-regulated serine/threonine protein kinase, which has been identi-

fied as a mediator of autophagic or apoptotic cell death [Cohen *et al*., 1997; Gozuacik *et al*., 2008]. DAPK is also known as a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death [Gozuacik *et al*., 2008]. DAPK phosphorylates the BH3 domain of Beclin-1 and promotes dissociation from Bcl-xl [Zalckvar *et al*., 2009].

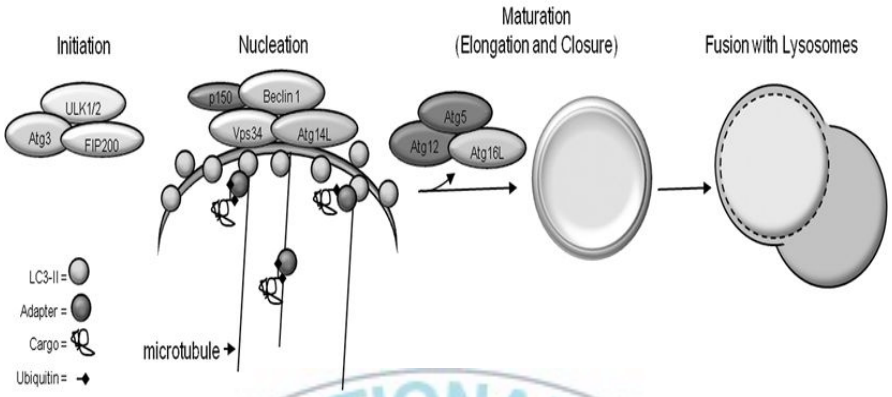

Figure 1.3. Simplified illustration of macroautophagy [Benbrook, 2012].

1.4. Apoptosis

Apoptosis, coined by Kerr *et al*. in 1972 and classified as type I programmed cell death (PCD), is a physiological process that leads to cellular self-destruction. The morphological characterization of apoptosis includes chromatin condensation, the fragmentation and removal of DNA and finally, phagocytosis [Kroemer *et al.*, 2009].

1.4.1. The two main pathways of apoptosis

There are two main pathways of apoptosis, the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic). When apoptotic ligands bind to death receptors the extrinsic pathway occurs through the activation of initiators such as caspases-8 and -10, and the activation of the executioners, caspases-3, -6, and -7, resulting in DNA fragmentation [Millan and Huerta, 2009; Slee *et al*., 2001; Walczak and Krammer, 2000]. The mitochondrion is an essential mediator of the intrinsic pathway. The arrival of signals leading to changes in permeability of the outer mitochondrial membrane causes the release of intermitochondrial apoptotic molecules into the cytosol [Zamzami *et al*., 1996; Schultz and Harrington, 2003].

Cytochrome c is one of the best-characterized pro-apoptotic molecules. It leads to the activation of caspases via the formation of the apoptosome [Adrain, 2001]. Following cytochrome c release from the mitochondria, caspases are activated and the cell undergoes apoptosis through the formation of apoptosomes, Apaf-1/caspase-9 complex [Li *et al*., 1997].

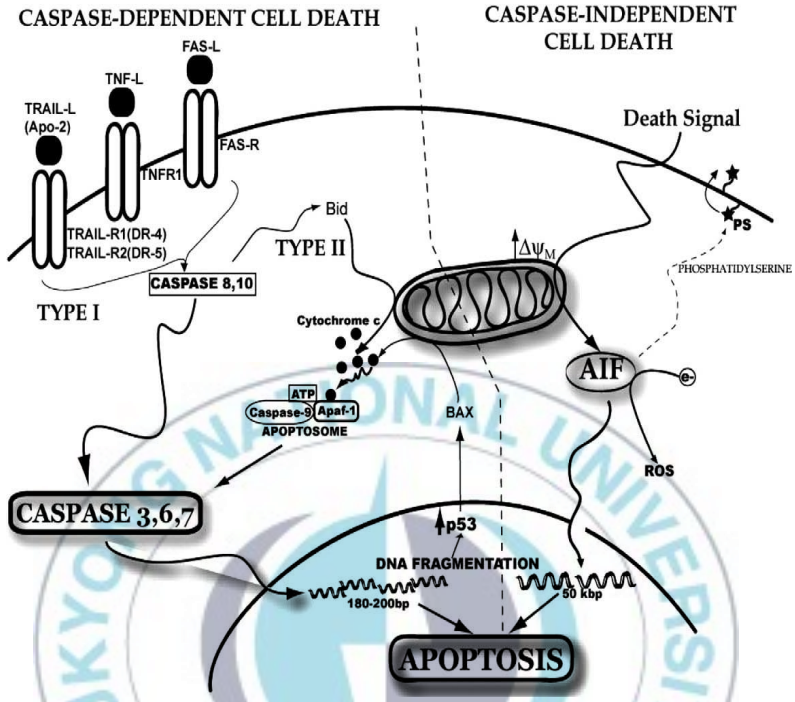

Figure 1.4. Caspase-dependent and -independent pathways of apoptosis. [Millan and Huerta, 2009].

1.4.2. Caspases

Caspases, cysteine proteases that are able to cleave proteins at aspartic acid residues, exist as zymogens. Some are activated through self-cleavage [Elmore, 2007; Thornberry and Lazebnik, 1998] while some activate others. Cleaved caspases acting in a proteolytic cascade eventually lead to the death of the cell [Nagata, 1997]. There are two groups of caspases that participate in apoptosis, initiator caspases (caspase-2, -8, -9, -10) and executioner or effector caspases (caspase-3, -6, -7). Furthermore, caspase-12 mediates ER-specific apoptosis [Elmore, 2007; Cohen, 1997]. Initiator caspases become activated in specialized protein complexes [Sprick and Walczak, 2004]. Caspase-9 is activated in apoptosomes while caspase-8 and caspase-10 are activated in death-inducing signaling complex (DISC) [Elmore, 2007; Baliga and Kumar, 2003]. Executioners result in DNA fragmentation [Slee *et al*., 2001]

1.4.3. Bcl-2 family members

Previous research reported that the release of cytochrome c from the mitochondria is an early event during apoptosis, and pro-apoptotic (Bid, Bad, Bim, Bax, Bak and Noxa) Bcl-2 family members induce the release of cytochrome c and anti-apoptotic (Bcl-2, Bcl-xL, A1 and Bcl-w) Bcl-2 protein family members inhibit the release of cytochrome c [Kluck *et al*., 1997; Yang *et al*., 1997]. The balance between the pro-apoptotic and anti-apoptotic Bcl-2 protein family members is an important factor contributing to cytochrome c release, and thus in determining the cell's fate [Green and Amarante-Mendes, 1998; Li *et al*., 1997]. Bax and Bak are also known to promote apoptosis by modulating ER and mitochondrial Ca^{2+} stores [Nutt *et al*., 2002].

1.5. The purpose of this research

The experiments reported in this study were designed to investigate the cytotoxic effect of AA on cancer cells and to find the potential signaling pathways which lead to cell death in AA-treated human lung adenocarcinoma A549 cells.

Chapter 2 focuses on the induction of ER stress by AA in human lung adenocarcinoma A549 cells.

chapter 3 focuses on the induction of autophagy by AA in human lung adenocarcinoma A549 cells.

chapter 4 focuses on the induction of apoptosis and the elucidation of the potential signaling pathway which leads to cell death.

The results support the view that AA induces ER stress, autophagy and apoptosis. For the first time it has been revealed that AA induces ER stress and autophagy in cancer cells. Furthermore our results show the activation of a mitochondrial intrinsic pathway, an increase of the expression of apoptosis inducing factor (AIF) and death-associated protein kinase (DAPK), and a decrease of the expression of Hsp70 in the AA-treated A549 human lung cancer cells. Based on our results, we suggest the possibility that, in AA-treated A549 cells, activation of the signaling pathways leading to cell death occurs via ER stress-induced autophagy and apoptosis.

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PART Ⅱ

Induction of Endoplasmic Reticulum Stress, Autophagy and Apoptosis by Anacardic Acid

Chapter 2.

Induction of endoplasmic reticulum stress in human lung adenocarcinoma A549 cells by anacardic acid

2.1. Abstract

Anacardic acid (2-hydroxy-6-pentadecylbenzoic acid, AA), a constituent of the cashew-nut shell, has a variety of beneficial effects in the treatment of cancers and tumors. However, the fact that AA induces ER stress in cancer cells is not known. The purpose of this research is to identify the induction of ER stress in A549 cells by AA. In this research, several results show that AA induces intracellular Ca^{2+} mobilization and ER stress. AA induces the ER stress-inducing factors, especially IRE1α, ATF6α and the hallmarks of UPR such as Grp78/Bip, caspase-12 and GADD153/CHOP. AA inhibits the expression of p-PERK and its downstream substrate, p-elF2α. In addition, the morphological analysis of intracellular organelles by TEM also shows that AA induces ER stress. For the first time, we have revealed that AA induces ER stress and autophagy in cancer cells.

2.2. Introduction

Cancer afflicts many people globally. Lung cancer especially is one of the three most common cancers in men and one of the three leading causes of cancer death in men and women in the United States. AA, a constituent of cashew-nut shell (*Anacardium occidentale*), inhibits the activation of nuclear factor-kappa B (NF-κB) in tumor cells and induces caspase-independent apoptosis in pituitary adenoma cells [Kubo *et al*., 1994; Sung *et al*., 2008; Sukumari-Ramesh *et al*., 2011]. However, the cell signaling pathway leading to death in cancer cells is not yet much elucidated.

The endoplasmic reticulum (ER) is a subcellular organelle that takes a part in the regulation of protein synthesis, folding and targeting as well as maintaining cellular calcium homeostasis. In the ER, synthesized proteins fold into their native conformation, followed by post-translational modifications such as glycosylation and the formation of intra- and intermolecular disulfide bonds [Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985; Fewell *et al*, 2001]. Through the quality-control process only correctly folded proteins are moved to the Golgi complex, while incompletely folded proteins are retained in the ER to complete the folding process or to be targeted for degradation [Ellgaard *et al*., 1999: Schröder and Kaufman, 2005].

The malfunction of ER stress response can be the causes of many diseases such as obesity, diabetes, Alzheimer's disease, hypercholesterolemia and Parkinsonism [Yoshida, 2007; Rutishauser and Spiess, 2002; Taylor, 1992; Koo *et al*., 1999; Kim and Arvan, 1998; Imai *et al*, 2001].

A variety of situation, such as pathogen infection, nutrient

deprivation, inflammation, alterations in ER lumenal Ca^{2+} or redox status, genetic mutation or the presence of toxic chemicals can cause the accumulation of unfolded proteins [Sakaki and Kaufman, 2008]. Malfolded or misfolded proteins cause ER stress and an ER stress response, called the unfolded protein response (UPR) [Kozutsumi *et al*., 1988; Zimmer *et al*., 1999]. In addition, the ER plays a key role in maintaining Ca^{2+} homeostasis within the cell and Ca^{2+} is known to directly regulate protein folding [Berridge, 2002; Corbe *et al*., 1999; Groenendyk and Michalak, 2005]. It is also well known that disruption of Ca^{2+} homeostasis in the ER can trigger ER stress [Rao *et al*., 2004].

Several molecular chaperones, BiP/GRP78 (binding protein/glucose regulated protein 78), calnexin (CNX) and calreticulin (CRT) as well as folding enzymes such as ER oxidoreductin-1 (Ero-1) and protein disulfide isomerases (PDI) participate in protein folding in the ER [Rasheva and Domingos, 2009].

Calnexin functions as a molecular chaperone with calreticulin during folding and quality control by comprising the calnexin/calreticulin cycle [Hammond and Helenius; 1993]. Together they interact with nascent and newly synthesized glycoproteins in the endoplasmic reticulum (ER) of eukaryotic cells [Parodi, 2000; Molinari *et al*., 2004]. Furthermore, prior study has shown the essential role of calnexin as a Ca^{2+} buffer, which is important for photoreceptor cell survival [Rosenbaum *et al*., 2006].

PDI and Ero-1 take part in oxidative folding that results in the formation of intra- and inter-molecular disulphide bonds, and generates H_2O_2 by transfer reducing equivalents to molecular oxygen [Sevier and Kaiser, 2008; Verfaillie *et al*., 2010]. BiP/GRP78 takes a role in promoting protein folding by inducing conformational changes in the

unfolded proteins [Hendershot *et al*., 1996].

Under ER stress, UPR is activated by three distinct ER stress sensors, inositol requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK), which activates downstream signaling effector [Rasheva and Domingos, 2009].

Although UPR is a protective process in ER stressed cells, in severe and prolonged ER stress, UPR induces autophagy [Kondo *et al*., 2005], a cellular degradation process implicated in both cell death and survival. In addition, prolonged stress-related UPR can result in cell death through the activation of the apoptotic pathway [Li *et al*., 2006] and severe ER stress can cause apoptotic cell death, usually by activating the intrinsic pathway [Szegezdi *et al*., 2006].

This chapter focuses on the endoplasmic reticulum (ER) stress by AA on A549 cells.

2.3. Materials and Methods

2.3.1. Cell culture and reagents

HEK293 (human embryonic kidney cell), A549 (human lung adenocarcinoma cell) and SK-N-SH (human neuroblastoma cell) cells were purchased from the American Tissue Culture Collection (Manassas, VA). Cells were cultured in DMEM, RPMI 1640 and MEM (Hyclone Laboratories, Logan) medium, respectively. A549 cells were incubated in the FBS-free RPMI 1640 medium for 24 hours and AA was added to the medium for more research. AA and Flou-3-AM were purchased from Calbiochem (San Diego, CA) and Invitrogen (Carlsbad, CA), respectively.

2.3.2. Cell cytotoxicity assay

Cells were seeded in 96-well plates at an initial density of approximately 0.5×10^5 ~ 1×10^5 cells in a medium supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 100 U/ml penicillin and 10 μg/ml streptomycin (PAA Laboratories GmbH, Pasching, Austria) in a humidified atmosphere containing 5% CO_2 at 37℃. Following 24 hours of incubation AA, dissolved in 100% Dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), was added to the culture medium at the different concentration. Cells were incubated for 24 hours and 10 μl of EZ-Cytox Cell Viability Assay Solution (WST-1^{TM}) (Daeil Lab Service, Seoul, Korea) was added for 4 hours at 37℃. The intensity was measured at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

2.3.3. Protein extraction and Western blot analysis

A549 cell lysates were prepared in a Radioimmunoprecipitation assay buffer (RIPA, Cell Signaling Technology, Danvers, MA, USA). After incubation for 30 min on ice, the lysate was centrifuged at 14,000 rpm for 20 min at 4℃ and the protein was determined by a Protein Quantification Kit (CBB solution; Biosesang Inc., Sungnam) with BSA as a standard. An aliquot from each sample (20~100 μg of total protein) was separated by electrophoresis through 10-12% SDS-polyacrylamide gels and blotted onto nitrocellulose. Proteins were detected by enhanced chemiluminescence (ECL) detection solution (Pierce, Rockford, IL, USA) using antibodies. The antibodies used in this research are anti-ATF6α, anti-p-PERK (Thr 981) and secondary antibodies conjugated with HRP rabbit goat (Santa Cruz Biotechnology), anti-caspase-12 (Abcam plc), anti-IRE1α, anti-Bip, anti-p-eIF2α, anti-CHOP, anti-β-actin, anti-PDI, anti-Ero1, anti-calnexin and secondary anti-rabbit and anti-mouse IgG (horseradish peroxidase-linked) (Cell Signaling Technology, Danvers, MA).

2.3.4. Detection of Ca^{2+} mobility

To detect the release of Ca^{2+} from the ER to the cytosol, Flou-3 AM calcium staining method was used [Kao *et al.*, 1989]. Cells were cultured in a glass bottom culture dish for 24 hours. After incubation with a graded concentration of AA for 24 hours, cells were stained with Flou-3 AM (1.5 μM) for 30 minutes at 37℃. After washing with PBS, cells were examined under an ECLIPSE 50i microscope (Nikon, Tokyo).

2.4. Results

2.4.1. Cytoxicity of AA

In order to investigate the cytotoxicity of AA, we used three cell lines, HEK293, A549 and SK-N-SH cells (Fig. 2.1). After 24 hours, the viability of AA-treated cells decreased depending on the cell lines. Due to our interest in lung cancer we performed further experiments using A549 cells. The IC50 value of AA-treated A549 cells was 2.75 ± 0.25 μg/ml (Fig. 2.1). Henceforth, 3.0 μg/ml of AA was used to investigate the molecular mechanism of cell death in A549. Time- and concentration- dependent changes of cellular morphology in AA-treated A549 cells were shown under microscopy, respectively (Fig. 2.2 and Fig. 2.3). The results shown that cell become round and cell density decreased according to AA treatment time and concentration in A549 cells.

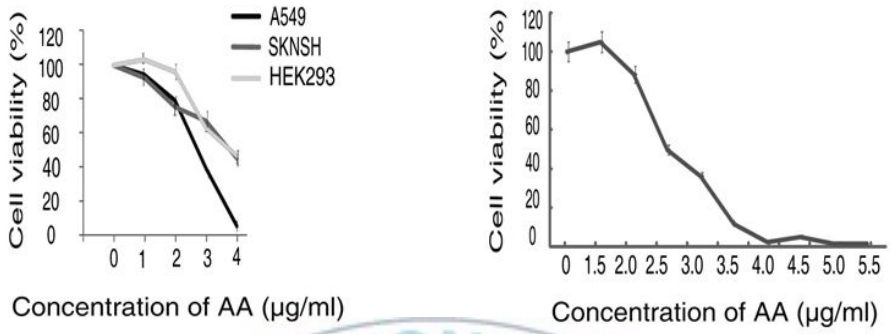

Figure 2.1. Cytotoxicity of AA. The viability of cells was examined with a WST-1 assay.

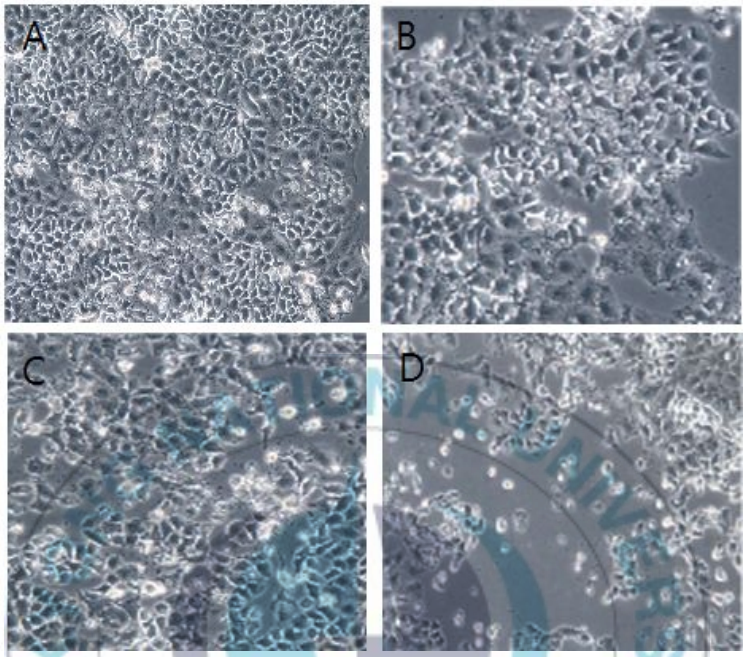

Figure 2.2. Time-dependent changes of cellular morphology in AA-treated A549 cells. A549 cells were incubated with 3.0 μg/ml of AA for the indicated time and analyzed under the microscope. (A) Non-treated cells; (B-D) AA-treated cells for 6 hours, 12 hours and 24 hours respectively.

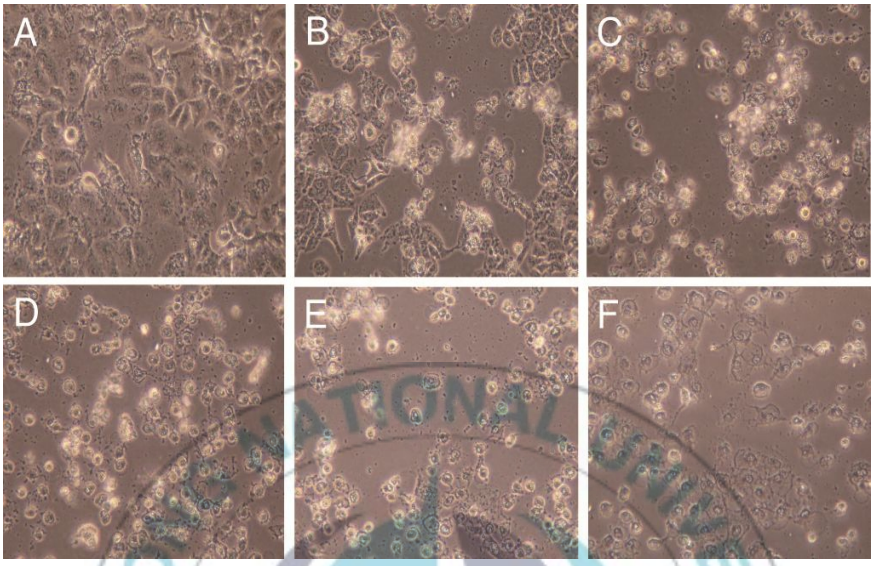

Figure 2.3. Concentration-dependent changes of cellular morphology in AA-treated A549 cells. A549 cells were incubated with the indicated concentrations of AA for 24 hours and analyzed under a microscope: (A) Non-treated cells; (B-F) 1.0, 2.0, 3.0, 4.0 and 5.0 μg/ml of AA- treated cells respectively.

2.4.2. Induction of Ca^{2+} mobilization

Because the release of Ca^{2+} from the ER and its increased concentration in the cytosol are known to be involved in cell death induction upon ER stress, we analyzed Ca^{2+} mobilization [Berridge, 2002]. By staining the cells with Fluo-3 AM we were able to show that the concentration of Ca^{2+} in the cytosol increased in a time-dependent manner with a plateau at 6 hours of treatment (Fig. 2.4).

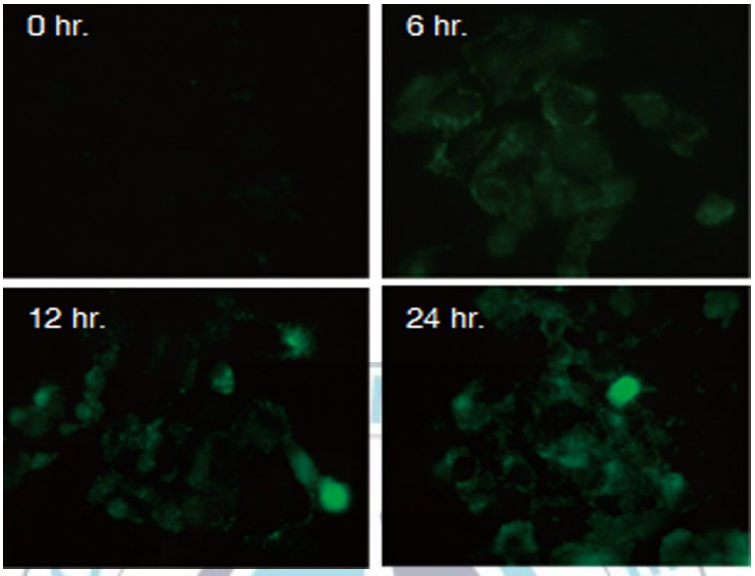

Figure 2.4. Mobilization of Ca^{2+} distribution by AA. A549 cells were treated with 3.0 μg/ml of AA for the indicated times. Intracellular Ca^{2+} mobilization was detected by using Fluo-3 AM and the result showed under fluorescence microscopy.

2.4.3. Expression of protein folding genes

To study the expression of proteins that participate in protein folding, we examined the expression of the molecular chaperone, calnexin (CNX), and folding enzymes such as ER oxidoreductin-1 (Ero-1) as well as protein disulfide isomerases (PDI). While the expression of calnexin increased, Ero-1 and PDI were almost unchanged at indicated times (Fig. 2.5).

2.4.4. Induction of ER stress in A549 cells

To study the effect of AA on unfolded protein response (UPR), several signatures of the UPR pathway were examined in A549 cells treated with or without AA. We examined the active form of PERK, p-PERK, with p-elF2α, as its downstream substrate. As shown in Fig. 3, the expressions of p-PERK and p-elF2α showed a gradual decrease in a time-dependent manner. The expression of IRE1α started to increase after 1 hour and rose steadily, with an increase of ATF6 cleavage after 4 hours of AA treatment. The expression of Bip began to increase after 12 hours and rose steadily with AA treatment time (Fig. 2.5). In addition, to study the effect of AA on ER stress-associated proapoptotic response, two ER stress-associated proapoptotic markers, caspase-12 and CHOP/GADD153, were examined (Fig. 2.5). When cells were exposed to AA, the expression of CHOP/GADD153 increased at 18 hours and cleaved caspase-12 increased at 6 hours.

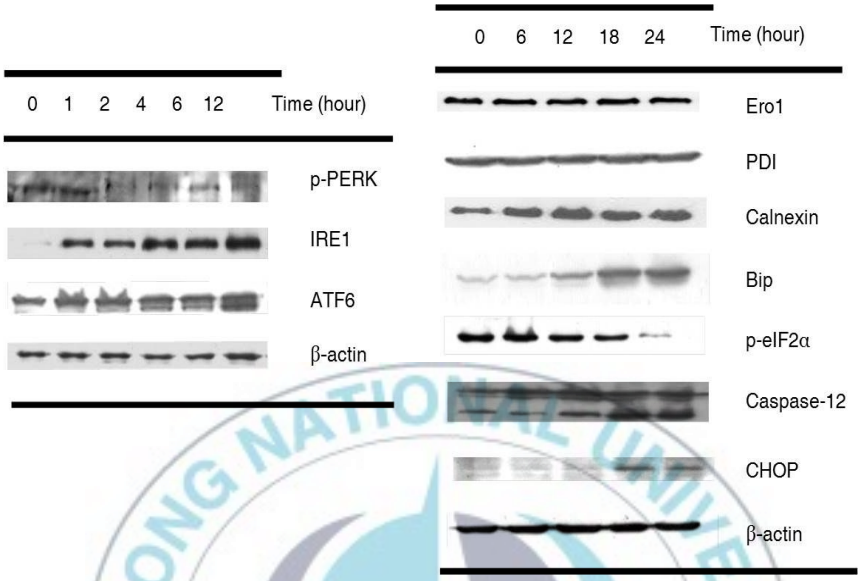

Figure 2.5. Expression of protein folding enzymes, ER stress transducers and ER stress-induced pro-apoptotic markers in A549 cells by AA. Whole-cell extracts were prepared and analyzed by Western blotting. Time-dependent expression of proteins is depicted.

2.5. Discussion

This chapter focused on the induction of ER stress in A549 cells by AA. Our results provide strong evidence to support the view that AA induces ER stress in A549 cells. The result of staining with Fluo-3 AM shows that AA induces the efflux of calcium from the ER to the cytosol in A549 cells. While the expression of oxidative folding enzymes such as PDI and Ero-1 do not change, calnexin takes part in the folding of glycosylated proteins and role in buffering Ca^{2+} in the cell, increased gradually by AA [Molinari *et al*., 2004; Rosenbaum *et al*., 2006]. Increased ER load leads to phosphorylation of calnexin (CNX) by activation of Erk1 through the IRE1-dependent signalling and to extended retention of misfolded proteins in the ER [Cameron *et al*., 2009; Chevet *et al*., 2010]. In our research, the increasing of misfolded proteins by AA could be cause of calnexin induction.

Disturbance of calcium homeostasis may be a turning point in triggering ER stress. After AA treatment, numerous ER stress signatures appeared. These include morphological evidence like ER membrane bleb as well as the changed expression of genes known as ER stressors. These genes can be grouped. One group consists of ER stress sensors such as p-PERK, IRE1α, ATF6 and the ER chaperone protein, Bip. Another group includes apoptotic proteins such as csapase-12 and CHOP/GADD153.

Grp78/Bip is a well-known regulator of the UPR response and is required for ER integrity and stress-induced autophagy in mammalian cells [Li *et al.*, 2008]. Upon ER stress, Grp78/Bip is released from three ER transmembrane signal transducers, PERK, IRE1α and ATF6. Then, binding with misfolded/unfolded proteins reduces the free

Grp78/Bip, thus triggering the UPR pathway [Lee, 2005].

PERK plays an important role in translational regulation and cell survival during the UPR [Harding *et al*., 2000]. Upon ER stress, activated PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2α), which transiently blocks most protein translation and reduces the influx of nascent proteins into the ER lumen, except for the transcriptional factor ATF4 [Shi *et al.*, 1998; Harding *et al*., 1999]. After release from Bip, ATF6 is cleaved at the Golgi complex. Cleaved ATF6 acts as an active transcription factor for the UPR target genes, including XBP-1, Bip and CHOP/GADD153 [Haze *et al.*, 1999; Yoshida *et al*., 2000].

The accumulation of misfolded proteins in the ER activates Janus N-terminal kinases (JNKs) and induces stress-induced oligomerization and activation of IRE1 [Srivastava *et al.*, 1999; Urano *et al*., 2000]. IRE1α provides a physical link between the apoptotic pathway and the UPR by modulating the UPR via direct interaction with proapoptotic BAX and BAK [Hetz *et al*., 2006].

In a recent study of ER stress using thapsigargin and tunicamycin, Grp78/Bip up-regulated and PERK, IRE1α and ATF6α activated [Rutkowski, 2006]. In addition, the up-regulation of Grp78/Bip played a role in mediating autophagy [Rovetta *et al*., 2012; Benbrook and Long, 2012].

In this research, the expression of p-PERK and p-eIF2α showed a gradual decrease in a time dependent manner. When this signaling pathway is decreased, the accumulation of unfolded proteins can overload the folding machinery during ER stress. The expression of cleaved ATF6, IRE1 and Grp78/Bip increased with AA. Increase of these proteins by AA also may affect the cell's fate, possibly leading

to the cell death, apoptosis or autophagy. We will discuss the induction of autophgy and apoptosis by AA in the following chapter.

CHOP, C/EBP-homologous protein, is an important proapoptotic transrciption factor that is induced by ER stressed cells [Marciniak *et al.*, 2004] and has been implicated in the control of translation and apoptosis. It is commonly accepted that CHOP/GADD153 expression is under the control of PERK, ATF6 and IRE1α signaling [Wang *et al.*, 1998; Yoshida *et al.*, 2000; Marciniak *et al.*, 2004]. In addition, CHOP induces the transcription of Bim, a proapoptotic member of the Bcl-2 protein family that directly causes cell death by increasing mitochondrial outer membrane permeability [Puthalakath *et al.*, 2007]. CHOP leads to the translocation of Bax from the cytosol to the mitochondria [Gotoh *et al.*, 2004] and sensitizes cells to ER stress by down-regulating Bcl-2 and perturbing the cellular redox state [McCullough *et al.*, 2001].

In our results, cleaved caspase-12 and CHOP/GADD153, known as mediators of endoplasmic reticulum-specific apoptosis, increase in away that could affect the cell's fate, especially it's death.

In conclusion, AA induces ER stress by disturbing the calcium homeostasis in A549 cells and activates UPR, eventually inducing the cell-signaling pathway that causes cell death.

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Chapter 3.

Induction of autophagy in human lung adenocarcinoma A549 cells by anacardic acid

3.1. Abstract

Autophagy has an important role in the cellular response to stress. We have demonstrated the induction of ER stress by AA in a prior chapter. Therefore, in this study we investigated the possibility of ER stress-induced autophagy by AA. The morphological analysis of intracellular organelles using TEM suggests that AA induces autophagy. TEM image showed the disruption of the inner mitochondrial membrane and morphological changes in the mitochondria and ER, including the formation of double-membrane autophagic vacuoles, autophagosomes and many diverse autolysosomes. Up-regulation of autophagy-related genes and the appearance of numerous fluorescent puncta observed in transfected cells with green fluorescent protein (GFP)-LC3 and GFP-Beclin-1 plasmid show the induction of autophagy in A549 cells by AA. The results of Western blot analysis show that the expression of Atg5-12 complex, Atg7, LC3B and especially the gradual increase of Beclin-1 and DAPK3, may play a key role in cell death by autophagy in AA-treated A549 cells.

3.2. Introduction

Autophagy (presently synonymous with macroautophagy) is an alternative, non-apoptotic route of programmed cell death, in which cytoplasmic components are delivered to the lysosome for bulk degradation. The morphological changes associated with this type of cell death include double-membrane autophagic vacuoles called autophagosomes [Reggiori and Klionsky, 2005], which degrade and recycle long-lived organelles and proteins by merging with lysosomes to form autolysosomes [Levine and Klionsky, 2004; Eskelinen, 2005]. Autophagy can function as either a cellular survival mechanism during starvation or a cell death mechanism when other cell death pathways such as apoptosis are deficient [Gozuacik and Kimchi, 2004].

The experiments reported in this chapter were designed to investigate the induction of autophagy by AA on human lung adenocarcinoma A549 cells and the results support the view that AA induces autophagy. For the first time we revealed that AA induces ER stress and autophagy in cancer cells. Based on our results, we suggest the possibility that activation of the signaling pathway leading to cell death occurs by ER stress-induced autophagy in AA-treated A549 cells.

3.3. Materials and Methods

3.3.1. Cell culture and reagents

A549 (human lung adenocarcinoma cell) cells were purchased from the American Tissue Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (Hyclone Laboratories, Logan) medium. A549 cells were incubated in the FBS-free RPMI 1640 medium or FBS-containing RPMI 1640 medium and anacardic acid (AA) was added to the medium for more research.

3.3.2. Protein extraction and Western blot analysis

A549 cell lysates were prepared in a Radioimmunoprecipitation assay buffer (RIPA, Cell Signaling Technology, Danvers, MA, USA). After incubation for 30 min on ice, the lysate was centrifuged at 14,000 rpm for 20 min at 4℃ and the protein was determined by a Protein Quantification Kit (CBB solution; Biosesang Inc., Sungnam) with BSA as a standard. An aliquot from each sample (20~100 μg of total protein) was separated by electrophoresis through 10-12% SDS-polyacrylamide gels and blotted onto nitrocellulose. Proteins were detected with enhanced chemiluminescence (ECL) detection solution (Pierce, Rockford, IL, USA) using antibodies against Atg5, Atg7, Atg12, LC3, Beclin-1, DAPK3 (Cell Signaling Technology, Danvers, MA, USA) and secondary antibodies conjugated with goat anti-rabbit (Santa Cruz, CA, USA). Anti-caspase-12 antibody was purchased from Abcam plc.

3.3.3. Transfection of GFP expressing vector

For examining the expression of GFP-fused protein, GFP-LC3B and

GFP-Beclin-1 expressing vectors were used. The full-length cDNAs of LC3B and Beclin-1 were amplified from a human liver cDNA library (Clontech, Mountain View, CA, USA) by polymerase chain reaction (PCR). The LC3B primers used for this experiment were 5'-CCGGAATTCCATGCCCTCAGACCGGCCTTT-3' (sense) and 5'-CGCGGATCCTCAGAAGCCGAAGGTTTCCTG-3' (antisense).

The Beclin-1 primers used for this experiment were 5'-AAGCTT ATGGAAGGGTCTAAGAC-3' (sense) and 5'-GGATCCTTTGTT ATAAAATTGTGAGG-3' (antisense). The purified PCR product was ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA). The insert was released by Hind Ⅲ and Bam HI, fused with N-terminal GFP tag in the pEGFP-N2 vector (Clontech). All plasmids were confirmed by DNA sequencing. For transfection, Fugene 6 reagent™ (Roche Diagnostics GmbH, Mannheim) was used and examined according to the manufacturer's protocol. The formation of autophagosomes in the cells was analyzed with an ECLIPSE 50i microscope.

3.3.4. TEM analysis of A549 cells

A549 cells were pre-fixed in pellets at 4℃ with 2.5% glutaraldehyde in a 0.1 M phosphate buffer and then post-fixed with 1% osmium tetraoxide (OsO4) in a 0.1 M phosphate buffer, pH 7.4. After fixing, cells were embedded in Epon 812 using general procedures. Approximately 70 μm of ultrasected specimens by Ultracut Reichert-jung were stained using uranyl acetate and lead citrate, and examined with Hitachi H600 TEM (Tokyo). All reagents used in the TEM experiment were purchased from Electron Microscopy Science (EMS).

3.4. Results

3.4.1. Morphological changes of the mitochondria and ER

The ultrastructure of the ER was observed by TEM (Fig. 3.1). Non-treated control cells showed normal ER morphology (A) while AA-treated cells showed dilation and bleb of the ER membrane in the several places (B).

In addition, the ultrastructure of the mitochondria was also observed in TEM (Fig. 3.2). Non-treated control cells showed normal morphology of mitochondria (A) while AA-treated cells showed significant morphological changes such as a disrupted inner membrane, curved form and dark stains in B, C and D respectively.

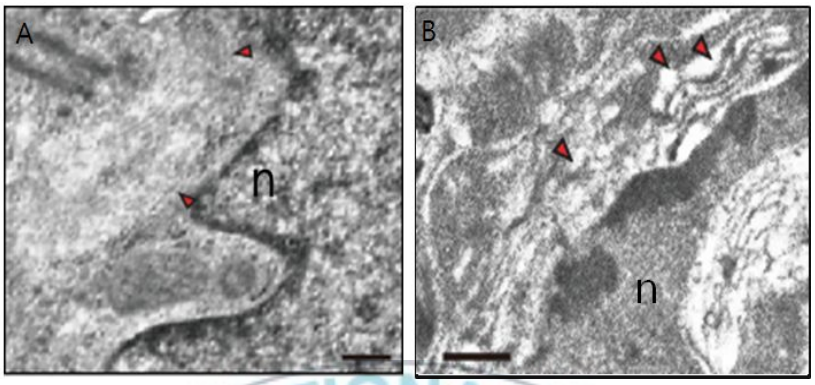

Figure 3.1. Marked Changes in the ER by AA. The ultrastructure of the ER was observed under TEM. (A) Non-treated cell, (B) Cells were incubated with 3.0 μg/ml of AA for 24 hours. Arrow heads show the appearance of ER membrane bleb after AA treatment. Bars in (A) and (B) denote 1 μm; n means nuclear.

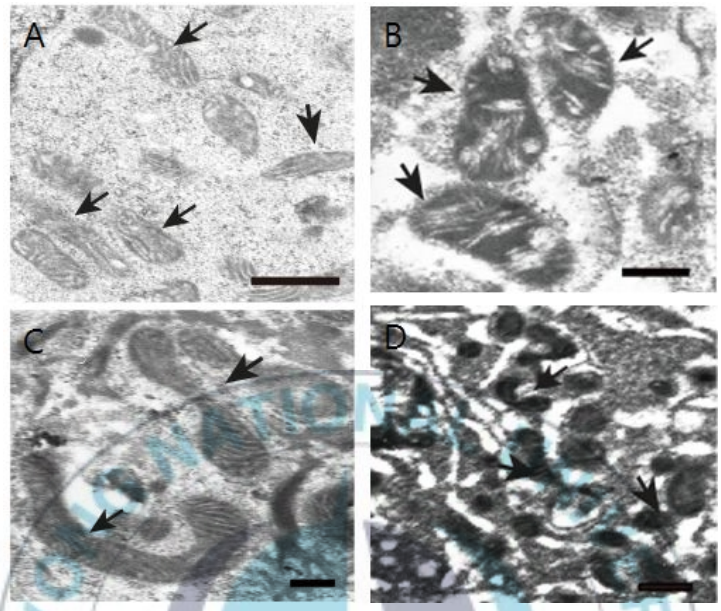

Figure 3.2. Morphological changes of mitochondria by AA. The ultrastructure of mitochondria was observed under TEM. (A) Non-treated cell, (B ~ D) Cells were incubated with 3.0 μg/ml of AA for 24 hours. Arrows indicate mitochondria. (B) shows disruption of the inner mitochondrial membrane. (C) and (D) show curved, shrunken and darkly stained mitochondria; Bars denote 500 nm.

3.4.2. Formation of autophagosomes in A549 cells

To identify the formation of autophagosomes, we examined the expression of transfected cells with green fluorescent protein (GFP)-LC3B (Fig. 3.3 A and B) and GFP-Beclin-1 (Fig. 3.3 C and D) plasmids by fluorescence microscopy. After exposure to AA for 24 hours, numerous fluorescent puncta were observed in AA-treated cells (Fig. 3.3 B and D), consistent with the induction of autophagy, while very few puncta were seen in untreated cells (Fig. 3.3 A and C).

Furthermore, TEM images clearly showed the formation of double and multilayered membrane bound autophagosomes (Fig. 3.4 A, B and C) as well as many diverse autolysosomes, including engulfed cell components and completely disrupted inner mitochondrial membranes (Fig. 3.4 D and E). The results are consistent with the view that AA induces autophagy.

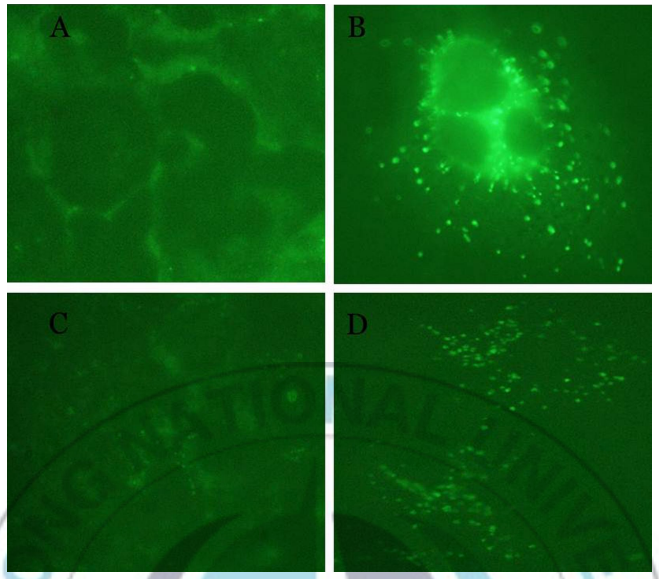

Figure 3.3. Formation of autophagosomes by AA. (A) Cells were transfected by either GFP-LC3B (A and B) or GFP-Beclin-1 (C and D) followed by treatment with (B and D) or without (A and C) AA for 24 hours. The results were examined at 1000 times magnification using a fluorescence microscope.

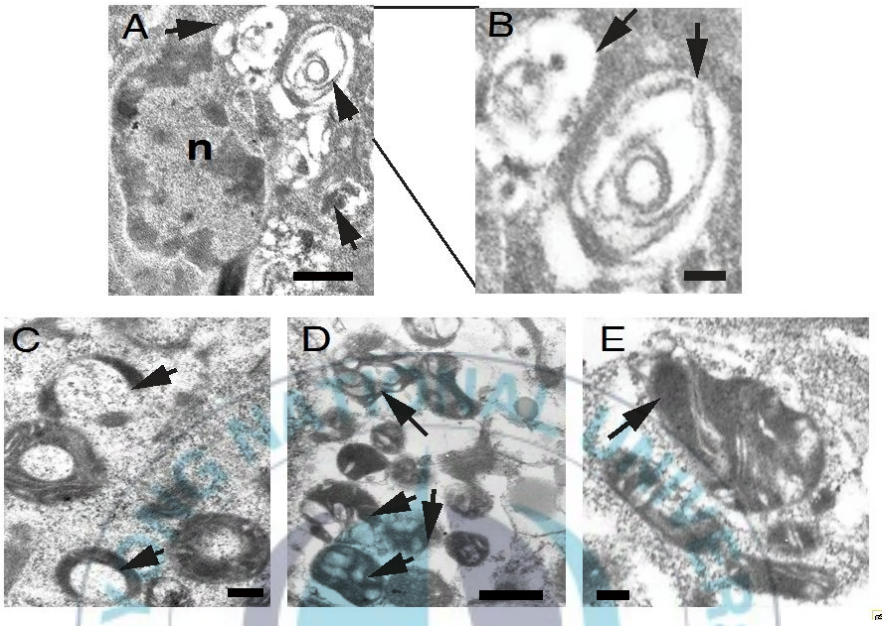

Figure 3.4. Fine structure of autophagosomes in AA-treated A549 cells. Double and multi-membrane autophagosomes were clearly seen in AA-treated cells (A and B). Arrows indicate diverse autophagosome (A ~ D) and mitophagy (E); n means nuclear. Bars in (A) and (D) denote 500 nm and (B), (C) and (E) denote 200 nm.

3.4.3. Induction of autophagy genes in A549 cells

We examined the expression of several autophagy-related genes including Atg5, Atg7, Atg12, LC3, Beclin-1 and DAPK3 (Fig. 3.5). LC3 I and LC3 II strongly increased after 6 hours and maintained a plateau after the cells exposed to AA. The expressions of Atg5-Atg12 complex and Atg7 were increased after the exposure to AA. Additionally, Beclin-1 and DAPK3 also gradually increased in a time-dependent manner in the AA-treated A549 cells.

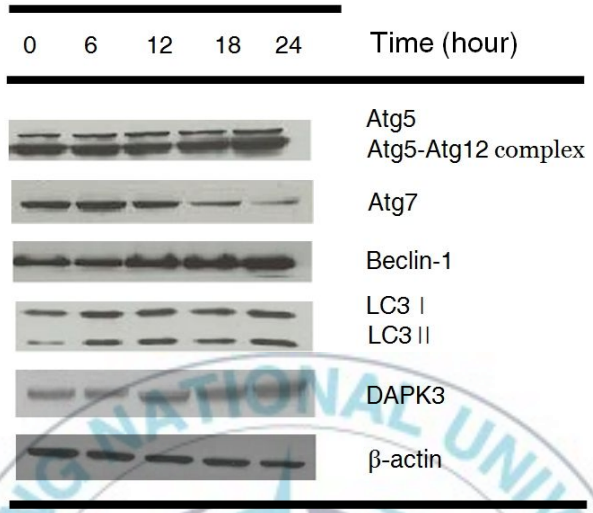

Figure 3.5. Induction of autophagy-related genes in A549 cells by AA. Whole-cell extracts were prepared and analyzed by Western blotting.

3.5. Discussion

In the previous chapter, we discussed the possibility that AA induces ER stress in A549 cells. Our results provide strong evidence to support the view that AA induces ER stress as well as autophagy in A549 cells after starvation. Accumulated unfolded proteins in the ER are removed by the proteasome-involved ERAD system and autophagy [Kruse *et al*., 2006; Werner *et al*., 1996]. In yeast, when the ERAD system is saturated, autophagy plays a role in removing both soluble and aggregated forms of unfolded proteins [Kruse *et al*, 2006]. The ER may assume a key role in the process of autophagy, but at the same time autophagy also takes a role in maintaining ER homeostasis. A recent study revealed that the PERK/eIF2α phosphorylation pathway is critical for ER stress-induced autophagy and the IRE1 signaling pathway is also required for activation of autophagy induced by ER stress [Ogata *et al*., 2006].

DAPK is a calcium/calmodulin-regulated serine/threonine protein kinase, which has been identified as a mediator of autophagic or apoptotic cell death [Cohen *et al*., 1997; Gozuacik *et al*., 2008]. DAPK is also known as a mediator of endoplasmic reticulum stress-induced caspase activation and autophagic cell death [Gozuacik *et al*., 2008]. DAPK phosphorylates the BH3 domain of Beclin-1 and promotes dissociation from Bcl-xl [Zalckvar *et al*., 2009]. Activated Beclin-1 positively regulates autophagy by activating type III PI3K protein and the formation of autophagosomes [Cao *et al*., 2009]. Like Beclin-1, microtubule- associated protein light chain 3 II (LC3II) also plays a key role in the process of autophagosome formation and biogenesis through two ubiquitin-like reactions: the conjugation of Atg12 and Atg5,

and the lipidation of LC3 by phosphatidylethanolamine (PE) [Ichimura *et al*., 2000]. In our results, we potentially conclude that the increase of DAPK3 by AA promotes the activation of Beclin-1 and the induction of autophagy. In a situation wherein Atg5 and Atg12 were expressed due to starvation, the influx of AA could induce excessive autophagy by the increasing expression of DAPK3 and other autophagy-inducing genes that cause cells to die. The images of autophagosomes presented by fluorescence microscopy and TEM also give us clear evidences that AA induces autophagy in A549 cells.

In conclusion, AA is a potent ER stressor that disturbs Ca^{2+} homeostasis as well as stimulates autophagy, which eventually leads to cell death. Here, we proposed the potential cell signaling pathways leading to cell death by ER stress and autophagy in AA-treated A549 cells in Fig. 3.6. Any disturbance of ER homeostasis (such as ER-Ca^{2+} depletion) induces ER stress and, to recover it, the UPR response and autophagy are activated. As a result of the failure of cell regulation, ER-stressed cells may undergo cell death through the overexpression of autophagy-related genes and induction of CHOP. For the first time, our research has revealed that AA induces ER stress and autophagy in lung cancer cells. We hope that this research will be helpful in developing a potential drug for lung cancer.

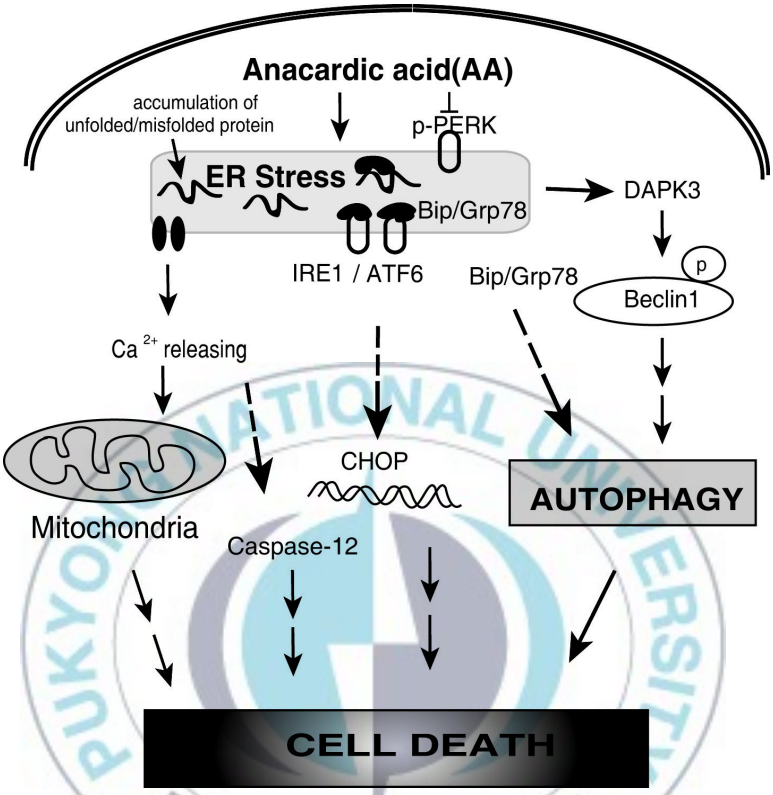

Figure 3.6. Proposal of ER stress and autophagy-mediated cell death pathway in AA-treated A549 cells.

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Chapter 4.

Induction of mitochondrial-mediated apoptosis in the A549 cells by AA

4.1. Abstract

In the prior chapter, we observed the possibility of ER stress and autophagy-induced cell death by AA. This chapter is focused on the induction of apoptosis by AA. To examine the molecular mechanism of cell death, apoptosis, in AA-treated A549 cells, we performed experiments such as transmission electron microscopy (TEM), Western blot analysis, fluorescence-activated cell sorting (FACS), genomic DNA extraction and nuclear staining with 4',6-diamidino-2-phenyl-indole (DAPI). Our results showed the possibility of mitochondrial-mediated apoptosis through the activation of apoptosis-inducing factor (AIF) and an intrinsic pathway executioners such as cytochrome c and Apaf-1. In addition, the expression of pro- and anti-apoptotic Bcl-2 family members also supports our conclusion. For the first time we revealed that AA induces caspase-independent apoptosis with no inhibition of cytotoxicity by pan-caspase inhibitor, Z-VAD-fmk, in A549 cells. This study will be helpful in revealing cell death mechanisms and in developing potential drugs for lung cancer using AA.

4.2. Introduction

Apoptosis is a physiological process characterized by chromatin condensation, nuclear fragmentation as well as the fragmentation and removal of DNA by phagocytosis [Kroemer *et al*., 2009]. Cell death is frequently thought to be 'caspase-independent' when it is not suppressed by pan-caspase inhibitors such as N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk). However, the efficiency of Z-VAD-fmk is different from that of caspases and it also inhibits calpains and cathepsins, especially at high concentrations (10 μM) [Kroemer *et al*., 2009].

Mitochondrial intermembrane flavoprotein AIF was originally characterized as a cell death mediator [Susin *et al*., 1999] and potentially acts as a prognostic marker and a target for radiochemotherapeutic intervention in CH27 human lung carcinoma cells [Leung *et al*., 2005]. AIF translocates from the mitochondria to the cytosol and then moves to the nucleus where it causes peripheral chromatin condensation and large-scale fragmentation of DNA [Susin *et al*., 1999; Ye *et al*., 2002; Zhang *et al*., 2002]. AIF is an important mitochondrial protein involved in both caspase-dependent and -independent pathways [Cregan *et al*., 2004]. One well-known mechanism by which to release AIF from the mitochondria is the activation of poly (ADP-ribose) polymerase-1 (PARP-1), which is a key molecule in AIF-induced cell death and mediates the release and translocation of AIF [Yu *et al*., 2002]. Calpain also takes part in the regulation of AIF cleavage in a Ca^{2+}-dependent context and in mitochondrial release of apoptosis-inducing factor [Cao *et al*., 2007].

While PARP-1 is involved in the release of AIF from mitochondria [Yu *et al*., 2002], heat shock protein 70 (Hsp70) negatively regulates AIF function by inhibiting its translocation to the nucleus [Gurbuxani *et*

al., 2003]. Furthermore, Hsp70 prevents apoptosis by inhibiting a downstream pathway of cytochrome c release, upstream of caspase-3 activation and Apaf-1 apoptosome formation [Gurbuxani *et al*., 2003; Li *et al.*, 2000; Saleh *et al.,* 2000]. Heat shock proteins also block caspase-dependent and -independent apoptosis in Jurkat T cells [Creagh *et al.*, 2000], and a depletion of Hsp70 produces apoptosis-like death in various types of tumor cells, including human oral carcinoma cells [Nylandsted *et al.*, 2002]. Heat shock proteins are important prognostic factors in malignant diseases due to their abundant expression in many cancer cells. Strap is known as the Hsp70 transcription cofactor [Xu *et al.*, 2008].

The experiments reported in this research were designed to investigate the apoptotic properties and the potential cell signaling pathways that lead to cell death in AA treated human lung adenocarcinoma A549 cells.

4.3. Materials and Methods

4.3.1. Cell culture and reagents

A549 (human lung adenocarcinoma), HEK293 (human embryonic kidn ey cell), HepG2 and SK-Hep1 (human hepatocarcinoma) were purchased from the American Tissue Culture Collection (Manassas, VA, USA). Ce lls were cultured in RPMI 1640 (A549), DMEM (HEK293), EMEM (H epG2 and SK-Hep1) (HyClone Laboratories, Logan, UT, USA) medium supplemented with 10% heat inactivated fetal bovine serum (FBS; HyCl one Laboratories), 100 U/ml penicillin and 10 μg/ml streptomycin (PAA Laboratories GmbH, Pasching, Austria) in a humidified atmosphere cont aining 5% CO_2 at 37˚C. A549 cells were incubated in the FBS-free RP MI 1640 medium for 24 h, and then anacardic acid (AA, Calbiochem, San Diego, CA, USA) was added to the medium. Pan-caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.3.2. Cell cytotoxicity assay

The exponential phase of cells was seeded in a 96-well plate at an initial density of $0.5x10^5$–$1x10^5$ cells in medium containing 10% FBS per well of 100 μl. Following 24 h of incubation, AA, dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich), was added to the culture medium at various concentrations. Cells were incubated for 24 h and 10 μl of EZ-Cytox Cell Viability Assay Solution (WST-1™; Daeil Lab Service, Seoul, Korea) was added and incubated for 4 h at 37˚C. The intensity was measured at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

4.3.3. Western blot analysis.

A549 cell lysates were prepared in a Radioimmunoprecipitation assay buffer (RIPA, Cell Signaling Technology, Danvers, MA, USA) and proteins were visualized using enhanced chemiluminescent (ECL) detection solution (Pierce Biotechnology, Rockford, IL, USA). Antibodies include anti-cleaved caspase-3, anti-cleaved caspase-7, anti-cytochrome c, anti-Bax, anti-Bad, anti-Bak, anti-Bcl-XL, anti-cleaved PARP, anti-FoxO1, anti-FoxO3a, anti-FoxO4, anti-calpain, anti-β-actin, secondary rabbit and mouse antibodies conjugated with HRP were purchased from Cell Signaling Technology. Anti-AIF, anti-Hsp70, anti-polyclonal Hsp27, anti-Noxa, anti-STRAP, anti-Bim and secondary antibodies conjugated with HRP rabbit goat were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.3.4. DAPI staining

4',6-diamidino-2-phenylindole (DAPI) staining was used for the morphological observation of apoptosomes. A549 cells were seeded in plates, and the cells were treated with or without AA (3 μg/ml) for 24 h, then the cells were washed with PBS. Two-to-three milliliters of diluted DAPI was added to the cells and incubated for 15 min at 37°C. The cells were rinsed once with methanol and the result was analyzed by fluorescence microscopy using an Eclipse 50i microscope.

4.3.5. FACS analysis

A549 cells were harvested by trypsinization and fixed with ice-cold ethanol (70%) for 5 h at 4°C, followed by resuspending with PBS containing RNase A (0.2 μg/ml) and incubation for 1 h at 37°C. Cells were stained with propidium iodide (40 μg/ml) for 30 min. The

distribution of sub-G1 DNA content was analyzed using the FACS Calibur apparatus (Becton-Dickinson, Mountain View, CA, USA).

4.3.6. Genomic DNA extraction.

For analyzing DNA fragmentation, genomic DNA was extracted using DNeasy Blood and Tissue kit purchased from Qiagen Inc. (Valencia, CA, USA) according to the manufacturer's protocol.

4.3.7. TEM.

For observation of more detailed morphological features, transmission electron microscopy (TEM) was used. Cultured A549 cells were pre-fixed in pellets at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer and then post-fixed with 1% osmium tetraoxide (OsO4) in 0.1 M phosphate buffer, pH 7.4. After fixing, cells were embedded in Epon 812 using routine procedures. Approximately 70 μm ultrasected specimens by Ultracut Reichert-jung were stained using uranyl acetate and lead citrate, and examined with Hitachi H600 TEM (Tokyo, Japan). All reagents used in the TEM experiment were purchased from Electron Microscopy Science (EMS).

4.4. Results

4.4.1. Cytotoxicity of AA.

In order to investigate the cytotoxicity of AA, normal cells (HEK293), and lung (A549) and liver (HepG2, SK-Hep1) cancer cells were treated with AA in a dose-dependent manner. After 24 h of exposure, the results of the cell viability assay showed that AA inhibits the proliferation of all cells used. Although AA inhibited cellular proliferation of HEK293 cells, the cytotoxicity was less than in cancer cells (Fig. 4.1). Considering our interest in lung cancer, we used A549 cells for further research and 3.0 μg/ml of AA was used for investigating the molecular mechanism of cell death in A549.

To study the effect of the caspase inhibitor, cells were pre-treated for 2 h with the cell-permeable and irreversible pan-caspase inhibitor Z-VAD-fmk (100 μM) and then exposed to AA for 24 h in the continued presence of Z-VAD-fmk. Viability was then assessed by MTT assay using WST-1™ and the results showed that Z-VAD-fmk did not inhibit the cell viability in AA-treated A549 cells (Fig. 4.2).

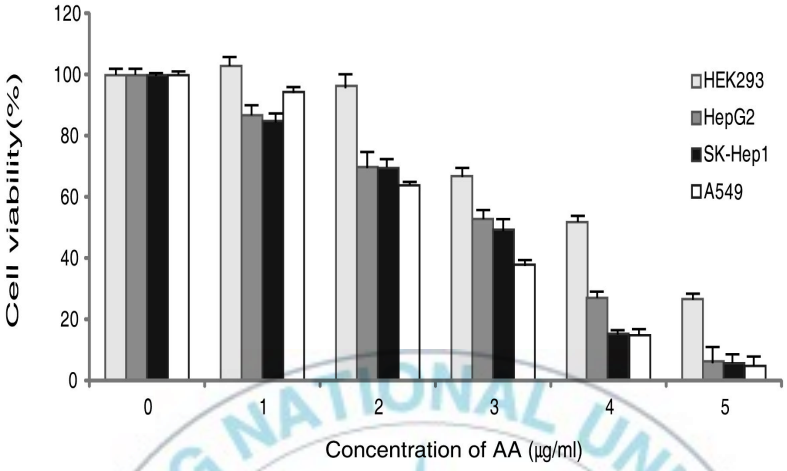

Figure 4.1. Cell viability in AA-treated cell lines. (A) Cell viability was examined with a WST-1 assay. Cell growth was inhibited on a dose-dependent manner in AA-treated cells.

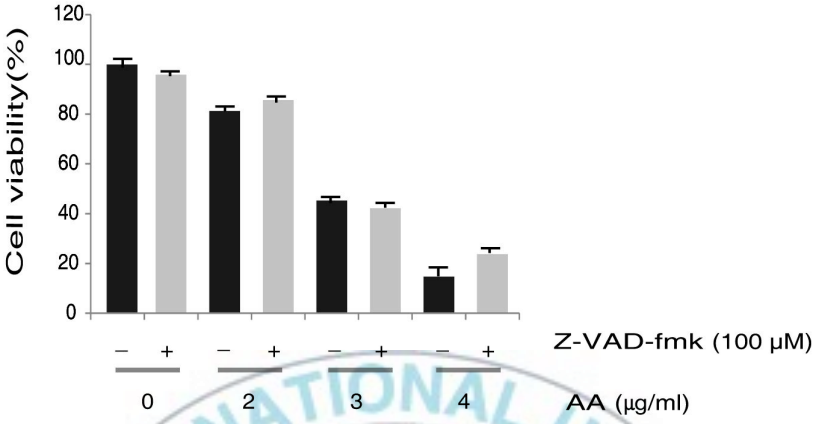

Figure 4.2. Cell viability in AA-treated A549 cells by pan-caspase inhibitor Z-VAD-fmk. The A549 cells were pre-treated for 2 h with the cell permeable and irreversible pan-caspase inhibitor Z-VAD-fmk (100 μM) and then exposed to 3.0 μg/ml of AA for 24 h in the continued presence of Z-VAD-fmk. Viability was then assessed by WST-1 assay.

4.4.2. The formation of apoptosomes in A549 cells by AA.

In order to further elucidate the nature of AA-induced cell death in A549, we investigated the nuclear morphology of AA-treated cells by using 4',6-diamidino-2-phenylindole (DAPI) staining (Fig. 4.3). The results showed totally different patterns between the AA-untreated (A) and -treated cells (B). AA-treated cells exhibited the formation of apoptosomes and nuclear fragmentation, indicating that AA induces apoptosis in A549 cells.

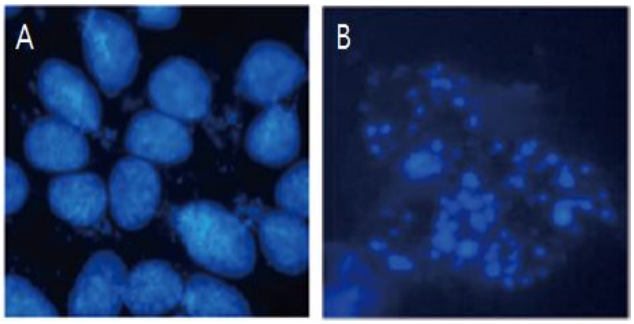

Figure 4.3. Induction of apoptosis in AA-treated A549 cells. The formation of apoptosomes by AA was investigated by DAPI staining; (A) untreated cells; (B) apoptosomes were seen in the cells treated with 3.0 μg/ml of AA for 24 h.

4.4.3. The induction of chromosomal DNA fragmentation in A549 cells by AA.

For further evidence of apoptosis induction, we analyzed the fragmentation of chromosomal DNA by using agarose gel electrophoresis (Fig. 4.4). The fragmentation of chromosomal DNA by AA increased according to the exposure time (Fig. 4.4 B, C and D), while it almost did not appear in the untreated cells to compare with the treated cells (Fig. 4.4 A).

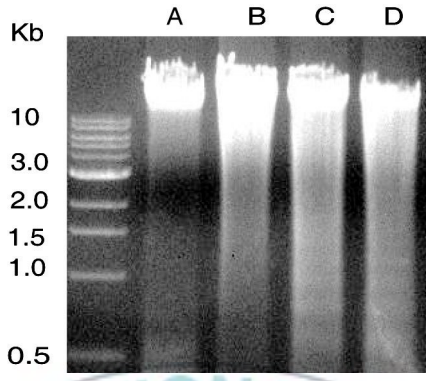

Figure 4.4. DNA fragmentation assay; (A) untreated cells; (B-D) AA- treated cells for 6, 12 and 24 h respectively. The results were obtained by 1.5% agarose gel electrophoresis.

4.4.4. The change of sub-G1 DNA content in AA-treated A549 cells.

Additionally, to study cell death induced by AA, we quantified the sub-G1 DNA content by fluorescence-activated cell sorting (FACS) analysis. After exposure to AA, the sub-G1 genomic DNA content was gradually increased at 6 h, 12 h and 24 h to 7.58%, 14.01% and 38.79% respectively (Fig. 4.5).

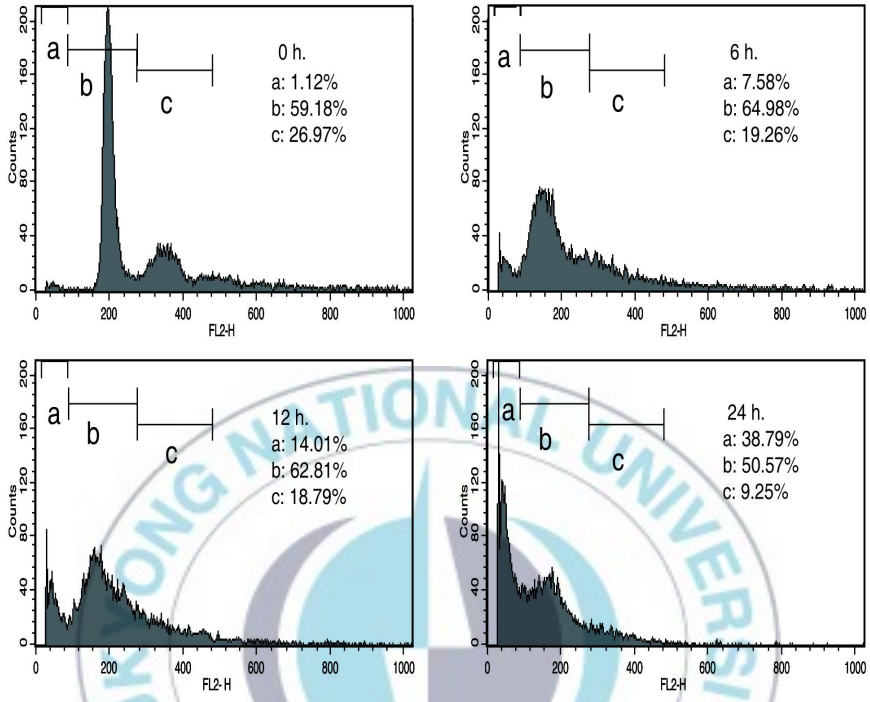

Figure 4.5. Quantification of sub-G1 DNA content in A549 cells. Cells treated with 3.0 μg/ml of AA for 0 h, 6 h, 12 h and 24 h were analyzed by FACS.

4.4.5. Chromatin condensation and nuclear cleavage in A549 cells by AA

TEM also showed apoptotic features such as chromatin condensation and nuclear fragmentation (Fig. 4.6 B). These results support the view that AA plays a major role in apoptosis induction in A549 cells.

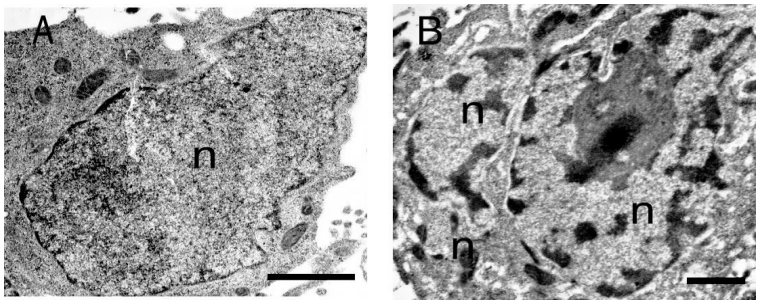

Figure 4.6. Induction of chromatin condensation and nuclear cleavage by AA under TEM. (A) untreated cells; (B) cells treated with 3.0 μg/ml of AA for 24 h; n, nuclear. Bars denote 1 μm.

4.4.6. The induction of apoptosome formation-related genes in A549 cells by AA

To determine the apoptosis-signaling pathway induced by AA, we analyzed the expression of several genes using Western blot analysis. Cleaved caspase-3, cleaved caspase-7, Apaf-1, caspase-9 and cytochrome c, known as the executioners of the intrinsic pathway. All proteins gradually increased in a time-dependent manners in AA-treated A549 cells (Fig. 4.7).

In addition, the expression of pro- and anti-apoptotic Bcl-2 family was determined. Pro-apoptotic members of the Bcl-2 family, Bim, Bad, Bak and Noxa increased, while the anti-apoptotic member, Bcl-xL, decreased (Fig. 4.7). The expression of Bak and Bad showed a gradual, time-dependent increase and the expression of Bax and Noxa showed even more increased levels at 12 h after AA exposure. Bim has three isoforms (BimS, BimL and BimEL), each with different intrinsic toxicities that promote apoptosis [O'Connor *et al*., 1998]. The cleaved form of Bim appeared at 6 h and the expression of Bcl-xl decreased gradually until 18 h after AA exposure (Fig. 4.7).

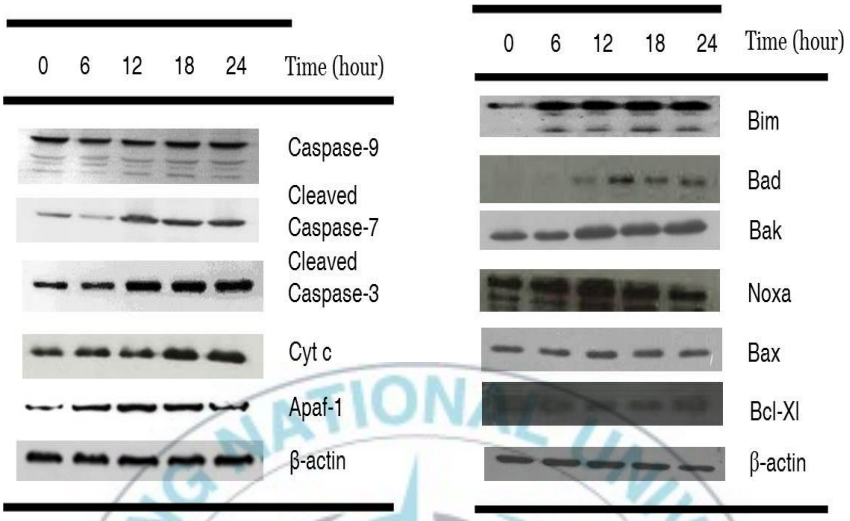

Figure 4.7. Time-dependent expression of intrinsic apoptosis related-proteins and pro/anti-apoptotic Bcl-2 family proteins in AA-treated A549 cells. Whole-cell extracts were prepared and analyzed by Western blotting.

4.4.7. Effects of AA on AIF-related cell death signaling pathway.

Because the inhibition of the pan-caspase inhibitor, Z-VAD-fmk, failed to prevent cell death, we analyzed the possibility of a caspase-independent pathway by apoptogenic molecules, namely, apoptosis-inducing factor (AIF). Poly (ADP-ribose) polymerase-1 (PARP-1), the mediator of AIF release, was also investigated. The expression of AIF showed a gradual increase, as did cleaved PARP-1 after 18 h (Fig. 4.8).

4.4.8. Decrease of the chaperone protein.

We examined the expression of several genes that encode proteins known as molecular chaperones by assisting the correct folding of nascent and stress-accumulated misfolded proteins [Beckmann *et al*., 1990]. We investigated the expression of Hsp70 and Strap, the stress-responsive activator of p300, and the results showed down-regulation of Hsp70 and Strap by AA (Fig. 4.8).

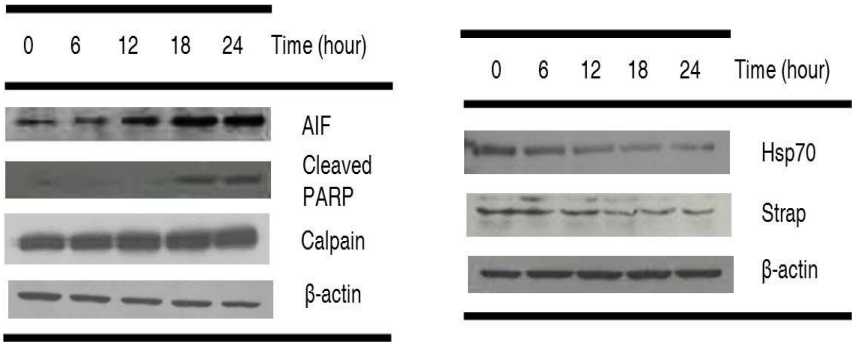

Figure 4.8. Time-dependent expression of the AIF signaling pathway-related proteins and anti-apoptotic chaperone protein, Hsp70, and its cofactor in AA-treated A549 cells. Whole-cell extracts were prepared and analyzed by Western blotting.

4.4.9. Activation of forkhead transcription factors by AA.

Members of the non-phosphorylated mammalian forkhead transcription factors (FoxOs) are involved in regulating the expression of genes associated with apoptosis. FoxO1, FoxO4 and FoxO3a are known as mammalian forkhead transcription factors and trigger the up-regulation of proteins such as Bim and NOXA [Obexer *et al*., 2007]. The expression of FoxO1, FoxO4 and FoxO3a in AA-treated A549 cells all increased with slightly different optimal expression times being depending on the subfamily (Fig. 4.9).

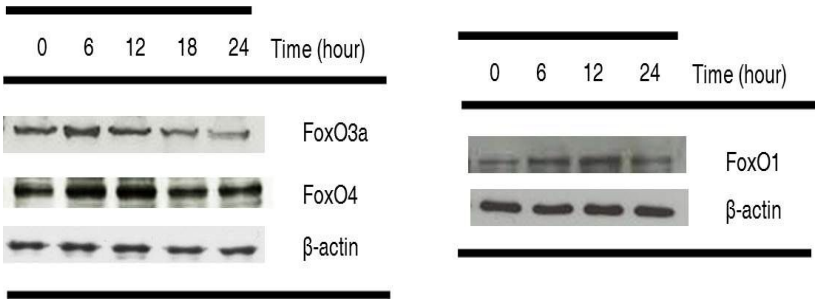

Figure 4.9. Time-dependent expression of FoxOs in AA-treated A549 cells. Whole-cell extracts were prepared and analyzed by Western blotting.

4.5. Discussion

This chapter focused on findings a cell signaling pathway leading to death in A549 cells by AA. The results provide strong evidence to support the view that AA induces apoptosis in a caspase-independent manner with no inhibition of cytotoxicity by pan-caspase inhibitor, Z-VAD-fmk, in A549 cells. In addition to the morphological features shown by TEM and microscopy, FACS analysis and the analysis of gene expression by western blotting demonstrate the induction of apoptosis by AA.

Previous research has reported that the release of cytochrome c from the mitochondria is an early event during apoptosis, pro-apoptotic Bcl-2 family members induce the release of cytochrome c and anti-apoptotic Bcl-2 proteins inhibit the release of cytochrome c [Kluck *et al*., 1997; Yang *et al*., 1997]. The balance between the pro-apoptotic (Bid, Bad, Bim, Bax, Bak and Noxa) and anti-apoptotic (Bcl-2, Bcl-xL, A1 and Bcl-w) Bcl-2 protein families is an important factor contributing to cytochrome c release, and in determining cell fate [Green and Amarante-Mendes, 1998; Li *et al*., 1997].

Following cytochrome c release, caspases are activated and the cell undergoes apoptosis via the formation of apoptosomes, Apaf-1/caspase-9 complex [Li *et al*., 1997].

Bax and Bak are also known to promote apoptosis by modulating ER and mitochondrial Ca^{2+} stores [Nutt *et al.*, 2002]. Therefore, we are also studying the possibility of apoptosis by ER stress. Increased expression of Bak may promote Ca^{2+} mobility in the cytosol.

The pro-apoptotic BH3-only protein Bim induces cell death by binding to the anti-apoptotic Bcl-2 family protein and Noxa is known

as a mediator of p53-induced apoptosis [Oda *et al*., 2000]. The expression of Bim and Noxa is regulated by the transcription factor Forkhead (FKHR) in the rhabdomyosarcoma family, which includes FoxO [Obexer *et al*., 2007]. FoxO transcription factors modulate the expression of genes involved in apoptosis, cell cycle, cell differentiation and other cellular functions [Huang and Tindall, 2007].

In this study, AA induced the expression of the pro-apoptotic Bcl-2 family proteins, Bim, Bad, Bak, Bax and Noxa and cytochrome c, while it reduced the expression of the anti-apoptotic Bcl-2 family protein, Bcl-XL, in A549 cells. The expression of FoxOs increased on AA-treated A549 cells. These results showed the possibility that the increase of pro-apoptotic BH3-only protein, Bim and Noxa by FoxOs and the decrease of anti-apoptotic protein, Bcl-XL, induce disruption of the outer mitochondrial membrane in AA-treated A549. Disruption of the mitochondrial membrane may induce the release of proapoptotic mediators such as AIF and cytochrome c from mitochondria. The expression of Apaf-1, cytochrome c, caspase-9, caspase-7 and caspase-3 show a gradual increase, thus proposing the possibility that AA induces cell death through the intrinsic apoptotic pathway.

Because pan-caspase inhibitor, Z-VAD-fmk, did not affect the result of the cytotoxicity, we examined the possibility of apoptosis by AIF-related cell death signaling pathway. As a phylogenetically conserved mitochondrial flavoprotein, AIF plays a key role in apoptosis through chromatin condensation and large-scale DNA fragmentation after translocation from the mitochondria to the nucleus [Susin *et al*., 1999; Ye *et al*., 2002].

The expression of AIF and PARP-1, which mediates of its release and translocation from mitochondria, increased, and Hsp70, a negative

regulator, decreased. Furthermore, the expression of calpain also exhibited a slight, gradual increase. Current results show the possibility that the decrease of Hsp70 and increase of pro-apoptotic protein AIF and PARP-1 promote chromatin condensation and DNA fragmentation causing the induction of apoptosis in AA-treated A549 cells. We suggest the potential apoptosis pathway induced by AA in A549 cells (Fig. 4.10).

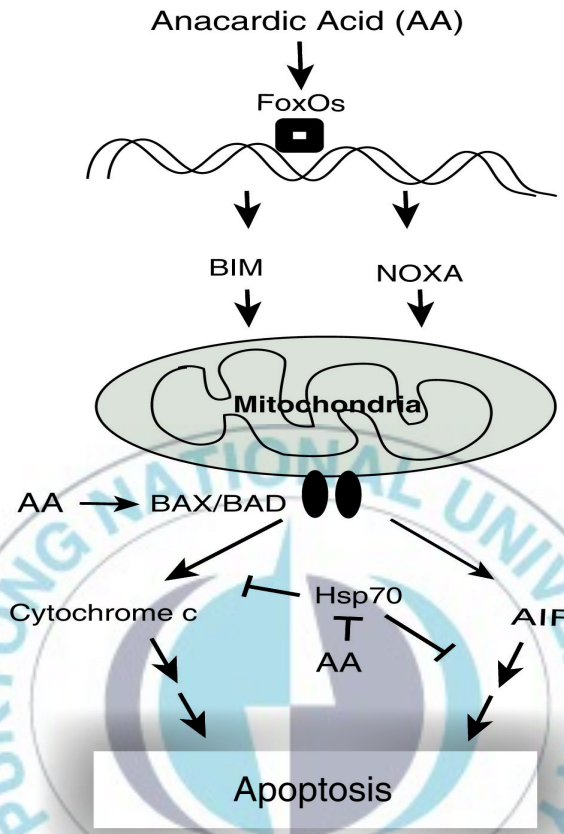

Figure 4.10. Potential apoptosis pathway in AA-treated A549 cells. The release of the intermembrane pro-apoptotic factors from the mitochondria by imbalances between pro- and anti-apoptotic Bcl-2 family members, decrease of Hsp 70 and increase of AIF could all play a key role in mitochondrial-mediated apoptosis in AA-treated A549 cells.

4.6. Conclusion

The purpose of this research is to examine the cytotoxic effect of AA on cancer cells and to investigate the potential cell death pathway caused by AA in A549 cells.

In starved condition, AA induces ER stress, autophagy and apoptosis. ER stress-induced cell death pathway in AA-treated A549 cells is characterized by the disruption of Ca^{2+} homeostasis, up-regulation of Bip/GRP78 and UPR sensors such as IRE1 and ATF6, down-regulation of the p-PERK pathway, activation of caspase-12 and GADD153.

In addition, over-activated autophagy by AA shows features such as the over-expression of autophagy-related genes including Beclin-1 and DAPK3 and the formation of autophagosomes. Furthermore, apoptosis in AA-treated A549 cells is characterized by the mitochondral-mediated intrinsic pathway and caspase-independent pathway with no inhibition of cytotoxicity by pan-caspase inhibitor, Z-VAD-fmk. The release of the intermembrane pro-apoptotic factors from the mitochondria by imbalances between pro- and anti-apoptotic Bcl-2 family members, decrease of Hsp70 and increase of AIF could all have a key role in apoptosis in AA-treated A549 cells.

In conclusion, based on our results, we suggest the potential signaling pathway leading to death in AA-treated A549 cells. All factors may effect the cell death through the integrated regulation of ER stress, autophagy and apoptosis. In addition, the degree of stress in the ER may be a turning point between life and death in AA-treated cells.

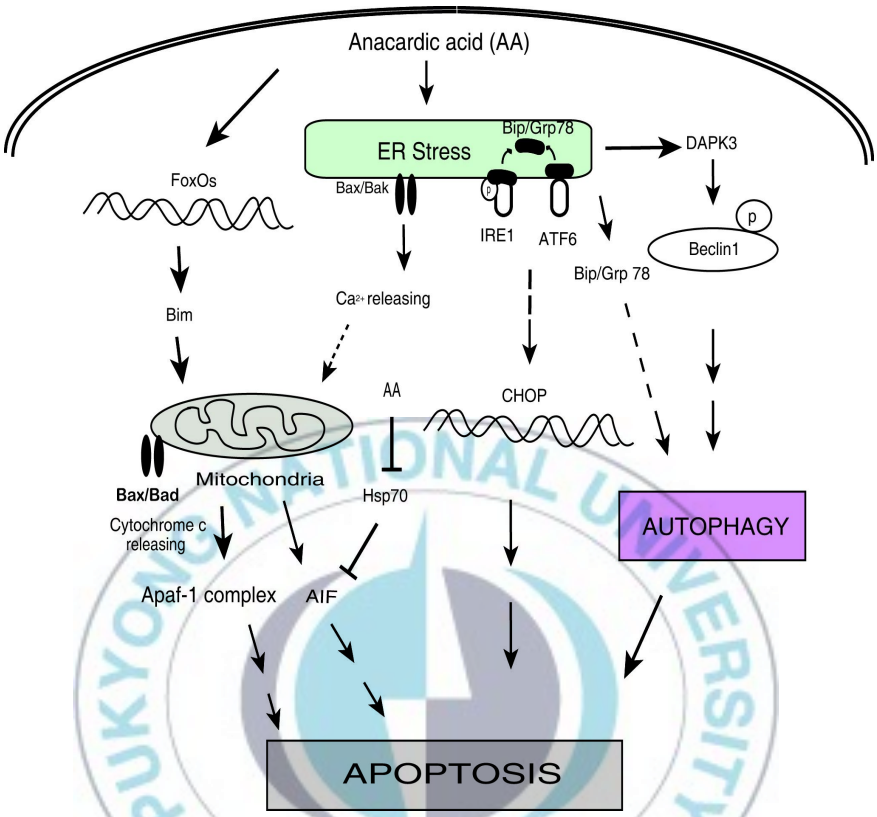

Figure 4.11. Proposal of integrated cell death signaling pathway in AA-treated A549 cells. The treatment of A549 cells with AA may trigger irreversible catastrophe, cell death, through the integration of ER stress, UPR, autophagy and apoptosis. (Signaling pathways key : → direct stimulatory modification; ⊣ direct inhibitory modification; →→ multistep stimulatory modification; ⇢ tentative stimulatory modification).

4.7. References

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국문 요약

Anacardic acid (2-hydroxy-6-pentadecylbenzoic acid, AA)는 캐슈넛의 껍질 성분으로 은행나무를 포함한 여러 종류의 식물들에서 발견되고 있다. AA는 항균효과와 항암효과 등 많은 유용한 성질을 가지고 있으며, 멕시코 등의 지역에서 위염, 위궤양, 위암 등의 치료를 위한 민간요법에 널리 이용되고 있으며 여러 연구자들에 의해 다양한 약리작용이 밝혀져 있다. AA의 NF-κB 조절 유전자 발현 억제 효과는 알려져 있으나, AA를 처리한 암세포가 죽음에 이르는 분자기전에 대해서는 거의 알려져 있지 않다. 그리하여 AA의 항암효과와 AA를 처리한 암세포의 사멸을 유도하는 신호전달 경로를 밝히기 위해 본 연구를 수행하였다.

본 연구에 이용된 모든 암세포에 대한 AA의 처리는 세포에 따라 차이는 있었으나, 모두 세포독성을 보였다. 특히, 본 연구는 폐암에 연구의 초점을 두고자 폐의 기저 상피에서 유래한 폐선암세포인 A549 세포를 이용하여 세포사멸에 이르는 분자기전을 연구하였다.

실험결과, AA가 UPR (unfolded protein response)과 ER (endoplasmic reticulum) stress, autophagy, apoptosis를 유발한다는 것을 관찰할 수 있었다. Ca^{2+} 분포의 변화와 전자현미경 관찰, Western blot, gene transfection, fluorescence activated cell sorting (FACS), 4', 6-diamidino-2-phenylindole (DAPI) 염색 등의 결과는 AA를 처리한 A549에서 UPR과 ER stress, autophagy, apoptosis가 일어남을 보여주는 증거들을 제공하였다.

ER로부터의 세포질내로의 Ca^{2+}유출과 ER stress 센서인 p-PERK의 감소로 인해 misfold되거나 unfold된 단백질이 ER 내강에 축적됨으로써, ATF6의 활성화와 IRE1α의 발현이 증가하여 UPR 경로가 활성화되었을 것으로 여겨진다. 또한, ER stress로부터 세포를 보호하기 위한 autophagy 관련 단백질들의 발현 뿐 아니라, AA에 의한 Beclin-1과 DAPK3의 지속

적인 증가가 autophagy를 심화시켜 세포사멸에 이르게 했을 것으로 생각된다.

특히 apoptosis는 광범위 caspase 저해제인 Z-VAD-fmk에 의해 그 세포독성이 영향을 받지 않았으며, 따라서 caspase 비의존적 특징과 두 가지 다른 경로에 의해 apoptosis가 유도될 가능성을 보여주었다. 실험결과, ER stress 후 UPR 활성화에 의한 CHOP이 관련된 경로와 apoptosis-inducing factor (AIF)와 cytochrome c와 같은 인자들이 활성화되는 미토콘드리아가 관여하는 내재성 경로임을 규명할 수 있었다.

본 연구는 AA가 폐암세포에서 ER stress와 autophagy를 유발한다는 것을 최초로 규명하였으며, AA를 처리한 A549 세포에서 일어나는 유효 세포사멸 경로를 제안하였다. 이 연구가 세포사멸 기전을 밝히고 폐암치료를 위한 약제 개발에 도움이 되기를 기대한다.

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살면서 저는 참 인복이 많다는 것을 느낍니다. 짧지 않은 시간 동안 많은 후배님들과 만남의 기쁨과 이별의 아쉬움을 겪었으며, 그들 모두가 포기하지 않고 계속 연구할 수 있는 힘이 되어 주었습니다. 먼저 실험실 생활에 적응할 수 있도록 모든 것을 도와준 철우, 자신들의 실험과 연구로 바쁜 와중에도 항상 웃으며 진심으로 도와주고 양보해 준 실험실의 보물들, 진수, 현일, 호진, 지영, 상보, 성자, 미정, 순진, 초원, 난희, 우석, 덕현, 은수, Kasin, 창원, 민석, 종용이, 지친 나에게 언제나 해맑은 웃음과 즐거움을 준 신미, 해진과 민재에게도 진심으로 고마움을 전합니다. 그리고 바이러스 실험실의 경용, Jeeva, 상은이 생화학실의 용배, 근우, 태혁이 미생물 유전학 실험실의 인형에게도 고마움을 전합니다. Coy에게 응원을 보내며, 실험실의 새로운 주인이 될 지연, 또 다른 민재, 영익, 전일, 기일, 형욱이에게도 진심어린 성원을 보냅니다.

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