# Studies on the preventive effects of chalcones on obesity and insulin resistance

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**DOCTORAL DISSERTATION**

# Studies on the preventive effects of chalcones on obesity and insulin resistance

**January 2014**

*Graduate School of Agricultural Science, Kobe University*

**Tianshun Zhang**

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# カルコンの肥満およびインスリン抵抗性予防効果に関する研究

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**ABBREVIATIONS USED**

(+)-*anti*-BaPDE, (+)-*anti*-7,8-dihydroxy-9,10-tetrahydrobenzo[a]pyrene

2-AA, 2-aminoanthracene

4HD, 4-hydroxyderricin

4NQO, 4-nitroquinoline-1-oxide

8-OHdG, 8-hydroxy-2'-deoxyguanosine

8-SHG, 8-mercaptoguanosine

AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride

ACC, acetyl CoA carboxylase

ACOX1, acy-CoA oxidase X1

AF-2, 2-(2-furyl-3-(5-nitro-2-furyl) acrylamide

AhR, aryl hydrocarbon receptor

ALP, alpinetin

AMP, adenosine 5'-monophosphate

AMPK, AMP-activated protein kinase

ARE, antioxidant response elements

Arnt, AhR nuclear translocator

B(a)P, benzo[a]pyrene

BE, black soybean seed coat extract

C/EBP, CCAAT/enhancer-binding protein

C3G, cyanidin 3-glucoside

CaMKKβ, Ca(2+)/CaM-dependent protein kinase kinase β

CAR, cardamonin

CD36, cluster of differentiation 36

ChREBP, carbohydrate-responsive element-binding protein

CPT-1, carnitine palmitoyltransferase-1

CYP1A1, cytochrome P4501A1

DRE, dioxin responsive element

EC, epicatechin

ERK, extracellular signal-regulated kinase

FAS, fatty acid synthase

FA, fatty acid

FKB, flavokawain B

GLUT4, glucose transporter type 4

GPAT-1, glycerol-3-phosphate acyl transferase-1

GST, glutathione S-transferase

HF, high fat

JNK, c-jun amino-terminal kinase

LC-MS/MS, liquid chromatography-mass spectrometry-mass spectrometry

LKB1, liver kinase B1

MAPK, mitogen-activated protein kinase

MN assay, micronucleus assay

NAFLD, nonalcoholic fat liver disease

NEFA, non-esterified fatty acid

Nrf2, nuclear factor-erythroid 2-related factor 2

p38, p38 MAPK

PC4-1, EC-(4β→6)-EC-(4β→8)-EC-(4β→8)-EC

PC4-2, cinnamtannin A2

PCB1, procyanidin B1

PCB2, procyanidin B2

PCC, procyanidin C

PC-rich BE, procyanidin-rich BE

PPARα, peroxisome proliferator-activated receptor alpha

PPARγ, peroxisome proliferator-activated receptor gamma

ROS, reactive oxygen species

RXRα, retinoid X receptor α

SREBP-1, sterol regulatory element binding protein-1

UCPs, uncoupling proteins

XAG, xanthoangelol

## General introduction

### Preface

Diet and lifestyle are closely related to human health [Hu *et al*., 2001; Knoops *et al*., 2004]. For example, the associated genotoxic carcinogens for some types of cancers and heart diseases causation are produced during the broiling and frying of creatinine-containing foods such as meats: The fat intake is associated with a higher incidence of the nutritionally linked metabolic syndrome and cancers [Weisburger, 2002]. Resent years, the composition of diet is changing dramatically and associated with excess intake of energy-dense foods and physical inactivity. The proportion of energy from fats and oil, and animal proteins has increased. In contrast, the amount of time spent on physical activity has been decreasing year-by-year [Eaton, 2006]. These lifestyle changes lead to an increase in patients suffering from life-style-related disease including obesity, nonalcoholic fatty liver disease (NAFLD), insulin resistance and even cancers. To prevent life style-related disease, natural products offer a bonanza for the development of novel therapeutic agents used to treat a variety of human diseases [Williamson and Manach, 2005]. There is currently a growing interest in the use of compounds from natural sources. Especially, polyphenols showed the prevention of obesity, diabetes, cardivascular disease, cancers and other diseases [Scalbert *et al*., 2005].

Among the polyphenols, the author focused on the chaclcones 4-hydroxyderricin (4HD), xanthoangelol (XAG), cardamonin (CAR) and flavokawain B (FKB). The chemical structures of these chalcones were shown in Fig. G1. Each has an aromatic ketone and an enone that forms the central core for a variety of important biological compounds. Chalcones were originally isolated from natural sources and have a variety of biological properties, including antioxidant, anti-inflammatory and anticancer activities [Dimmock *et al*., 1999; Birari *et al*., 2011]. Several synthetic chalcone derivatives are also known to inhibit diabetic complications [Lim *et al*., 2001]. However, the molecular mechanisms of chalcones on prevention of obesity and insulin resistance are still not yet fully understood. In this dissertation, the author introduced the novel molecular targets of 4HD, XAG, CAR and FKB for prevention of obesity and insulin resistance. In the next part, the author explained obesity and metabolic syndrome briefly as a background of this dissertation.

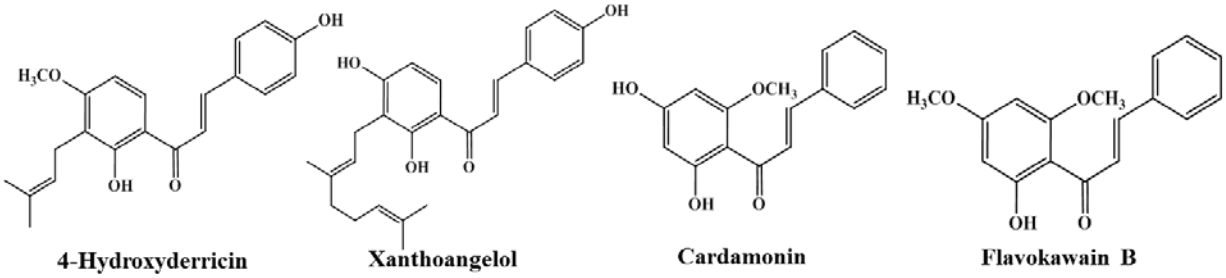


Fig. G1. The chemical structures of 4-hydroxyderricin, xanthoangelol, cardamonin and flavokawain B.

**Obesity and metabolic syndrome**

Obesity has increased at an alarming rate in recent years and is now a worldwide public health problem. Obesity is defined as an abdomen increase in fat, though obtaining not necessarily in body weight gain. It may have an adverse effect on health, leading to reducing life expectancy and increased health problems [Haslam *et al*., 2005]. Obesity is not a single disorder but a heterogeneous group of conditions with multiple causes. Body weight is determined by an interaction between genetic, environmental and psychosocial factors acting through the physiological mediators of energy intake and expenditure [Friedman *et al*., 2000]. At an individual level, a combination of excessive food energy intake and a lack of physical activity are thought to explain most cases of obesity. A limited number of cases are due primarily to genetics, medical reasons or psychiatric illness [Bleich *et al*., 2008].

Obesity is a medical condition, in which body fatness is increasing accompanied by profound changes in physiological function of adipose tissue, skeletal muscle and liver. These changes are, to a certain extent, dependent on the regional distribution of adipose tissue. The intra-abdominal visceral deposition of fat in adipose tissue is a major contributor to the development of elevated plasma insulin and insulin resistance, diabetes, NAFLD and other metabolic disease [Friedman *et al*., 2000; Reaven *et al.*, 2011]. Skeletal muscle is the most important tissue for maintaining glucose homeostasis under insulin-stimulated conditions [DeFronzo *et al.*, 1988]. Moreover, it is known that skeletal muscle is a primary site of insulin resistance in type2 diabetes mellitus [Bogardus *et al.*, 1984; Bonadonna *et al.*, 1990, Bouzakri *et al*., 2005]. Under the condition of obesity, the inflammation adipockines and free fatty acids (FAs), which secreted from adipose tissue, decreased skeletal insulin sensitivity with reduced glucose uptake and increased the risk of insulin resistance, [Kim *et al*., 2004; Ueki *et al*., 2004; Friedman *et al*., 2000]. Liver is a pivotal organ in regulating the metabolism especially

lipid metabolism. Obesity is associated with an increased risk of steatosis in liver. Under the conditions of obesity, the rate of hepatic FA uptake from plasmas and *de novo* FA synthesis are greater than the rate of FA oxidation and export. The excessive amount of intrahepatic triglyceride represents an imbalance between complex interactions of metabolic events, which increased the risk of NAFLD [Targher *et al.*, 2007; Ahmed *et al.*, 2009]. The obesity and the profound changes in physiological functions of adipose tissue, skeletal muscle and liver are leading to the metabolic syndrome.

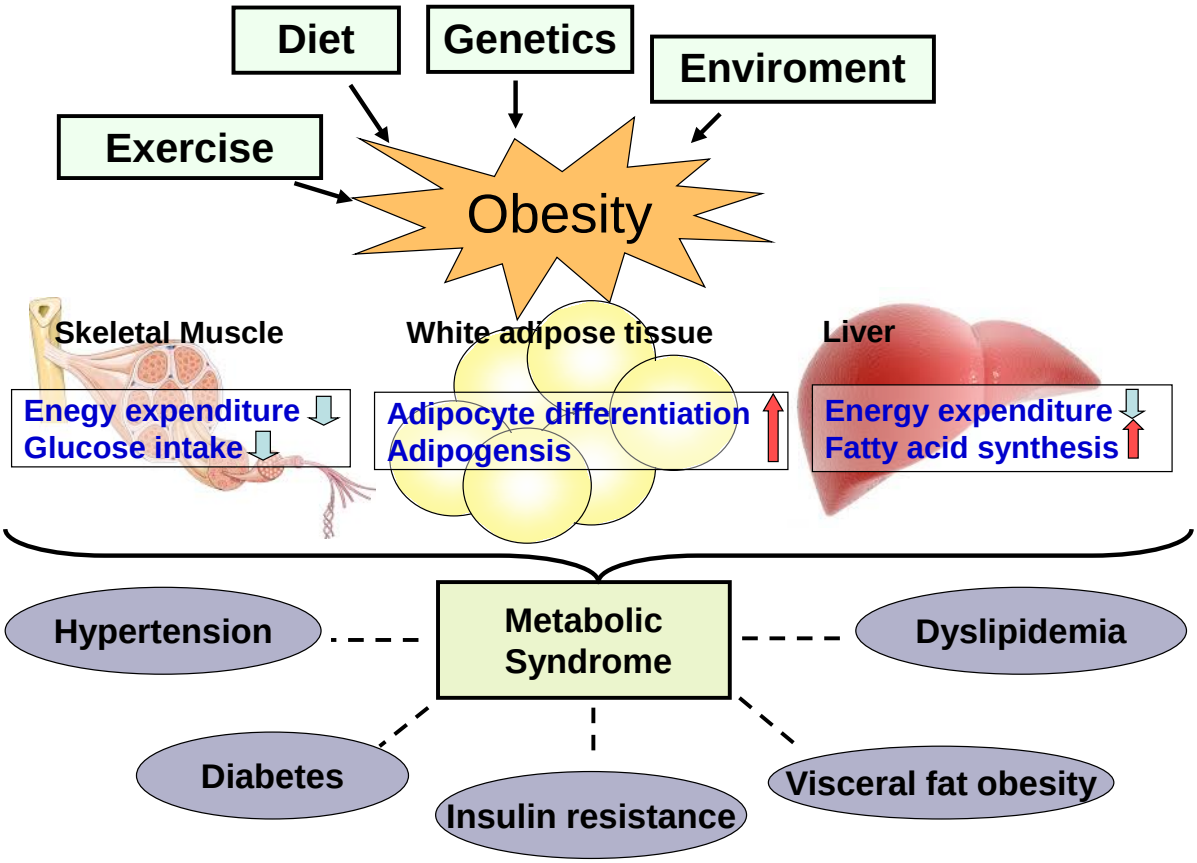


Fig. G2. Obesity and metabolic syndrome.

The metabolic syndrome is a common metabolic disorder. The relationship of obesity and metabolic syndrome is illustrated in Fig. G2. The metabolic syndrome can be characterized by a cluster of manifestations, such as insulin resistance, hypertension, NAFLD, and glucose intolerance. These medical disorders increase a risk of developing cardiovascular diseases and diabetes mellitus [Eckel *et al.*, 2005; Lakka *et al.*, 2002; Marchesini *et al.*, 2003]. Obesity is associated with a significant risk factor for metabolic syndrome [Hajer *et al.*, 2008;

Ligibel, 2011]. Thus, weight loss has been recognized to the major health beneficial way for overweight people and also increases life expectancy in people having metabolic syndrome [Goldstein, 1992]. Weight control medications are becoming popular in today's society, but all the medications controls have side effects [Bray *et al.*, 2009]. Therefore, safe and effective weight loss methods are expected. Certain phytochemicals have been reported to prevent and reduce the risk of obesity [Gonzalez-Castejon and Rodriguez-Casado, 2011]. Dietary phytochemicals may be another cure for weight control and management of metabolic syndrome.

**Adipocyte differentiation**

Adipocyte differentiation is deeply involved in the onset of obesity. 3T3-L1 cells, isolated from non-clonal Swiss 3T3-L1 cells, supply for a well-characterized cell culture model for the study of adipocyte-specific differentiation [Green *et. al.*, 1975]. During adipogenesis, fibroblast-like preadipocytes differentiate into lipid-laden and insulin-responsive adipocytes. This process occurs in several stages and involves a cascade of transcription factors. Among them, peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) are considered to be the master regulator or the crucial determinants of adipocyte fate [Tontonoz *et al*., 1994; Lefterova *et al*., 2009]. The role of PPARγ as the master regulator of adipogenesis is supported by overwhelming evidence from both *in vivo* and *in vitr*o studies. Important early evidence of the critical role of PPARγ in regulating adipogenesis came from Spiegelman and collaborators [Tontonoz *et al*., 1994; Tontonoz *et al.*, 1994], who had investigated to elucidate the transcription factors regulating expression of the adiposes pecific FA binding protein aP2/FABP4. Similar to a subset of other nuclear receptors, PPARγ binds DNA as a heterodimer with retinoid X receptor α (RXRα). Analysis of the regulatory regions of PPARγ target genes has identified a PPARγ-response element, the consensus sequence of which consists of two direct repeats of the hexamer 'AGGTCA' separated by a single nucleotide (called a 'DR-1' sequence) [Lefterova *et al*., 2009; Tontonoz and Spiegelma, 2008]. On the other hand, C/EBPα functions as a principal player in adipogenesis also resulted from gain-of-function studies in cultured cells [Freytag *et al*., 1994] as well as establishment of appropriate knockout mice. Whole-body C/EBPα-knock mice, which die shortly after birth because of liver defects and hypoglycemia, fail to accumulate lipid in white or brown

adipocytes [Wang *et al.*, 1995]. Before the discovery of PPARγ and C/EBPα as the master regulators of adipogenesis, many researchers tried to identify the mechanism of adipocyte differentiation. The cascade of transcription factors eventually leads to expression of PPARγ and C/EBPα, which is established by McKnight and associates [Cao *et al.*, 1991; Yeh *et al.*, 1995]: two other members of the C/EBP family, C/EBPβ and C/EBPδ, are expressed earlier than C/EBPα during adipogenesis in 3T3-L1 cells and they are responsible for regulating C/EBPα and PPARγ expression. The model of PPARγ and C/EBP transcriptional action during adipogenesis is illustrated in Fig. G3. [Lefterova *et al.*, 2009]

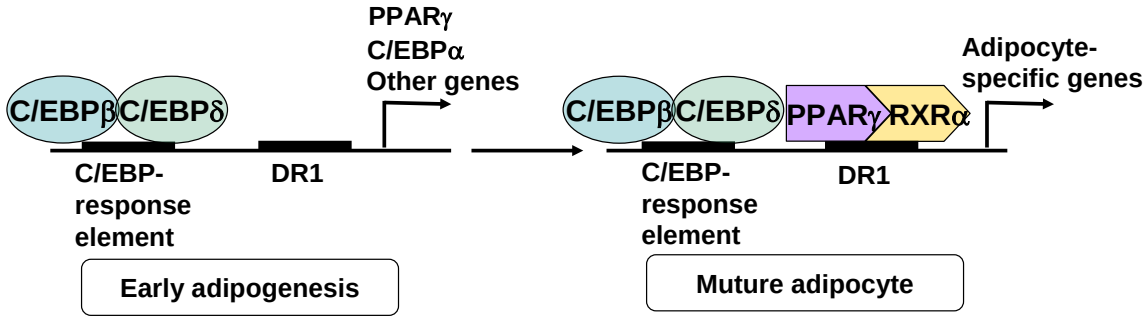


Fig. G3. Model of PPARγ and C/EBP transcriptional action during adipogenesis. During the early stages of adipogenesis, C/EBPβ and -δ activate expression of PPARγ, C/EBPα and probably other adipogenic genes. Downstream genes characteristic of terminally differentiated adipocytes are regulated by binding of PPARγ as aheterodimer with RXRα at thousands of locations, with C/EBPα and β frequently occupying C/EBP-response elements nearby.

**Hepatic steatosis**

Hepatic steatosis caused by the activation of FAs uptake, enhanced *de novo* lipogenesis, and lowing of lipid catabolism. The rate of transport of FAs is the first essential step in the accumulation of fat in the hepotocytes [Gasbarrini *et al.*, 2005; Bradbury, 2006]. Cluster of differentiation 36 (CD36) is one of the characterized FA transporters for FAs uptake [Su *et al.*, 2009]. CD36 is expressed in a wide variety of cells including macrophages, adipocytes, myocytes, enterocytes and hepatocytes [Hajri *et al.*, 2002]. Hepatic CD36 expression is normally weak, but its expression is increased in rodents with fatty liver [Bonen *et al.*, 2007]. The expression of CD36 increased concomitantly with hepatic lipid content in different animal models of liver steatosis [Buque *et al.*, 2010; Degrace *et al.*, 2006]. *De novo* lipogenesis is mediated by sterol regulatory element binding protein-1 (SREBP-1) which is a

key lipogenic transcription factor, and nutritionally regulated by glucose and insulin [Goldstein *et al*., 2008; Zhou *et al*., 2001]. SREBP-1 preferentially regulates the lipogenic process by activating genes for FA and triglyceride synthesis, which are involved in hepatic steatosis [Sanyal, 2005]. It is also known that peroxisome proliferator-activated receptors (PPARs) mediate the critical transcriptional regulation of genes associated with lipid homeostasis [Lee *et al*., 2003]. Especially, PPARα is most abundantly expressed in the liver and has the effect of diminishing circulating triglycerides and preventing hepatic steatosis through increasing hepatic β-oxidation [Braissant and Wahli, 1998]. Therefore, the CD36, SREBP-1 and PPARα are the important molecular targets for prevention of hepatic steatosis.

**AMPK**

Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) is a key modulator to maintain the cellular as well as whole-body energy balance. Fig. G4 shows the roles of AMPK in the control of whole-body energy metabolism and AMPK signaling pathway. In the liver and white adipose tissue, phosphorylation of AMPK reversibly and inactivates acetyl CoA carboxylase (ACC) through phosphorylation of this enzyme. Glycerol-3-phosphate acyl transferase-1 (GPAT-1) and carnitine palmitoyltransferase-1 (CPT-1) are the enzymes involving in FA metabolism. GPAT-1 esterifies FAs to glycerol to form triglyceride for storage. The activation of AMPK phosphorylates GPAT-1 and inhibits an activity of this enzyme as the same as ACC. In contrast, CPT-1 transfers cytosolic long-chain fatty acyl CoA into the mitochondria for oxidation and is allosterically inhibited by malonyl CoA [Lage *et al.*, 2008; Ejaz *et al.*, 2009;]. AMPK also mediates suppression of lipogeneic gene expression such as FA synthase and ACC through decreasing the functions of transcription factors SREBP-1 [Foretz *et al.*, 2005; Zhou *et al.*, 2001] and carbohydrate-responsive element-binding protein (ChREBP) [Kawaguchi *et al.*, 2002; Merrill *et al.*, 1997]. In the skeletal muscle, AMPK is activated during exercise, which is involved in contraction-stimulated glucose transport and FA oxidation, as a result of acute decreases in the ratios of ATP/AMP and phosphocreatine/creatine. In the heart, AMPK activity increases during ischaemia and functions to sustain ATP, cardiac function and myocardial viability [Assifi *et al*., 2005]. AMPK represents a potential target for the treatment of obesity, insulin resistance and type II diabetes. Physiological, pharmacological and genetic data clearly demonstrate that manipulating the activity of AMPK and its upstream kinases, liver kinase B1

(LKB1) and Ca(2+)/CaM-dependent protein kinase kinase β (CaMKKβ), in different tissues has a profound impact on feeding, body weight, glucose homeostasis and insulin sensitivity in rodents and humans [Lage *et al*., 2008].

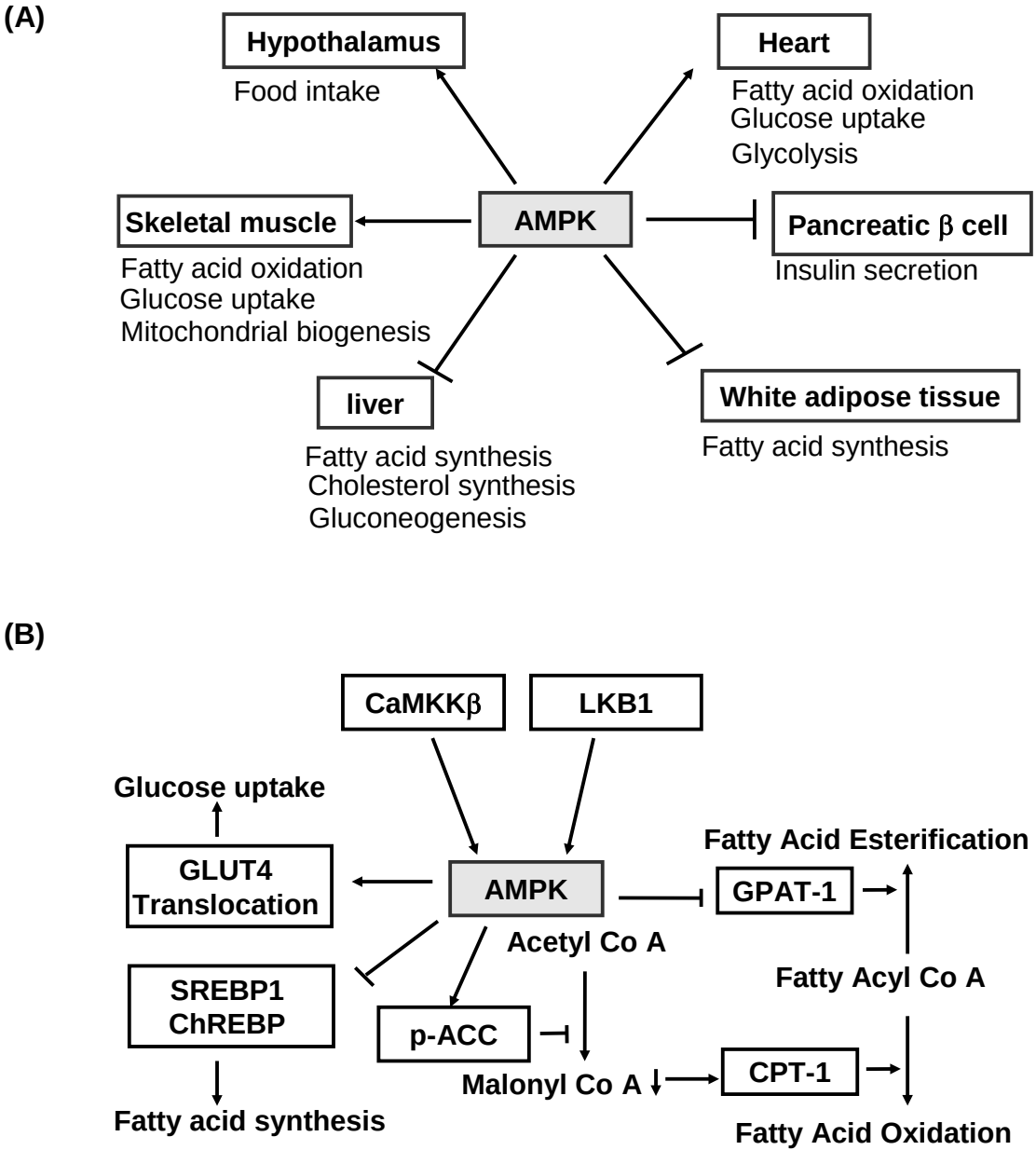


Fig. G4. Roles of AMPK in the control of whole-body energy metabolism (A) and AMPK signaling pathway (B).

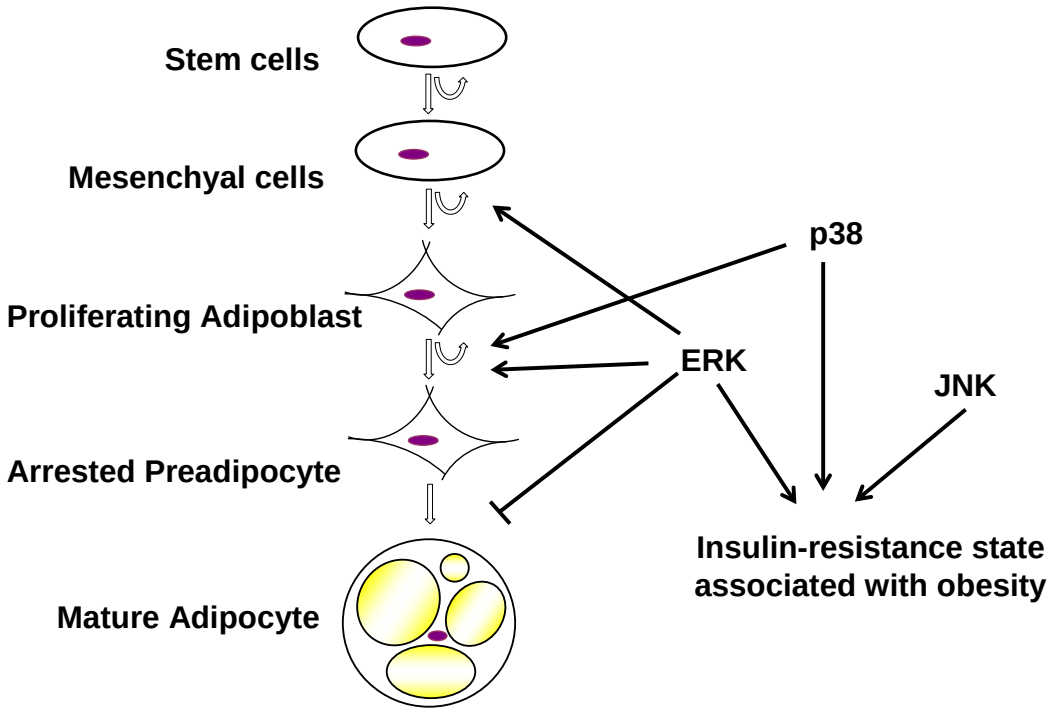


Fig. G5. Invovement of the MAPKs at the various steps of adipogenesis.

**MAPKs**

Mitogen-activated protein kinase (MAPK) signalling occurs in response to almost any change in the extracellular or intracellular milieu that affects the metabolism of the cell, organ or the entire organism [Gehart *et al*., 2010]. Most of the knowledge comes from the study of three groups of MAPKs: extracellular signal-regulated kinases (ERKs); c-Jun amino-terminal kinases (JNKs); and p38 MAPK (p38). These kinases are serine/threonine kinases regulated by phosphorylation cascades organized in specific modules. All modules comprise three additional protein kinases activated in series and leading to activation of a specific MAP kinase: a MAP kinase kinase, represented by MEK or MKK proteins, which phosphorylates a specific MAPK, and a MAP kinase kinase kinase (MAPKKK), represented by Raf and MEKK proteins, which phosphorylates a specific MAPKK [Robinson and Melanie, 1997; Gehart *et al*., 2010]. Activation of MAPKs is associated with detrimental events in obesity and diabetes that contribute to disease progression [Bost *et al*., 2005; Gehart *et al*., 2010]. MAPK pathways are able to regulate adipogenesis at each steps of the process, from stem cells to adipocytes (the various effects are summarized in Fig. G5) [Bost *et al*., 2005]. ERK pathway is involved in throughout adipogenesis, demonstrating both positive and negative

effects. Activation of ERK was necessary for adipocytes differentiation, and the reduction of ERK expression in pre-adipocytes led to decrease in adipocyte differentiation [Sale *et al*., 1995]. However, the activation of ERK conversely attenuates differentiation by phosphorylation of PPARγ during adipocyte differentiation [Camp *et al*., 1999; Hu *et al*., 1996]. Therefore, ERK activation is tightly and temporally controlled: it has to turn on for pre-adipocytes, while shut off to avoid PPARγ phosphorylation during adipocyte differentiation. It has been known that JNK is involved in insulin signaling pathway [Hirosumi *et al*., 2002; Sabio *et al*., 1996]. However, the effect of JNK on adipogenesis was still not clear, although the activation of JNK inhibits adipocyte differentiation through phosphorylation of PPARγ and negatively regulating its transcriptional activity [Camp *et al*., 1999]. It is reported that p38 activity is required for adipocyte differentiation [Engelman *et al*., 1998]. Obesity-related insulin resistance is an important contributor to the metabolic disturbance that define metabolic syndrome. Many studies have causally implicated MAPKs in the development of insulin resistance. ERK1 knokout mice are protected from diet-induced obesity and insulin resistance [Bost *et al*., 2005]. Global deletion of JNK or its specific deletion in adipose tissue, skeletal muscle and brain has been show to attenuate diet-induced insulin resistance in mice fed a high-fat (HF) diet [Sabio and Davis, 2010]. p38 is requited for glucose transporter type 4 (GLUT4)-mediated glucose uptake in response to insulin [Antonescu *et al*., 2005; Ribe *et al*., 2005]. p38 is required for glucagon- fasting-mediated suppression of hepatic lipogenesis, possibly through the inhibition of SREBP-1 transcription [Xiong *et al*., 2007]. These results indicate that MAPKs are important targets for regulating obesity and obesity-related disease.

**The aim and profile of this dissertation**

The aim of this dissertation is elucidation of molecular mechanisms behind the preventive effects of chalcones on obesity and insulin resistance. Briefly, the author investigated the mechanisms of chalcones 4HD, XAG, CAR and FKB on adipocyte differentiation in 3T3-L1 cells and FAs-induced steatosis in HepG2 cells. Moreover, the author demonstrated the preventive effects of Ashitaba extract, which contains two main chalcones 4HD and XAG, on HF diet-induced obesity and insulin resistance in C57BL/6 mice.

In Section A of Chapter 1, the author screened for the polyphenols, which decreased

intracellular lipid accumulation in 3T3-L1 cells. It was found that 6 effective polyphenols, namely 4HD, XAG, CAR, FKB, glabridin and 1,4-anthraquinone, inhibited lipid accumulation without cytotoxity. In Section B of Chapter 1, the mechanism of Ashitaba chalcones 4HD and XAG on adipocytes differentiation was investigated. The author, first, evaluated the anti-adipogenic effect of two chalcones 4HD and XAG on intracellular lipid accumulation during the differentiation of 3T3-L1 cells. To clarify the underlying molecular mechanisms, the author, further, investigated the effects of these chalcones on the expression of adipocyte-specific transcription factors, C/EBPs and PPARγ, and phosphorylation of AMPK and MAPK that involved in the expression of these transcription factors. As the results, 4HD and XAG inhibit adipocytes differentiation through AMPK and MAPK pathways, resulting in the down-expression of adipocyte-specific transcription factors. In order to confirm the inhibitory effects of chalcones on lipid accumulation, the author investigated another two chalcones CAR and FKB (a 4'-*O*-methoxylated CAR analog) and alpinetin (ALP) (an inactive flavonone) on adipocyte differentiation in Section C of Chapter 1. The author, first, evaluated the anti-adipogenic effect of CAR, FKB and ALP on intracellular lipid accumulation during the differentiation of 3T3-L1 cells. To clarify the underlying molecular mechanisms, the author, further, investigated the effects of these compounds on the expression of adipocyte-specific transcription factors, C/EBPα and PPARγ, and the phosphorylation level of ERK. As the results, CAR and FKB but not ALP inhibited lipid accumulation through down-expression of adipocyte-specific transcription factors. The inhibitory effects of the two chalcones are involved, at least in part, in ERK activation.

In Chapter 2, the author established an experimental model of hepatocellular steatosis with a fat overaccumulation profile using a mixture of FAs [palmitic acid (C16:0) : oleic acid (C18:1) = 1:2]. Then the author studied the effect of 4HD, XAG, CAR and FKB on FAs-induced lipid accumulation. To clarify the underlying mechanism, the effects of these chalcones on the expression of SREBP-1 and PPARα, and the activation of LKB-1/AMPK and MAPKs were investigated. As the results, these chalcones decreased the expression of SREBP-1 and increased the expression of PPARα. These chalcones increased AMPK and LKB-1 activation without the activation of MAPKs.

In Chapter 3, the author first detected the content of the two main chalcones 4HD and XAG in Ashitaba extract using LC-MS/MS. Then the author investigated whether Ashitaba extract prevented obesity and insulin resistance in HF diet-fed C57BL/6 mice. The effects of

Ashitaba extract on body, white adipose tissue weight, plasma and liver lipid levels, plasma glucose, insulin and adiponectin levels were investigated. Moreover the author demonstrated the underlying preventive molecular mechanism of Ashitaba extract on prevention of HF diet-induced obesity and insulin resistance.

Based on the obtained results, the author assumes that 4HD, XAG, CAR and FKB suppress adipocytes differentiation and FAs-induced lipid accumulation *in vitro*. Ashitaba extract rich in 4HD and XAG is able to prevent HF diet-induced obesity and insulin resistance. Taken together the findings in this dissertation, the author concludes that chalcones should be of benefit to prevent obesity and insulin resistance.

## Chapter 1

## Chalcones suppress differentiation of preadipocytes to adipocytes via AMPK and MAPK pathways

### CHAPTER INTRODUCTION

Adipocytes differentiation is deeply involved in the onset of obesity. During fibroblast-like preadipocytes differentiate into lipid-laden adipocytes, peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs) are known to be the master regulators or the crucial determinants of adipocyte fate [Tontonoz *et al*., 1994; Lefterova *et al*., 2009]. Glucose transporter type 4 (GLUT4), of which expression is regulated by C/EBPs, plays an important role in glucose uptake in adipocytes [Kaestner *et al*., 1990]. Adenosine 5'-monophosphate activated protein kinase (AMPK) is a key modulator to maintain the cellular as well as whole-body energy balance. In adipocytes, phosphorylation of AMPK reversibly and inactivates acetyl CoA carboxylase (ACC) through phosphorylation of this enzyme. Glycerol-3-phosphate acyl transferase-1 (GPAT-1) and carnitine palmitoyltransferase-1 (CPT-1) are the enzymes involved in fatty acid metabolism. GPAT-1 esterifies fatty acids to glycerol to form triglyceride for storage. The activation of AMPK also phosphorylates GPAT-1 and inhibits an activity of this enzyme as the same as ACC. In contrast, CPT-1 transfers cytosolic long-chain fatty acyl CoA into the mitochondria for oxidation and is allosterically inhibited by malonyl CoA [Ejaz *et al*., 2009]. Moreover, the activation of AMPK also inhibited the differentiation of 3T3-L1 cells by down-regulating the expression of C/EBPβ, δ, α, and PPARγ [Gao *et al*., 2008]. The extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 MAPK (p38) are the members of Mitogen-activated protein kinases (MAPKs) that play a pivotal role in many essential cellular processes including adipocytes differentiation. ERK is necessary for preadipocytes differentiation into adipocytes, but the sustained activation of the ERK inhibits the differentiation [Sale *et al*., 1995; Sakaue *et al*., 2004]. Activation of JNK also inhibits adipocytes differentiation through phosphorylation of PPARγ and negatively regulating its transcriptional activity [Camp *et al*., 1999]. However, p38, not the same as ERK and JNK, is important for promotion of 3T3-L1 cells differentiation [Engelman *et al*., 1998].

Natural products, especial polyphenols, are expected for prevention and treatment of obesity and other human diseases [Williamson and Manach, 2005; Rayalam, *et al*., 2008; Scalbert, *et al*., 2005]. Chalcones, which have an aromatic ketone and an enone, are

containing in various plants and have been reported to have a variety of beneficial functions for health promotion, including antioxidant, anti-inflammatory and anticancer activities. Several synthetic chalcones also show a series of biological activity [Dimmock, *et al*., 1999; Bandgar, *et al*., 2009]. However, the preventive effects and molecular mechanisms of chalcones on obesity are not fully understood yet.

This chapter was divided into 3 sections. In Section A, the author searched for the effective compounds capable of inhibiting the lipid accumulation in 3T3-L1 cells among 49 polyphenols. Then in Section B, the author investigated inhibitory effect of Ashitaba chalcones 4HD and XAG on lipid accumulation and clarified the underlying molecular mechanisms of these two chalcones on adipocytes differentiation. In Section C, the author selected another two effective chalcones CAR and FKB, and alpinetin (ALP) as a reference compound to confirm the inhibitory effects of chalcones on adipocytes differentiation.

## Section A

## Effects of polyphenols on lipid accumulation in 3T3-L1 adipocytes

## INTRODUCTION

Obesity is a condition in which adipocytes accumulate a large amount of fat and become enlarged. It is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in adipose tissue. 3T3-L1 preadipocytes are known to differentiate into mature adipocyte-like cells *in vitro*. It is a well-characterized cell culture model for the study of adipocyte specific differentiation [Green and Kehinde, 1975]. Intracellular accumulation of lipid droplets can be observed during cell differentiation.

Polyphenols are the most abundant antioxidants in diet. The research on the effects of polyphenols on human health has developed considerably recent years. It strongly supports a role for polyphenols in the prevention of obesity and insulin resistance [Williamson and Manach, 2005; Rayalam, *et al*., 2008; Scalbert, *et al*., 2005]. Flavonoids, a class of plant secondary metabolites or yellow pigments, can be visualized as two benzene rings which are joined together with a short three carbon chain. The flavonoids consist of several major subgroups: chalcone, flavone, flavonol, flavanone and isoflavonoids [Peterson *et al*., 1998]. Research on dietary flavonoids has shown them to have a broad spectrum of biological activities including anti-cancer, antioxidant, anti-inflammatory [Cook *et al*., 1998]. The anthraquinones, with two ketone groups added at posi-tion C9 and position C10, occur naturally in plants. The anthraquinones are also reported to active against certain diseases [Choi *et al*., 2005; Huang *et al*., 1998; Malterud *et al*., 1993]. As mentioned above, polyphenols have shown many biological effects, but, the effect of polyphenols on lipid accumulation in adipocytes is not fully understood yet. Thus, the author searched for the effective compounds capable of inhibiting lipid accumulation in 3T3-L1 cells among 49 polyphenols (Section A).

## MATERIAL AND METHODS

### Materials

Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Nissui Pharmaceutical (Tokyo, Japan). Calf serum and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD) and Biological industries (Kibbutz Beit Haemek, Israel),

respectively. The cell proliferation reagent WST-1 substrate was from Roche Diagnostics (Mannheim, Germany). 4HD and XAG were purified from “Ashitaba Chalcone Powder” as a commercial product of Japan Bio Science Laboratory (Osaka, Japan). CAR and ALP were isolated from the seeds of *Alpinia katsumadai* Hayata, while FKB was synthesized by direct aldol condensation of acetophenone with benzaldehyde as described previously [Yamamoto *et al*., 2011]. Biochanin A, caffeic acid and chalcone were purchased from LKT Laboratories (St. Paul, MN) and xanthohumol were obtained from Alexis Biochemicals (San Diego, CA). Chlorogenic acid and prunetin were from Sigma (St. Louis, MO) and eupatilin was from PhytoLab (Vestenbergsgreuth, Germany). Other polyphenols were products of Extrasyntese (Genay, France).

**Cell culture and treatments**

3T3-L1 cells were maintained and differentiated in a $CO_2$ incubator with 5% $CO_2$/95% air at 37°C. Adipocytes differentiation was induced as described previously [Huang *et al*., 2006]. Briefly, the cells were grown up to the contact inhibition stage in DMEM containing 10% (*v/v*) calf serum. The cells were cells maintained at post-confluent stage for 24 h in DMEM containing 10% (*v/v*) FBS. Differentiation was than induced by a MDI (0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone and 10 µg/mL insulin) cocktail and 100 µM ascorbic acid phosphate for 3 days in the same medium. Then, the cells were cultured in DMEM containing 10% FBS, 10 µg/mL insulin and 100 µM ascorbic acid phosphate for another 3 days. Thereafter, the cells were cultured in DMEM containing 10% FBS and 100 µM ascorbic acid phosphate.

To search for polyphenols capable of inhibiting intracellular lipid accumulation, post-confluent 3T3-L1 cells in the 96-well plates were first treated with the 49 polyphenols or DMSO alone as a vehicle control at a final concentration of 0.25% for 3 or 8 days. The cell viability assays and sudan II staining were carried out at Day 3 and 8, respectively.

**Sudan II staining**

Post-confluent 3T3-L1 cells were plated in 96-well plates and were treated with the indicated polyphenol or DMSO alone as a vehicle at a final concentration of 0.25%. The cells were washed twice with ice-cold phosphate-buffered saline (PBS), fixed with 100 µL of 4% (*w/v*) paraformaldehyde in PBS for 1 h at 4°C, and stained with 100 µL of 0.5% (*w/v*) Sudan

II solution in 60% (*v/v*) isopropanol for 1 h at room temperature. The cells were washed twice with distilled water to remove excess stain and a photograph was taken at a magnification of 200×. Thereafter, oil droplets present in the stained cells were dissolved in isopropanol containing 4% (*v/v*) Nonidet P-40, and absorbance of the dissolved solution was measured at a wavelength of 490 nm.

**Cell viability assay**

The cell viability was determined by WST-1 assay and crystal violet staining assay, as previously described [Zhang *et al*., 2013]. After treating the cells with 1, 5, 10, or 30 μM of the indicated polyphenol, the cells were incubated with WST-1 reagent in DMEM medium for 3 h. The absorbance of the medium was measured at 450 nm with a reference wavelength of 630 nm using a Wallac multilabel counter (1420 ARVO Sx; PerkinElmer, Waltham, MA, USA). Then, the cells were washed three times with PBS and stained with 0.2% (*w/v*) crystal violet in 2% (*v/v*) ethanol for 10 min. The cells were washed three times with distilled water, and the stained dye was dissolved in 0.5% (*w/v*) sodium dodecyl sulfate in 50% (*v/v*) ethanol. Absorbance was measured at 570 nm with a reference wavelength of 630 nm using a Wallac multilabel counter.

**Statistical analysis**

All data are expressed as the mean ± standard deviation (SD) of at least three independent determinations for each experiment. Statistical significance was analyzed using the Dunnett's test, and probability levels of 0.05 and 0.01 were considered to indicate statistical significance.

**RESULTS**

**Inhibitory effects of polyphenols on lipid accumulation in 3T3-L1 cells with treatment for 8 days**

The author initially searched for the inhibitory effect of polyphenols on the intracellular lipid accumulation in 3T3-L1 cells by Sudan II staining. The author tested 46 compounds, including 7 chalcons (4HD, XAG, CAR, FKB, xanthohumol, butein, and chalcone), 6 flavones (tangeretin, luteolin, diosmetin, apigenin, baicalein, chrysin, and eupatilin), 7 flavonols (3-hydroxyflavone, fisetin, quercetin, galangin, robinetin, isorhamnetin, and

kaempferol), 7 isoflavones (glabridin, prunetin, genistein, daidzein, equol, daidzin, and biochanin A), 8 flavanones (bavachinin, sakuranetin, pinostrobin, ALP, isosakuranetin, eriodictyol, flavanone, and naringenin), 8 anthraquinones [1,4-anthraquinone, 1,4-dimethylanthraquinone (1,4-DAQ), rhein, emodin, sennoside A, aloe-emodin, chrysophanol, and danthron], 1 quinone (shikonin), 1 stilbene (resveratrol), 1 phenylpropanoid (caffeic acid), 1 quinic acid (chlorogenic acid), and 1 hydroxycinnamic acid (artepillin C). The cells were treated with each of these compounds at concentrations of 10 or 30 μM for 8 days. As the results, all chalcones tested, tangeretin, luteolin, 3-hydroxyflavone, glabridin, prunetin, bavachinin, sakuranetin, 1,4-anthraquinone, 1,4-DAQ, rhein and shikonin significantly suppressed lipid accumulation (Fig. 1A-1.). However, some of these active compounds were thought to be cytotoxic based on the morphological features observed under a microscope. Moreover, it has been known that expression of adipocyte-specific transcription factors play an important role in the early stage of adipocytes differentiation [Lefterova and Lazar, 2009]. Hence, the author further investigated the effects of active compounds (at 1, 5, 10, and 30 μM) on lipid accumulation in 3T3-L1 cells during the first 3 days of treatment.

**Inhibitory effects of polyphenols on lipid accumulation in 3T3-L1 cells with treatment for 3 days**

During the differentiation of preadipocytes to adipocytes, the cells were treated with effective compounds (1, 5, 10, 30 μM) for the first 3 days. As shown in Fig 1A-2, 4HD, XAG, CAR, FKB, xanthohumol, butein, glabridin, bavachinin, 1,4-anthraquinone, 1,4-dimethylanthraquinone and shikonin dose-dependently decreased the lipid accumulation. 4HD, XAG, CAR, FKB, glabridin and 1,4-anthraquinone at 5 μM significantly decreased the lipid accumulation to 67%, 74%, 71%, 76%, 76% and 67%, respectively.

**Cell viability assay of effective compounds in 3T3-L1 cells**

3T3-L1 cells were treated with 18 effective compounds at 1, 5, 10 and 30 μM during MDI-induced differentiation. After treatment, cell viability was determined by both WST-1 assay and crystal violet staining assay. As shown in Fig. 1A-3, Both WST-1 assay and crystal violet staining assay revealed that some compounds caused a reduction in cell viability at 30 μM, but 5 μM and lower than 5 μM, neither compound showed any cytotoxicity except shikonin.

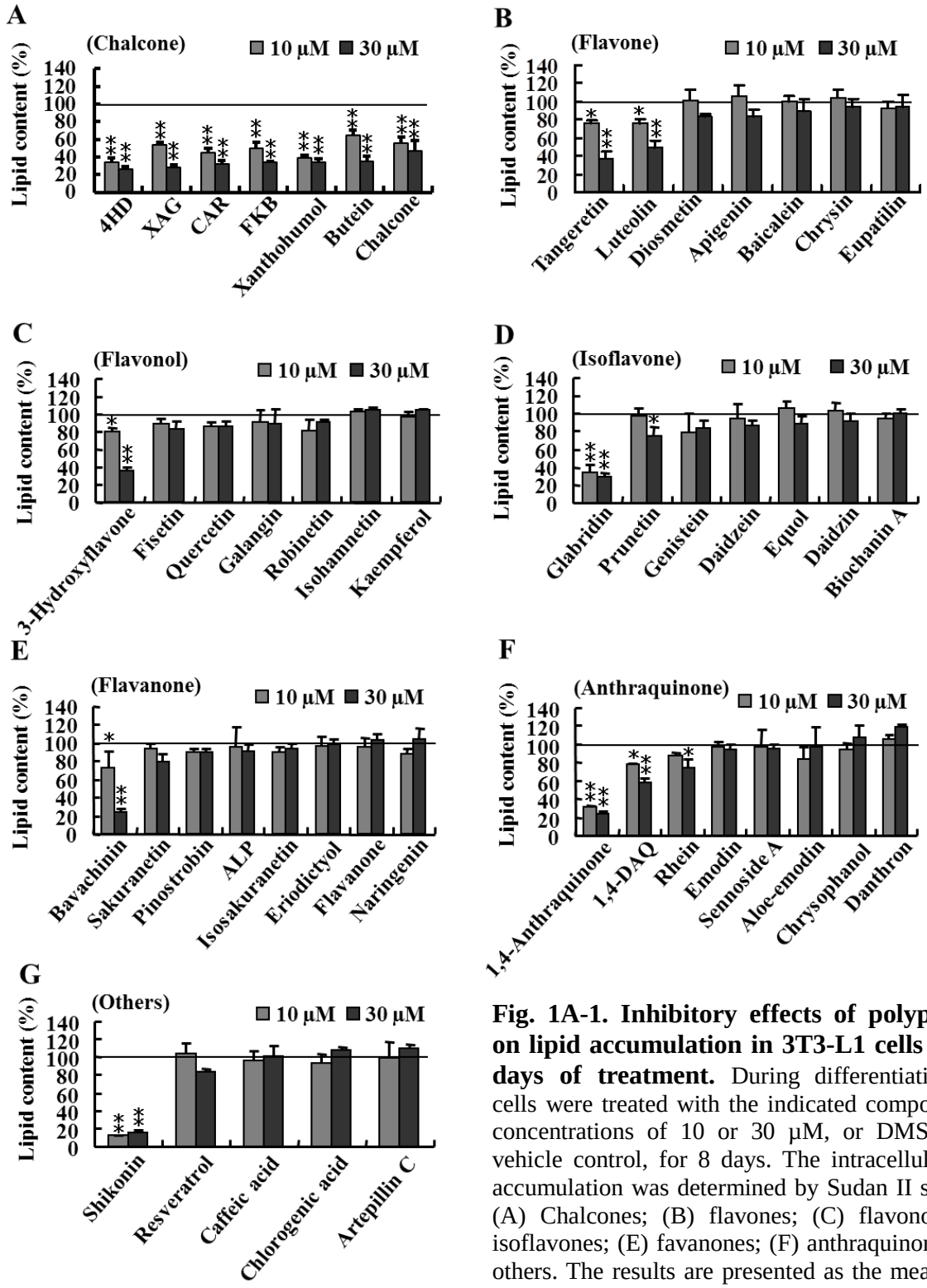


**Fig. 1A-1. Inhibitory effects of polyphenols on lipid accumulation in 3T3-L1 cells over 8 days of treatment.** During differentiation, the cells were treated with the indicated compounds at concentrations of 10 or 30 μM, or DMSO as a vehicle control, for 8 days. The intracellular lipid accumulation was determined by Sudan II staining. (A) Chalcones; (B) flavones; (C) flavonols; (D) isoflavones; (E) favanones; (F) anthraquinones; (G) others. The results are presented as the mean ± SD of three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO-treated cells (Dunnett's test).

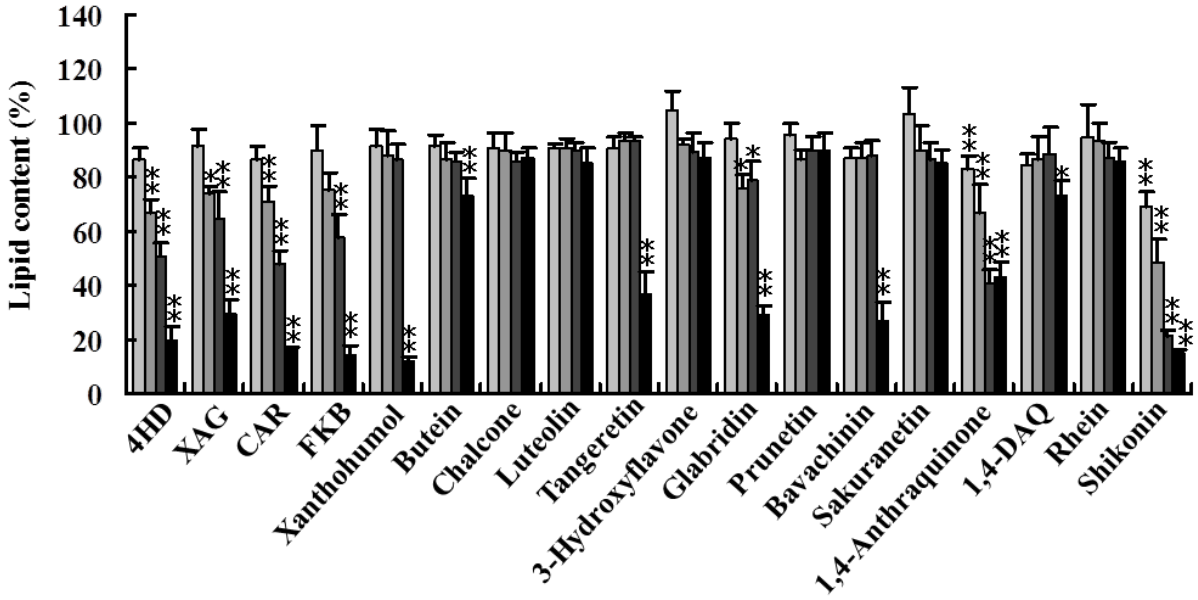


**Fig. 1A-2. Effects of polyphenols on lipid accumulation and viability of 3T3-L1 cells over 3 days of treatment.** The cells were treated with the indicated compounds at concentrations of 1, 5, 10, or 30 μM, or DMSO as a vehicle control, for the first 3 days. Intracellular lipid accumulation was determined by Sudan II straining at Day 8. The results are presented as the mean ± SD of three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO-treated cells (Dunnett's test).

## DISCUSSION

Obesity is associated with a significant risk factor for metabolic syndrome [Hajer *et al*., 2008; Ligibel, 2011]. Certain polyphenols have been reported to prevent and reduce the risk of obesity [Gonzalez-Castejon and Rodriguez-Casado, 2011]. In this section, the author searched for effective polyphenols capable of inhibiting lipid accumulation in 3T3-L1 cells. Of 49 polyphenols tested, the author found 4HD, XAG, CAR, FKB, glabridin and 1,4-anthraquinone inhibited lipid accumulation in 3T3-L1 cells without cytotoxity at 5 μM. 4HD, XAG, CAR and FKB, which are chalcones, showed strong inhibitory effects on lipid accumulation. The α,β-unsaturated carbonyl group in the 4HD, XAG, CAR and FKB structures may play an important role in the inhibition of lipid accumulation, because the α,β-unsaturated carbonyl group in chalcones is known to regulate cell functions, such as the NF-E2-related factor 2/Kelch-like ECH-associated protein system [Kim and Surh, 2006].

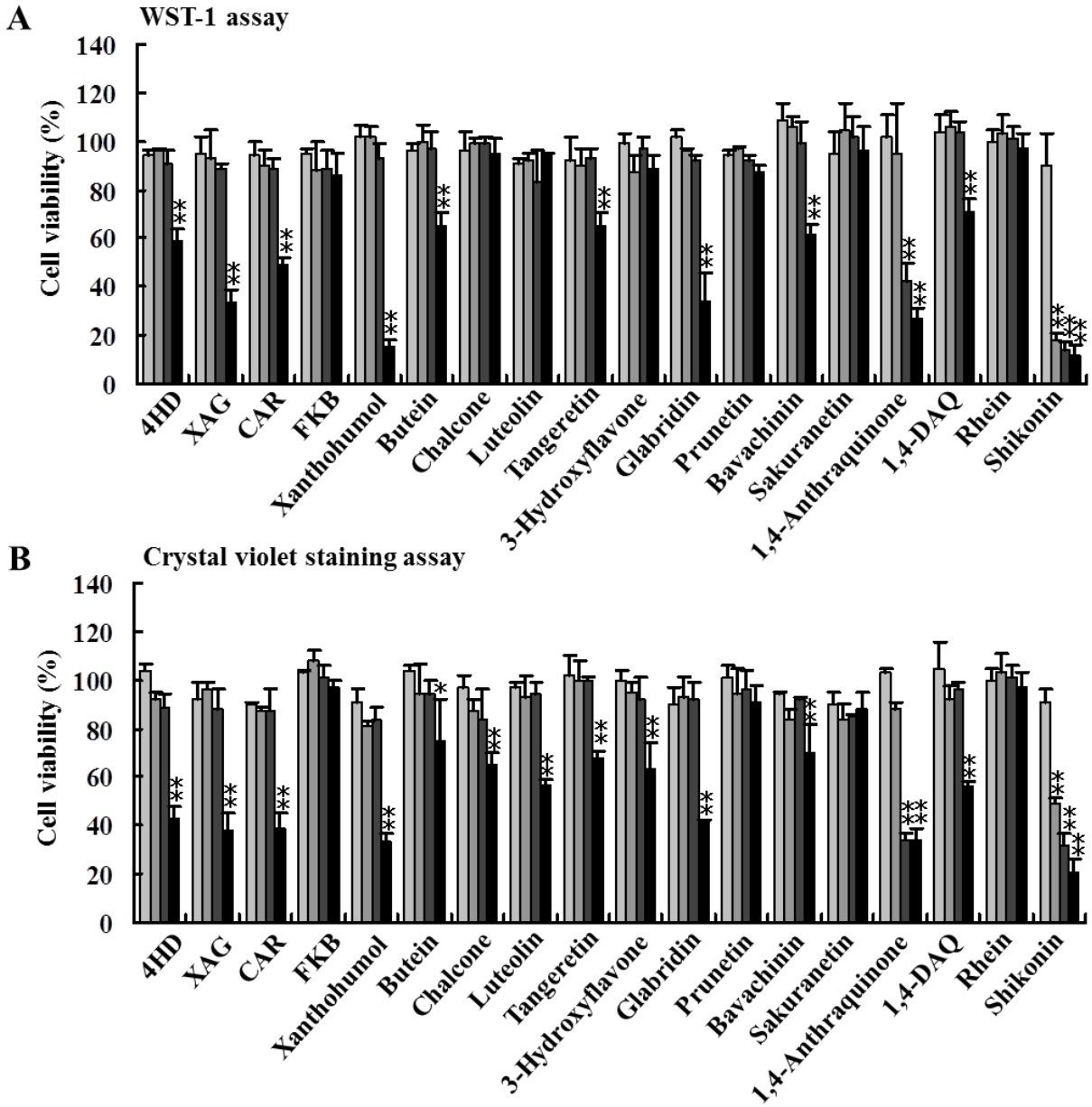


**Fig. 1A-3. Effects of polyphenols on viability of 3T3-L1 cells over 3 days of treatment.** The cells were treated with the indicated compounds at concentrations of 1, 5, 10, or 30 μM, or DMSO as a vehicle control, for the first 3 days. Cell viability was measured by WST-1 assays (A) and crystal violet staining assays (B), respectively. The results are presented as the mean ± SD of three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO-treated cells (Dunnett's test).

The author's finding indicates that α, β-unsaturated chalcones may be potential target molecules capable of inhibiting adipocytes differentiation and obesity. Broader screening tests are needed to determine the structure–activity relationship. To clarify the effect chalcones on differentiation of predadipocytes to adipocytes, the author demonstrated the underlying

molecular mechanism of 4HD, XAG, CAR and FKB on adipocyte differentiaton in following Section B and C.

**Section B**

**4-Hydroxyderricin and xanthoangelol from Ashitaba (*Angelica keiskei*) suppress differentiation of preadiopocytes to adipocytes via AMPK and MAPK pathways.**

## INTRODUCTION

Certain phytochemicals have been reported to reduce the risk of obesity [Rayalam *et al*., 2008; Lu *et al*., 2012; Alappat and Awad, 2010; Gourineni *et al*., 2012]. *Angelica keiskei*, a Japanese herb and Japanese name is "Ashidaba", has been used as a traditional medicine for so many years. It contains two main phytochemicals 4HD and XAG, and they have been reported to exert various biological activities such as anti-tumor [Kimura *et al*., 2004; Akihisa *et al*., 2012], anti-inflammatory [Ohkura *et al*., 2011] and anti-diabetes [Enoki *et al*., 2007; Kawabata *et al*., 2011] activities. However, the preventive effects of 4HD and XAG on obesity are not yet reported to date.

Results in the Section A showed that two chalcones 4HD and XAG inhibited lipid accumulation effectively. In this section B, the author, first, evaluated the anti-adipogenic effect of 4HD and XAG on intracellular lipid accumulation during the differentiation of 3T3-L1 cells. To clarify the underlying molecular mechanisms, we, further, investigated the effects of these chalcones on the expression of adipocyte-specific transcription factors, C/EBPs and PPARγ, and phosphorylation of AMPK and MAPKs that involved in the expression of these transcription factors.

## MATERIAL AND METHODS

### Materials

Antibodies for β-actin, PPARγ, C/EBPα, C/EBPβ, C/EBPδ, GLUT4, horseradish peroxidase-conjugated anti-rabbit IgG and anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), p-AMPK, AMPK, p-ACC, ACC, p-ERK, ERK, p-JNK, JNK, p-p38, p38 Compound C, PD98059 and SP600125 were purchased from Cell Signaling Technology (Beverly, MA). The PCR primer pairs were synthesized as shown in Table 1B-1.

### Cell culture and treatments

Cell culture was performed as described in Chapter 1A. To search for the inhibitory effect of 4HD and XAG on the intracellular lipid accumulation , post-confluent 3T3-L1 cells in the 96-well plates were first treated with 4HD and XAG or DMSO alone as a vehicle

control (a final concentration at 0.25%) for 3 days. Sudan II staining was carried out at Day 0, 3, 6, 8, and 10. To investigate the molecular mechanism by which the compounds inhibited adipocyte differentiation, 3T3-L1 cells were plated on a 35-mm dish and were treated with each compound for 3 days. Then, cells were prepared for protein and mRNA analysis at the times indicated in each figure. For treatments with the inhibitors for kinases, post-confluent 3T3-L1 cells were simultaneously treated with the chalcones and inhibitors [PD98059 (20 μM), SP600125 (5 μM), and compound C (5 μM), respectively], during MDI-induced adipocytes differentiation. Expression level of C/EBPβ mRNA was determined on Day 1, while that of C/EBPα and PPARγ was determined on Day 5. Lipid accumulation was assessed on Day 7.

Table 1B-1. Primer sequences used for real-time PCR amplification

| Gene | 5'-3' primer sequence | Reference |
|---|---|---|
| PPARγ | F: ACGTGCAGCTACTGCATGTGA | [Ejaz *et al*., 2009] |
| | R: AGAAGGAACACGTTGTCAGCG | |
| C/EBPα | F: GGAACTTGAAGCACAATCGATC | [Ejaz *et al*., 2009] |
| | R: TGGTTTAGCATAGACGTGCACA | |
| C/EBPβ | F: GGGGTTGTTGATGTTTTTGG | [Gao, *et al*., 2008] |
| | R: CGAAACGGAAAAGGTTCTCA | |
| C/EBPδ | F: GATCTGCACGGCCTGTTGTA | [Huang, *et al*., 2006] |
| | R: CTCCACTGCCCACCTGTCA | |
| GAPT-1 | F: GGCATCTCGTATGATCGCAT | [Ejaz *et al*., 2009] |
| | R: GCAAAATCCACTCGGACGTA | |
| CPT-1 | F: AGAGAAGCCTGCCAGTTTGT | [Ejaz *et al*., 2009] |
| | R: AAAGAGGTGACGGTCAATCC | |
| 18S rRNA | F: CATGGCCGTTCTTAGTTGGT | [Ferguson *et al*., 2010] |
| | R: CGCTGAGCCAGTCAGTGTAG | |

**Sudan II staining**

Sudan II staining was performed as described in Chapter 1A.

**RNA isolation and real-time quantitative PCR analysis**

The 3T3-L1 cells plated in 35-mm dishes were harvested with 200 μL of TRlzol Reagent (Invitrogen, Carlsbad, CA, USA), transferred to a microtube, and homogenized. The homogenate was mixed with 100 μL chloroform and incubated at room temperature for 10 min. The mixture was centrifuged at 12,000 × *g* for 15 min at 4°C and the aqueous phase was transferred to another microtube. To precipitate RNA, the aqueous phase was mixed with 400 μL of isopropyl and incubated for 10 min at room temperature. The mixture was centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellet containing RNA was dried at room temperature. The pellet was dissolved in 30 μL of deionized-distilled water. After isopropanol precipitation, RNA was re-dissolved in 40 μL of deionized-distilled water. The dissolved samples were purified by digesting the residual DNA using DNase I (Roche, Basel, Switzerland) according to the manufacturer's instructions. The quality and concentration of total RNA were measured by a spectrophotometer. Purified RNA (20 ng) was subjected to a quantitative RT-PCR amplification using One Step SYBR® PrimeScript™ Plus PCR-RT Kit (Takara Bio, Shiga, Japan). Reactions were run in a RT-PCR system (TaKaRa PCR Thermal Cycler Dice, Takara Bio Inc). The relative gene expression level was calculated using the comparative cycle threshold method (Livak and Schmittgen, 2001), using the expression of the ribosomal RNA (18S rRNA) gene as an internal control.

**Western blotting analysis**

Preparation of the cell lysate and western blotting were performed according to the previous report [Furuyashiki *et al*., 2004]. Specific immune complexes were detected with the ATTO Light-Capture II Western Blotting Detection System. The density of specific bands was calculated using ImageJ image analysis software (NIH, Bethesda, MD, USA).

**Statistical analysis**

All data are expressed as the mean ± standard deviation (SD) of at least three independent determinations for each experiment. Statistical significance was analyzed using the Dunnett's test, and probability levels of 0.05 and 0.01 were considered to indicate statistical significance

## RESULTS

### 4HD and XAG inhibited lipid accumulation during differentiation of 3T3-L1 adipocytes

In order to examine the effects of 4HD and XAG on the adipocytes differentiation, 3T3-L1 cells undergoing MDI-induced differentiation were treated with 4HD and XAG (5 μM). Sudan II staining revealed that 4HD and XAG significantly inhibited the lipid accumulation (Fig. 1B-1A). When the sudan II staining was also carried out at Day 0, 3, 6, 8 and 10, it was confirmed that 4HD and XAG significantly suppressed lipid accumulation after Day 6, and accumulated lipids decreased to approximately 40% and 70%, respectively, on Day 10 (Fig. 1B-1).

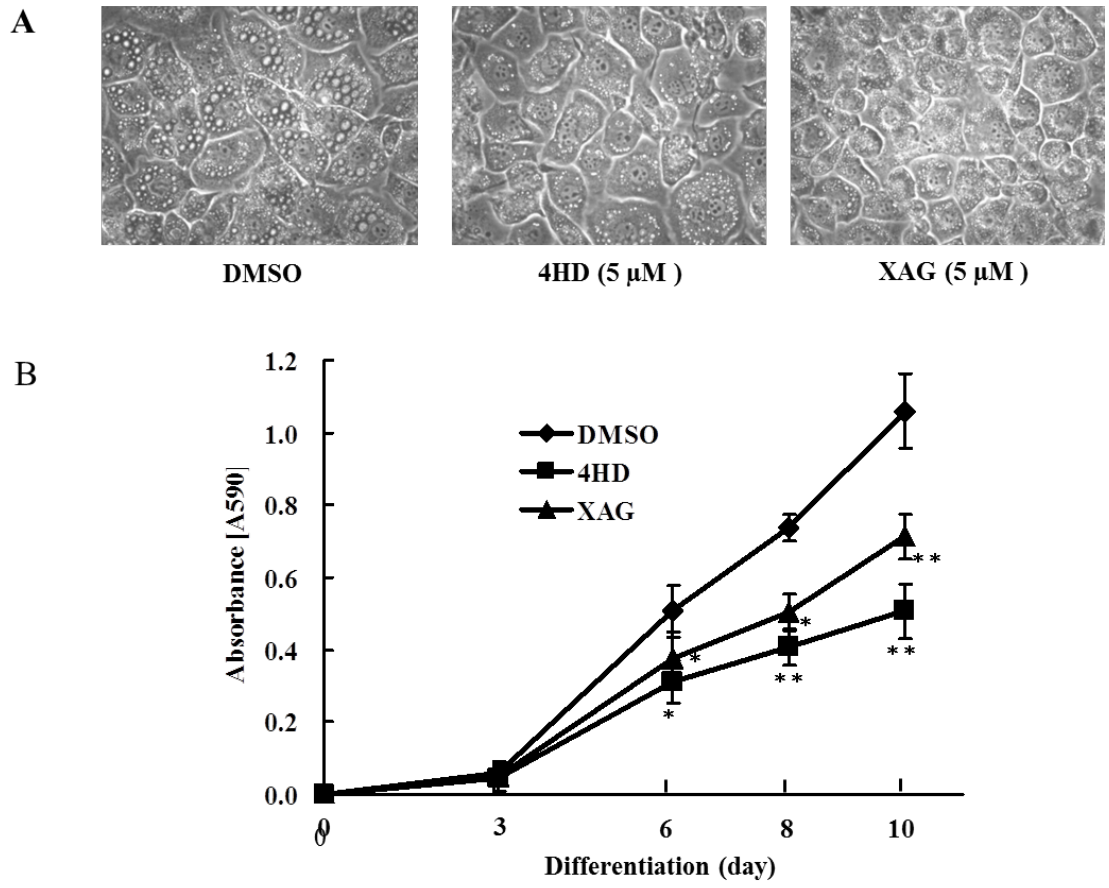


**Fig. 1B-1. Suppression of 4HD and XAG on lipid accumulation in 3T3-L1 cells.** 3T3-L1 cells were treated with 4HD, XAG at 5 μM or DMSO as a vehicle control during MDI-induced adipocytes differentiation. (A) The intracellular lipid accumulation was determined by sudan II straining at Day 8. (B) The intracellular lipid accumulation was stained by Sudan II straining at Day 0, 3, 6, 8 and 10. The results represent as the mean ± SD from three independent experiments. Asterisks indicate significant difference from control treated with DMSO alone at each time point by Dunnett's test. * $p<0.05$; * * $p<0.01$.

**Effects of 4HD and XAG on adipocyte-specific transcription factors expression during differentiation of 3T3-L1 adipocytes**

To determine whether the decrease in the lipid droplet accumulation was due to down-regulation of adipocyte-specific differentiation markers, the expression of C/EBPα, PPARγ and GLUT4 were determined by quantitative RT-PCR and western blotting analysis. 4HD and XAG at 5 μM significantly down-regulated mRNA levels of C/EBPα and PPARγ from 48 h (Fig. 1B-2A). Moreover, these two chalcones decreased C/EBPα (27-74%) and PPARγ (9-83%) on both Day 3 and 8. Meanwhile, 4HD and XAG also significantly down-regulated the protein expression of a late stage maker GLUT4 on Day 8 by 17% and 44%, respectively (Fig. 1B-2B). It was noteworthy that inhibition effect of 4HD was higher than that of XAG, consisting with their inhibitory degree of lipid accumulation (Fig. 1B-1).

The author, then, evaluated the effects of 4HD and XAG on expression of C/EBPβ and C/EBPδ transcription factors that are upstream of C/EBPα and PPARγ. The author found that these two chalcones decreased the C/EBPβ expression not only mRNA level from 6 hours to 24 hours (Fig. 1B-3A) but also the protein level by 56% and 43%, respectively, at 24 hours (Fig. 1B-3B). However, there is no different changes between in C/EBPδ expression neither mRNA nor protein level.

**4HD and XAG modulation of energy and lipid metabolism related enzymes.**

AMPK is a major regulator of energy metabolism, and its phosphorylation is involved in regulation of adipocytes differentiation [Ejaz *et al*., 2009]. The author, therefore, investigated whether 4HD and XAG regulated adipocytes differentiation and energy metabolism through AMPK pathway in 3T3-L1 cells. Western blotting analysis revealed that these chalcones enhanced the activation of AMPK by phosphorylation of its α-subunit (4HD: 1.63-fold on Day 1; 1.35-fold on Day 3 and XAG: 1.45-fold on Day 1; 1.31-fold on Day 3 compared with DMSO treated cells at each time point) (Fig. 1B-4A). In turn, activation of AMPK significantly increased the phosphorylation level of ACC (4HD: 1.45-fold on Day 3; 1.67-fold on Day 8 and XAG: 1.33-fold on Day 3; 1.37-foldon Day 8) (Fig. 1B-4B), down-regulated its down-stream events GAPT 1 mRNA (4HD: 71 %; 75 %; 92 % and XAG: 81 %; 79 %; 88 % on Day 1, 3 and 8, respectively) (Fig. 1B-4C), while up-regulated CPT-1 mRNA (4HD: 1.16-fold; 1.25-fold; 1.23-fold and XAG: 1.14-fold; 1.24-fold; 1.27-fold on Day 1, 3 and 8, respectively) (Fig. 1B-4D).

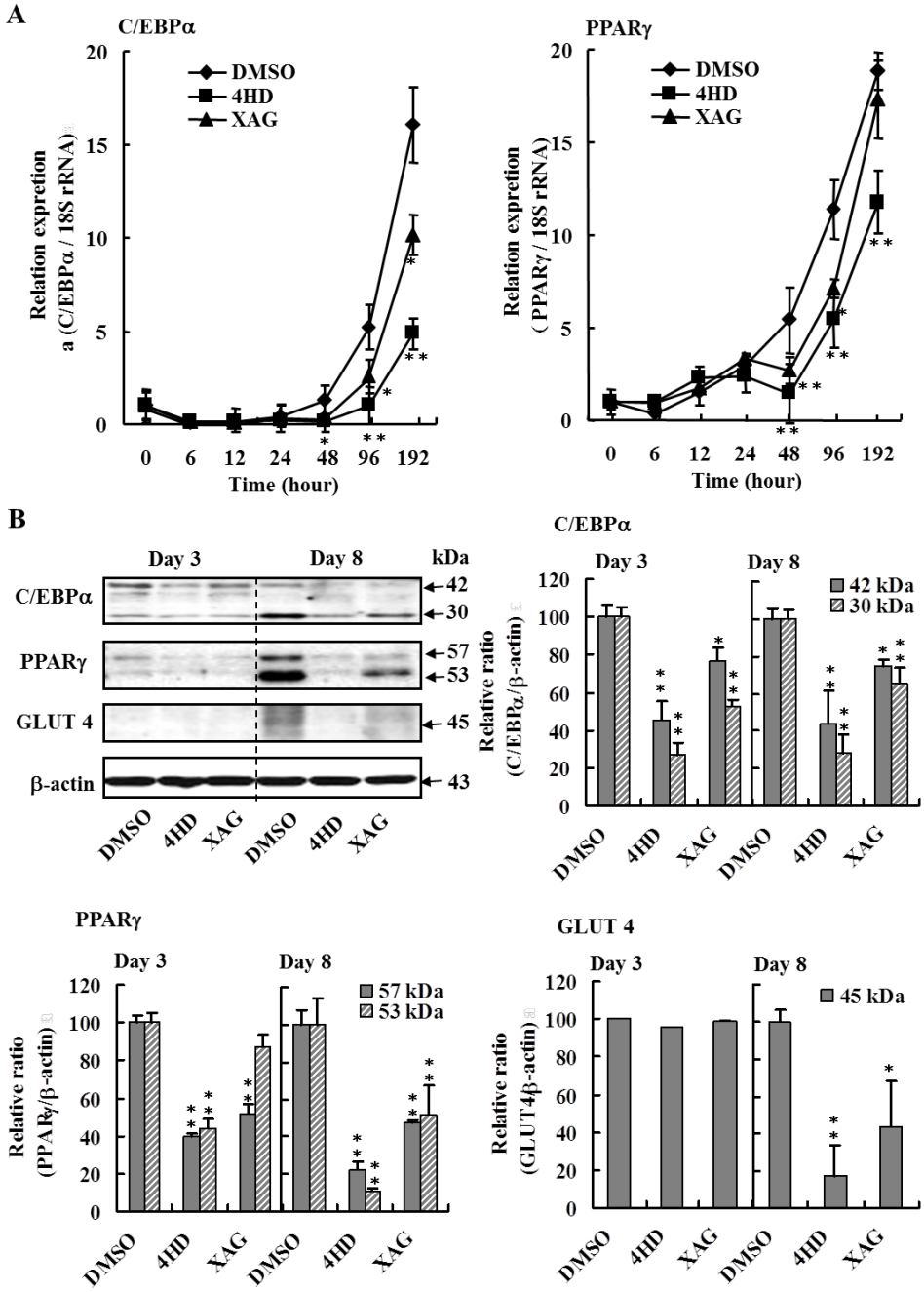


**Fig. 1B-2. 4HD and XAG down-regulated the expression of C/EBPα, PPARγ and GLUT4 during 3T3-L1 adipocytes differentiation.** The cells were treated with 4HD and XAG at 5 μM or DMSO as a vehicle control during MDI-induced adipocytes. (A) mRNA expression of C/EBPα and PPARγ were detected by quantitative RT-PCR. (B) Protein expression of C/EBPα, PPARγ and GLUT4 was determined by western blotting analysis. Typical results are shown from representative three independent experiments. Band density was quantified and normalized with β-actin. The results represent as the mean ± SD from three independent experiments. Asterisks indicate significant difference from control treated with DMSO alone at each time point by Dunnett's test. * $p<0.05$; * * $p<0.01$.

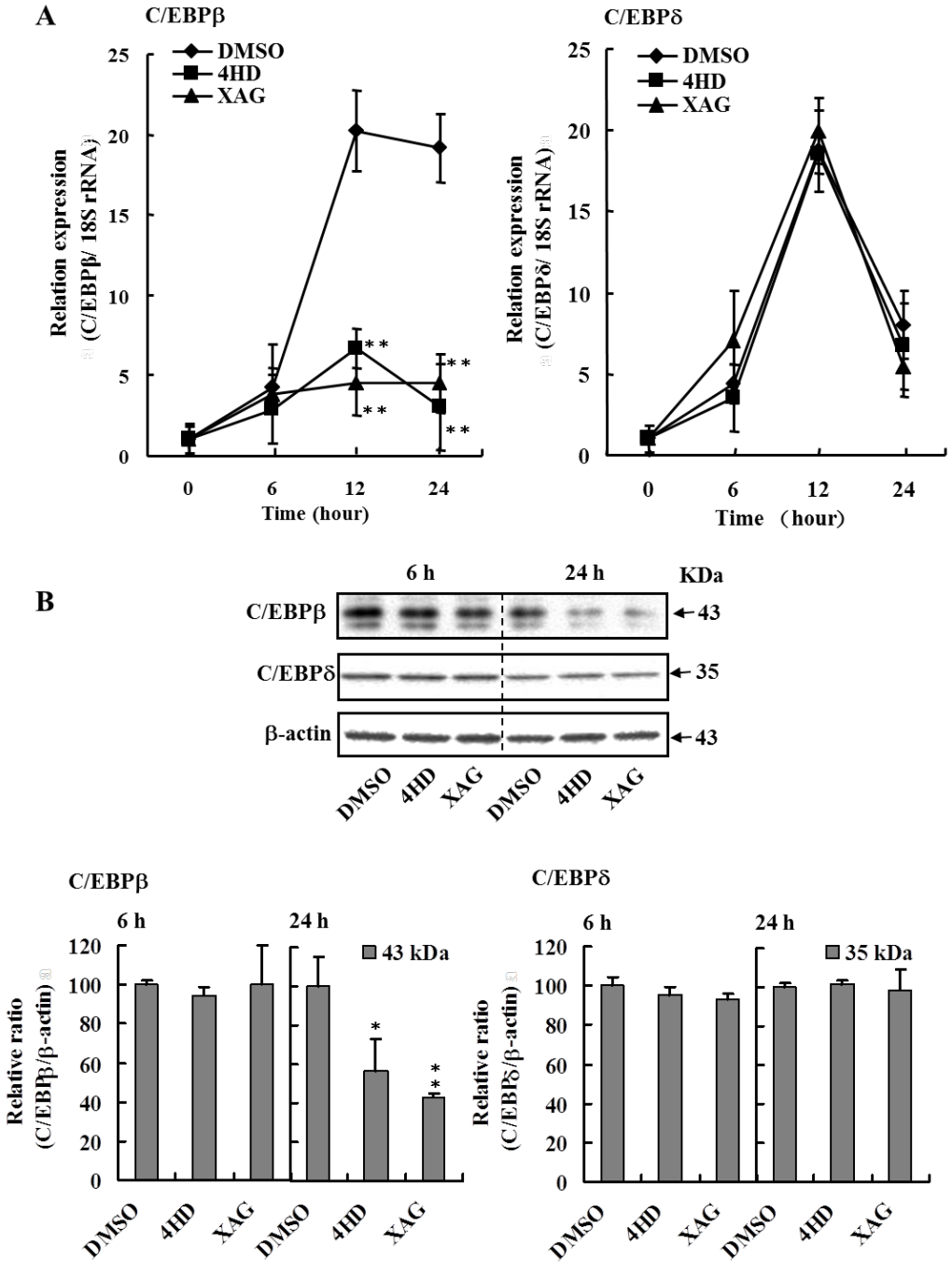


**Fig. 1B-3. Effect of 4HD and XAG on expression of C/EBPβ and C/EBPδ during 3T3-L1 adipocytes differentiation.** The cells were treated with 4HD and XAG at 5 μM or DMSO as a vehicle control for the first 24 h. (A) mRNA expression of C/EBPβ and C/EBPδ were detected by quantitative RT-PCR. (B) Protein expression of C/EBPβ and C/EBPδ was determined by western blotting analysis. Typical results are shown from representative three independent experiments. Band density was quantified and normalized with β-actin. The results represent as the mean ± SD from three independent experiments. Asterisks indicate significant difference from control treated with DMSO alone at each time point by Dunnett's test. * $p<0.05$; * * $p<0.01$.

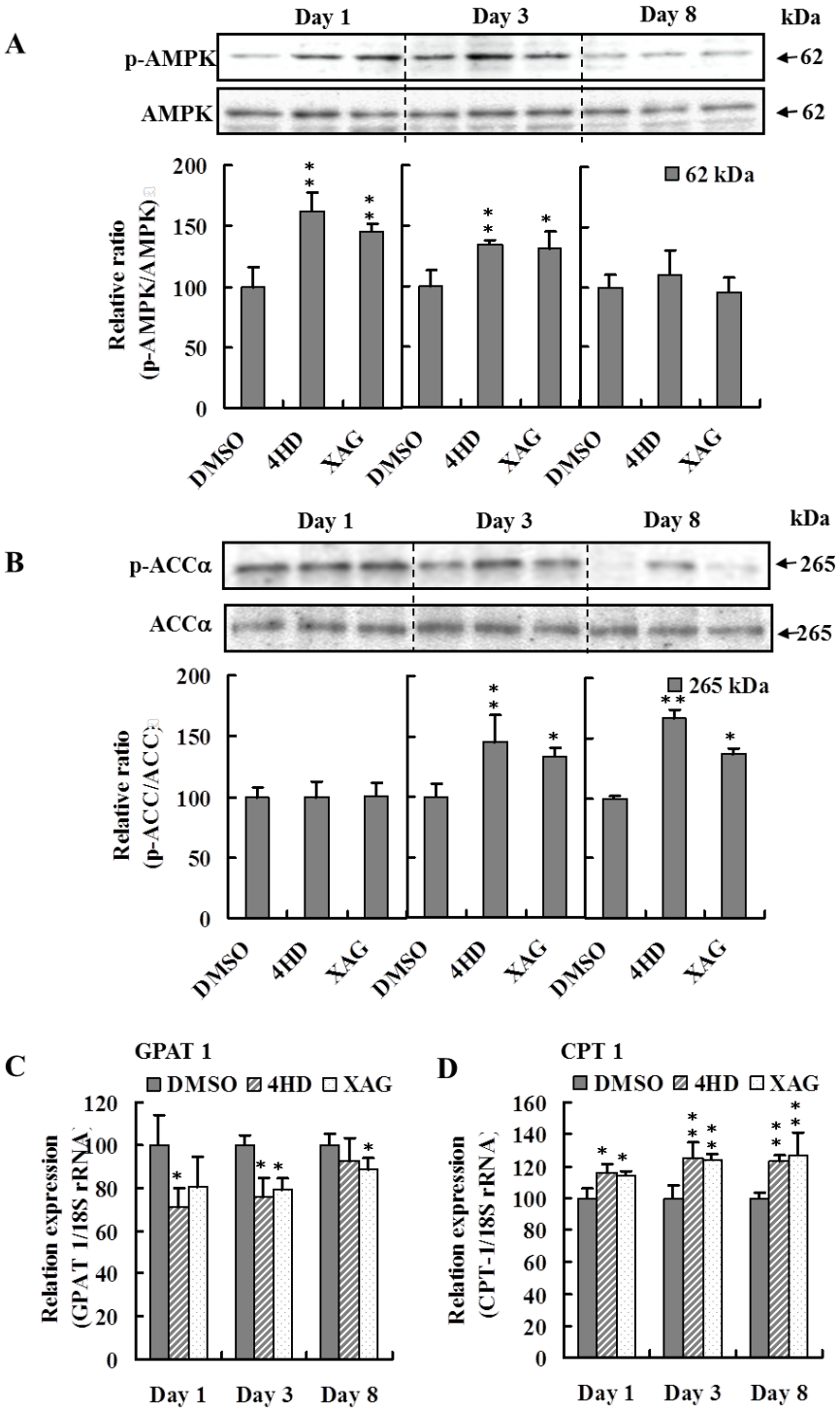


**Fig. 1B-4. Effects of 4HD and XAG on activation of AMPK signaling in 3T3-L1 adipocytes.** The cells were treated with compounds at 5 μM or DMSO as a vehicle control during MDI-induced adipocytes. (A and B) AMPK, p-AMPK, p-ACC and ACC were examined by western blotting. Typical results are shown from representative three independent experiments. Band density of p-AMPK and p-ACC was quantified and normalized with AMPK and ACC, respectively. (C and D) The mRNA expression of enzymes GPAT-1 and CPT-1 were examined using quantitative RT-PCR. The results represent as the mean ± SD from three independent experiments. Asterisks indicate significant difference from control treated with DMSO alone at each time point by Dunnett's test. * $p<0.05$; * * $p<0.01$.

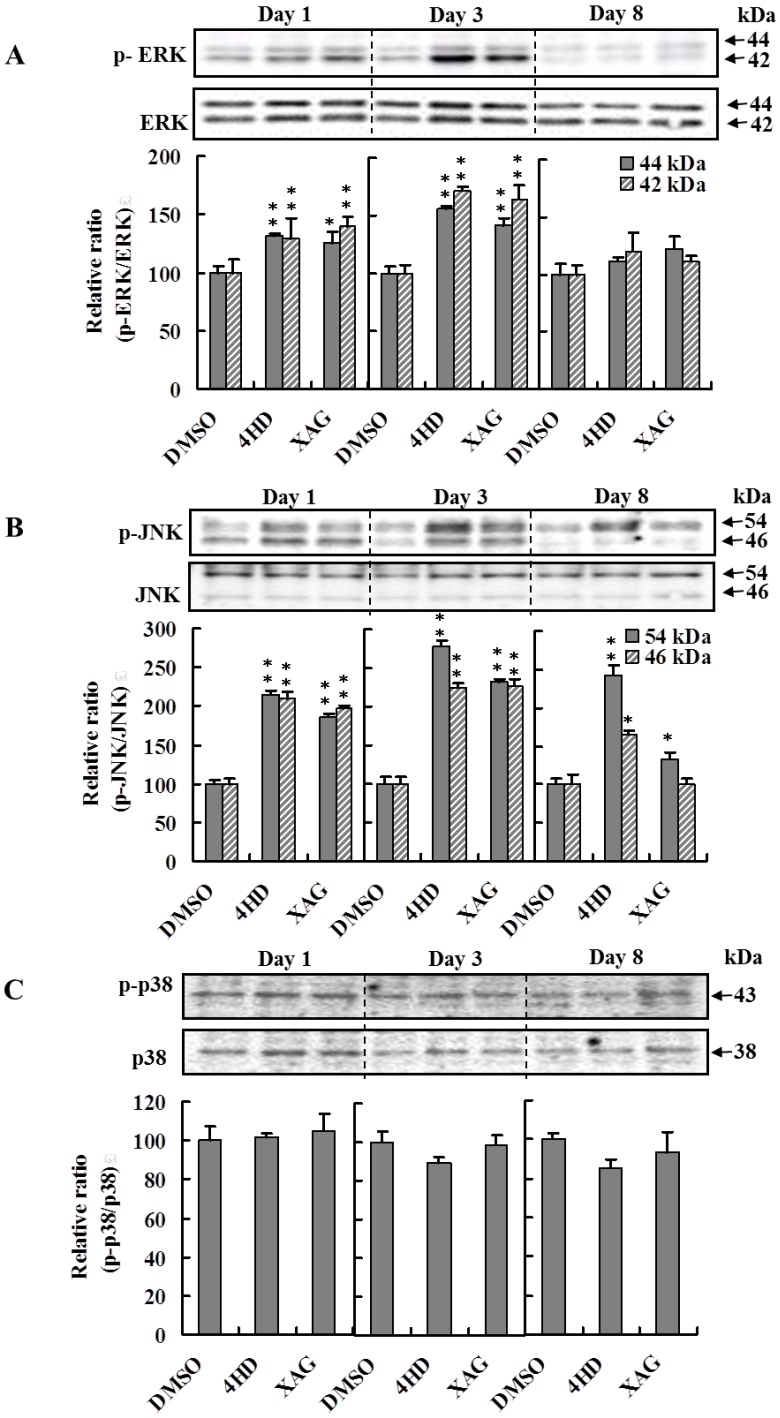


**Fig. 1B-5. Effects of 4HD and XAG on activation of MAPK signaling in 3T3-L1 adipocytes.** The cells were treated with compounds at 5 μM during MDI-induced adipocytes. ERK, JNK and p38 MAPK and their phophorylation level were examined by western blotting. Typical results are shown from representative three independent experiments. Band density of p-ERK, p-JNK and p-p38 MAPK was quantified and normalized with ERK, JNK and p38 MAPK, respectively. The results represent as the mean ± SD from three independent experiments. Asterisks indicate significant difference from control treated with DMSO alone at each time point by Dunnett's test. * $p<0.05$; * * $p<0.01$.

**The effect of 4HD and XAG on MAPK pathway during adipocytes differentiation**

MAPK pathway is required for series of metabolic events and is associated with modulation effect on obesity including adipocytes differentiation. To investigate the role of the MAPK pathway in 4HD- and XAG-inhibiting adipocytes differentiation, phosphorylation levels of ERK, JNK and p38 were estimated by western blotting analysis. These chalcones increased the level of ERK phosphorylation (4HD: 1.28 to 1.71-fold and XAG: 1.26 to 1.64-folds) on both Day 1 and 3 (Fig. 1B-5A). Moreover, they also enhanced the level of JNK phosphorylation (4HD: 1.69 to 2.79-fold and XAG: 1.00 to 2.33-folds on Day 1, 3 and 8) (Fig. 1B-5B) during the differentiation of 3T3-L1 cells. However, they did not bring any alter to p38 phosphorylation level (Fig. 1B-5C).

**AMPK, ERK, JNK inhibitors blocked inhibition effects of 4HD and XAG on adipocytes differentiation.**

To confirm the involvement of AMPK, ERK and JNK in 4HD- and XAG-inhibiting adipocytes differentiation accurately, the author employed Compound C (AMPK inhibitor, 5μM), PD98059 (ERK inhibitor, 20 μM) and SP600125 (JNK inhibitor, 5μM) and treated to the cells. The author have found that 4HD and XAG decreased the C/EBPβ expression both mRNA and protein level on Day 1 (Fig. 1B-3). These chalcones significantly down-regulated the expression of C/EBPα and PPARγ from Day 2 (Fig. 1B-2), thereby significantly decreased the lipid acuumulation from Day 6 (Fig 1B-1B). Moreover, it has been known that C/EBPβ is the initial event that subsequently leads to increase the expression of C/EBPα, PPARγ and probably other adipogenic factors [Tontonoz *et al*., 1994; Wu *et al*., 1999]. Thus, the author determined the mRNA level of C/EBPβ mRNA on Day 1 and that of C/EBPα and PPARγ was determined on Day 5 (Fig. 1B-7). Lipid accumulation was also assessed on Day 7 (Fig. 1B-6). The author found that treatment with the inhibitors for AMPK, ERK and JNK increased the lipid accumulation comparing with control group. In addition, these inhibitors abolished effects the 4HD- and XAG-caused suppression of lipid accumulation (Fig. 1B-6). Furthermore, the author found that the inhibitors increased the expression of C/EBPα (Compound C: 3.92-fold; PD98059: 3.12-fold; SP600125: 3.11-fold and PD+SP: 4.56-fold) and PPARγ (Compound C: 2.87-fold, PD98059: 2.18-fold, SP600125: 2.65-fold and PD+SP: 2.86-fold). The 4HD- and XAG-caused down-regulation of the expression of C/EBPα (4HD: 41 % and XAG: 58 % compared with DMSO treated cells in Control group) and PPARγ

(4HD: 24 % and XAG: 62%) was canceled by treatment with Compound C, PD98059 and SP600125 (Fig. 1B-7 upper and middle panels). In addition, Compound C and PD98059 but not SP600125 increased the expression of C/EBPβ (Compound C: 1.76-folds; PD98059: 1.53-folds; SP600125: 1.09-folds and PD+SP: 1.34-folds). The 4HD- and XAG-caused down-regulation of the expression of C/EBPβ (4HD: 34 % and XAG: 39 %) was also canceled by treatment with Compound C and PD98059, but not SP600125 (Fig.1B-7 bottom panel). These results strongly suggested that 4HD and XAG inhibited adipocytes differentiation via AMPK, ERK and JNK signaling pathways.

**4. Discussion**

Obesity is the main reason for metabolic syndrome. Many studies have been documented that adipocytes differentiation and lipid accumulation are associated with the development of obesity. Certain dietary phytochemicals are considered to be a prescription for anti-obesity by inhibition of differentiation and regulation of lipid metabolism [Rayalam *et al*., 2008; Lu *et al*., 2012; Alappat and Awad, 2010; Gourineni *et al*., 2012]. In this section, the author demonstrated that 4HD and XAG, the major active chalcones of the Japanese herb Ashitaba, reduced lipid accumulation during differentiation of 3T3-L1 cells (Fig. 1B-1) through down-regulation of adipocyte-specific transcription factors, C/EBPs and PPARγ (Figs. 1B-3 and 4), involving in the activation of AMPK and MAPK signaling pathways (Figs. 1B 5-7).

C/EBPs and PPARγ are the master regulators during the adipocyte differentiation. PPARγ forms a heterodimer with retinoic acid X-receptor (RXR) [Kliewer *et al*., 1992], and regulates the transcription of adipocyte-specific genes [Tontonoz *et al*., 1994]. C/EBPα functions as another principal player in adipogenesis and is most abundant in mature adipocytes, where plays a crucial role in insulin-dependent glucose uptake [Wu *et al*., 1999]. In this adipogenic process, GLUT4, which is regulated by C/EBPα, participates in glucose uptake in adipocytes [Lu *et al*., 2012]. At the early stage of adipocyte differentiation, activations of C/EBPβ and C/EBPδ are the initial events that subsequently lead to increase the expression of C/EBPα, PPARγ and probably other adipogenic factors [Cao *et al*., 1991; Yeh *et al*., 1995]. The author's founding revealed that 4HD and XAG dramatically down-regulated C/EBPα, PPARγ and GLUT4 expression accompanied by the decreased C/EBPβ but not C/EBPδ expression (Figs. 1B-2 and 3). However, there is no evidence to make clear that down-regulated C/EBPα and PPARγ expression directly associates with reducing C/EBPβ expression level. Further

study is needed to investigate this issue. These results indicated that 4HD and XAG suppressed C/EBPβ expression at the early stage, thereafter suppressed PPARγ, C/EBPα and GLUT4 expression during adipocytes differentiation.

AMPK is a key enzyme to maintain cellular as well as body energy balance. Intense research in recent years has revealed the critical roles that AMPK plays in modulating ever-expanding many biological pathways. The broad spectrum of activities of AMPK in lipid and glucose metabolism makes it a very attractive target for obesity, diabetes and metabolic syndrome [Zhang *et al*., 2009; Luo *et al*., 2005]. ACC, which is one of the targets molecular of AMPK [Ejaz *et al*., 2009], is an essential enzyme for the synthesis and consumption of fatty acids. The author's results revealed that 4HD and XAG enhanced the phosphorylation of AMPK and ACC during the MDI-induced adipocytes differentiation (Fig. 1B-4A and B). In the downstream events, GPAT-1 and CPT-1 are the enzymes involved in synthesis of glycerol lipids and in oxidation of fatty acid, respectively [Ejaz *et al*., 2009]. The author's results showed that these chalcones caused down-regulating GPAT-1 and up-regulating CPT-1 expression (Fig. 1B-4C and D), respectively. These results suggested that 4HD and XAG inhibited adipogenesis through AMPK activation, thereby suppressed ACC activation and affected the gene expression of GPAT-1 and CPT-1, which in turn inhibited lipid accumulation in adipocytes. To clarify whether the 4HD- and XAG- inhibited adipocytes differentiation was dependent on the AMPK activation, the author employed Compound C as an AMPK inhibitor. The results showed that Compound C entirely cancelled the inhibitory effects of 4HD and XAG on lipid accumulation of adipocytes (Fig. 1B-6). In addition, the effects of down-regulation of C/EBPβ, C/EBPα, and PPARγ were also cancelled by treatment with compound C. The previous study showed that the activation of AMPK inhibited the differentiation of 3T3-L1 cells by down-regulating the expression of C/EBPs and PPARγ [Kimura *et al*., 2004]. Thus, the author's findings strongly proved that 4HD- and XAG-driven inhibition of adipocytes differentiation was involved in AMPK activation.

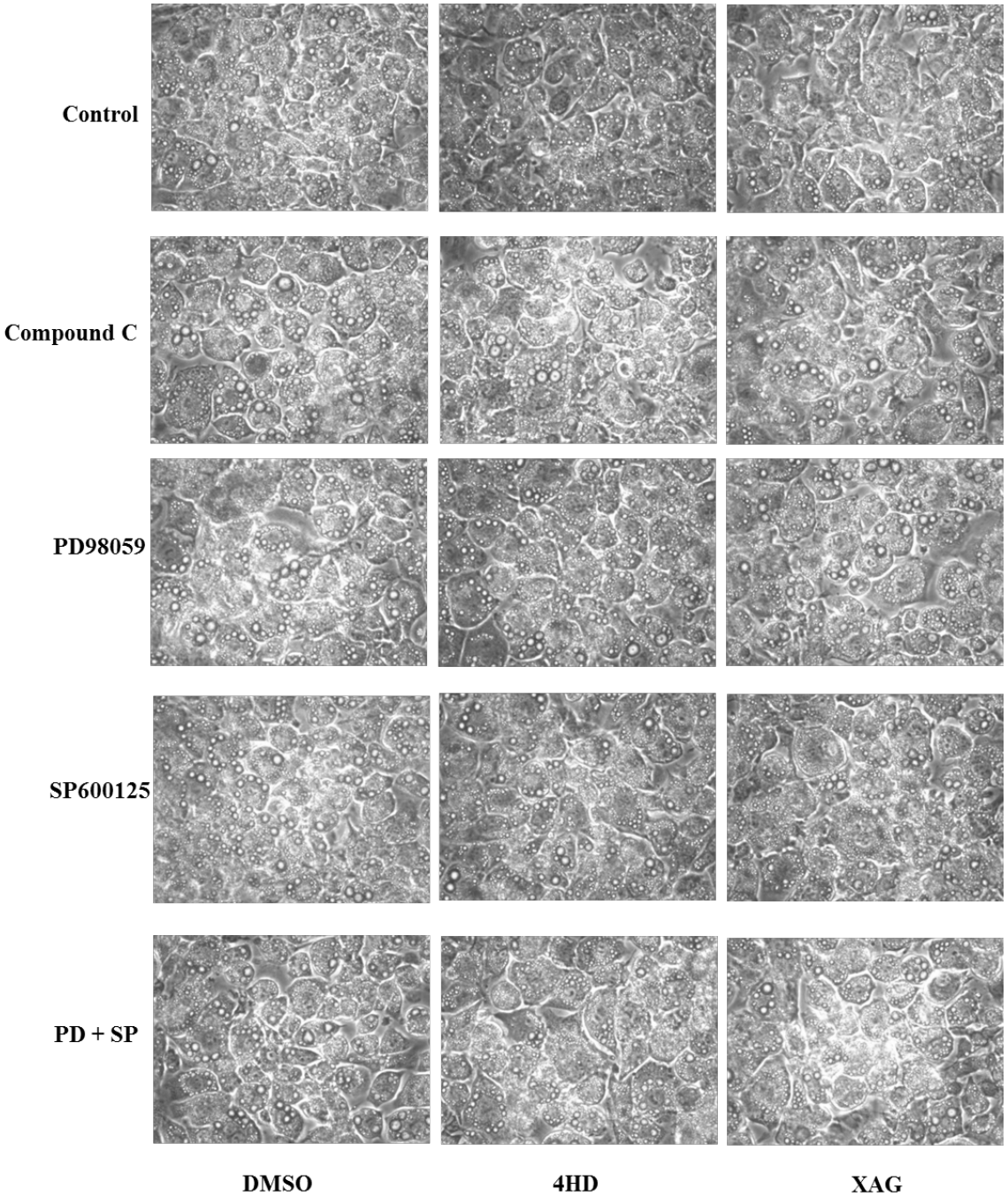


**Fig. 1B-6. Effect of AMPK, ERK, JNK inhibitors on 4HD- and XAG-caused suppression of lipid accumulation in 3T3-L1 cells.** Post-confluent 3T3-L1 cells were treated with 4HD and XAG (5 μM) and simultaneously treated with Compound C (5 μM), PD98059 (20 μM), SP600125 (5 μM) and PD98059 + SP500125 (PD+SP), respectively, for first 3 days. Lipid accumulation was assessed by Sudan II staining at Day 7.

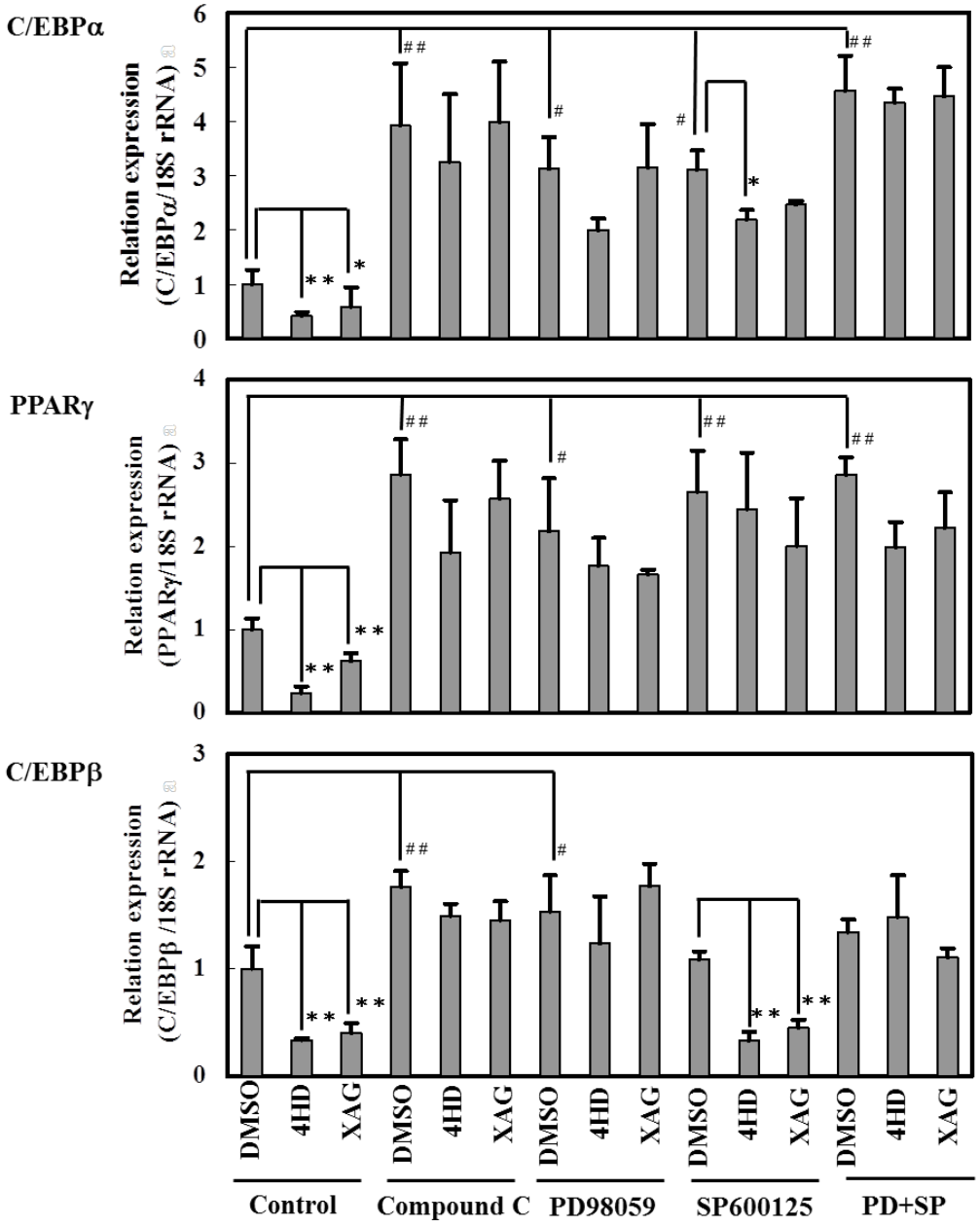


**Fig. 1B-7. Effects of AMPK, ERK, JNK inhibitors on 4HD- and XAG- caused suppression of CEBPβ, C/EBPα and PPARγ expression.** Post-confluent 3T3-L1 cells were treated with 4HD and XAG (5 μM) and simultaneously treated with Compound C (5 μM), PD98059 (20 μM), SP600125 (5 μM) and PD98059 + SP500125 (PD+SP), respectively, for first 3 days. mRNA levels of C/EBPα and PPARγ were determined at Day 5 while mRNA level of C/EBPβ was determined at Day 1. The results represent as the mean ± SD from three independent experiments. Asterisks indicate significant difference from DMSO treated cells in each group by Dunnett's test. * $p<0.05$; * * $p<0.01$. Octothorpes indicate significant difference between inhibitors treated cells to DMSO treated cells of Control group. # $p<0.05$; ## $p<0.01$.

MAPKs are intracellular signaling pathways that play a pivotal role in many essential cellular processes including differentiation [Sakaue *et al*., 2004]. Most of the knowledge comes from the study of three groups of MAPKs: ERK, JNKs and p38 MAPK [Bost *et al*., 2005]. It was reported that ERK activation was necessary for adipocytes differentiation, and the reduction of ERK expression in pre-adipocytes led to decrease in adipocytes differentiation [Kimura *et al*., 2004]. However, the activation of ERK conversely attenuates differentiation by phosphorylation of PPARγ during adipocytes differentiation [Akihisa *et al*., 2012; Hu *et al*., 1996]. Therefore, ERK activation is tightly and temporally controlled: it has to turn on for pre-adipocytes, while shut off to avoid PPARγ phosphorylation during adipocytes differentiation. The author's results showed that 4HD and XAG enhanced ERK phosphorylation during MDI-induced adipocytes differentiation (Fig. 1B-5A), while they brought no effect to ERK activation in preadipocytes (data was not shown). When the ERK inhibitor PD98059 was treated to the cells with the chalcones during MDI-induced differentiation, the inhibition effects of 4HD and XAG on lipid accumulation (Fig. 1B-6) and on the expression of C/EBPβ, C/EBPα and PPARγ were reserved (Figs. 1B-7). It has been known that JNK is involved in insulin signaling pathway [Hirosumi *et al*., 2002; Sabio *et al*., 2009]. However, the effect of JNK on adipocytes differentiation was still not clear. In the present study, the author attempted to clarify this effect using a JNK inhibitor SP600125 and found phosphorylation of JNK inhibited adipocytes differentiation. In addition, the author also found that SP600125 abolished the inhibitory effects of 4HD and XAG on lipid accumulation (Fig. 1B-6) and on the expression of C/EBPα and PPARγ but not C/EBPβ (Fig. 1B-7). Previous studies reported that activation of JNK inhibits adipocyte differentiation by enhancing phosphorylation of PPARγ and negatively regulating its transcriptional activity [Ohkura *et al*., 2011; Hu *et al*., 1996]. These results indicate that the inhibitory effects of 4HD and XAG on adipocytes differentiation is also depend on enhancing phosphorylation of JNK and decreasing expression of C/EBPα and PPARγ. p38 MAPK positively regulates adipocytes differentiation through the phosphorylation of C/EBPβ [Enoki *et al*., 2007]. However, 4HD and XAG did not alter p38 MAPK activation. Taken together all the results, 4HD and XAG inhibited adipocytes differentiation through activating ERK and JNK resulting in down-regulation of adipocyte-specific transcription factors, C/EBPs and PPARγ.

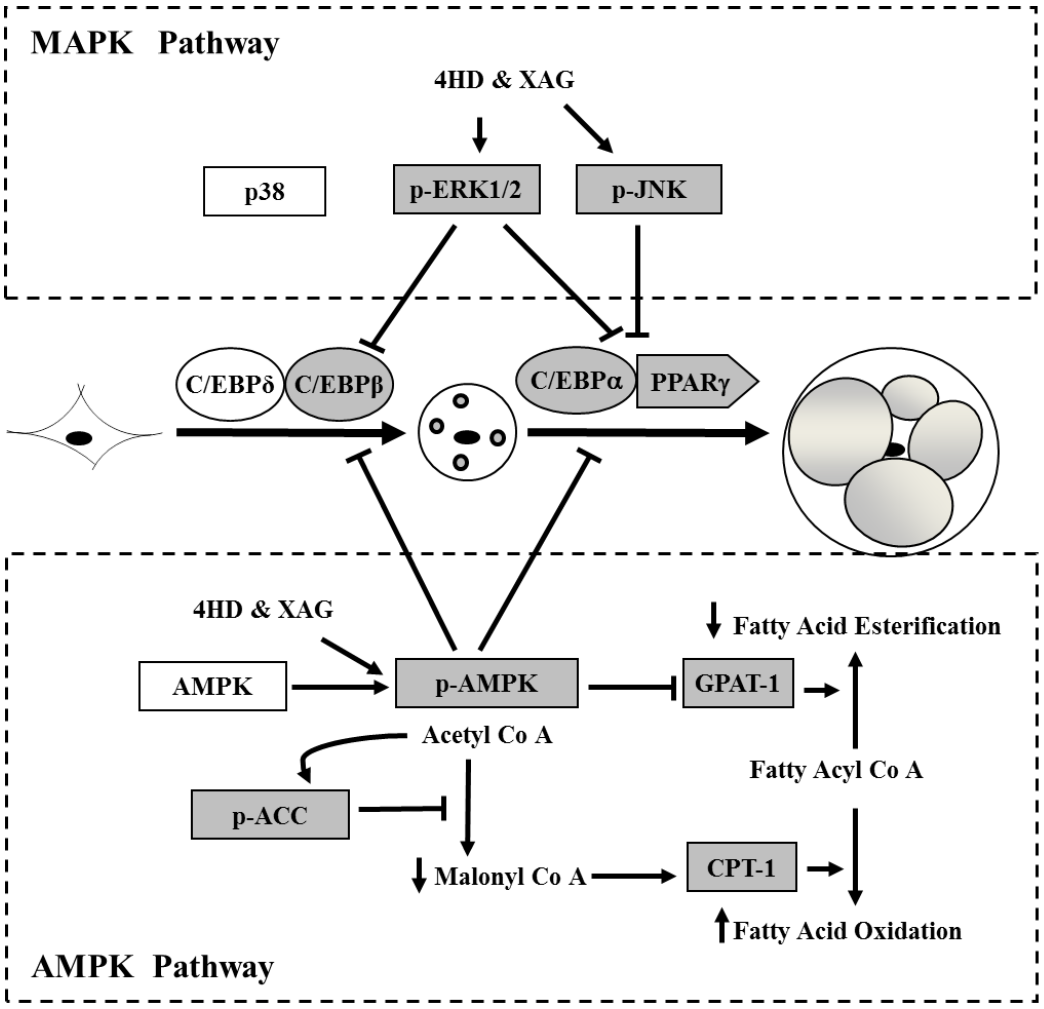


**Fig. 1B-8. The hypothetical scheme of mechanism that 4HD and XAG inhibit adipocytes differentiation by AMPK and MAPK pathways.** (Upper part) 4HD and XAG enhanced ERK and JNK activation by increasing their phosphorylation level but did not alter p38 activation. (Bottom part) 4HD and XAG enhanced AMPK and ACC activities by increasing their phosphorylation levels. The phosphorylated AMPK suppresses expression of GPAT-1 while increases expression of CPT-1. The phosphorylated ERK, JNK and AMPK result in suppression of adipocyte-specific transcription factors expression.

The author's study demonstrated the inhibition effects of Ashitaba chalcones 4HD and XAG on adipocytes differentiation which is deeply involved in the onset of obesity. Obesity is associated with certain metabolic disorder diseases such as type 2 diabetes, cardiovascular diseases, hypertension, and the certain forms of cancer [Kaul *et al*., 2012; Vernooij *et al*., 2012; Vucenik and Stains, 2012; Ligibel, 2011]. The author's founding indicates that 4HD and XAG should be effective compounds for prevention and improvement of obesity and obesity-induced metabolic disorder diseases. Moreover, the author found 4HD and XAG regulated adipocytes differentiation through activation of AMPK, ERK and JNK. These

factors could be available as the molecular targets for anti-obesity therapeutics. However, the mechanisms of between these factors for modulating adipocytes differentiation did not elucidate in present study. It should be an issue for the future study. In addition, to apply the anti-obesity effects to clinical treatment, animal studies should also be performed in the future.

In conclusion, 4HD and XAG are able to inhibit adipocytes differentiation through down-regulating expression of C/EBPβ, C/EBPα and PPARγ in 3T3-L1 adipocytes. As shown in Fig.1B-8, these effects are involved in activation of AMPK, ERK and JNK signal pathways. On the one hand, 4HD and XAG enhanced ERK and JNK activation by increasing their phosphorylation levels. On the other hand, 4HD and XAG enhanced phosphorylation of AMPK and its downstream ACC. The phosphorylation of AMPK also suppressed the expression of glycerol synthesis enzyme GPAT-1 and increased expression of fatty acid oxidative enzyme CPT-1. The phosphorylation of ERK, JNK and AMPK result in suppression of adipocyte-specific transcription factors, C/EBPs and PPARγ. The author's finding provide a possibility that 4HD and XAG are of great benefit to prevent obesity and obesity-related disorders.

**Section C**

## The chalcones cardamonin and flavokawain B inhibit the differentiation of preadipocytes to adipocytes by activating ERK

### INTRODUCTION

In Section B, the author demonstrated the underlying mechanism of two chalcones 4HD and XAG on adipocyte differentiation. To confirm the inhibitory effects of chalcones on adipocytes differentiation, the author focused on another two effective chalcones CAR and FKB in this section. In addition to these chalcones, the author also evaluated ALP, a flavanone with the same molecular weight with CAR, used as a reference compound. CAR and FKB were reported to have some biological activity [Kuroyanagi *et al*., 2011; Tang *et al*., 2012; He *et al*., 2005; Chen *et al*., 2013]. However the effects of these chalcones on adipocyte differentiation and obesity are still not clear to date.

In this section, the author determine the underlying molecular mechanism of CAR and FKB on adipocyte differentiation, the author examined the effects of these chalcones on the expression of the adipocyte-specific transcription factors PPARγ and C/EBPα, and on ERK phosphorylation in differentiating 3T3-L1 cells.

### MATERIAL AND METHODS

#### Materials

The PCR primer pairs were synthesized as shown in Table 1C-1. Other materials were performed as described in Section A and B.

Table 1C-1. Primer sequences used for RT-PCR amplification

| Gene | 5’-3’ primer sequence | Reference |
|---|---|---|
| PPARγ | F: ACGTGCAGCTACTGCATGTGA | [Ejaz et al., 2009] |
| | R: AGAAGGAACACGTTGTCAGCG | |
| C/EBPα | F: GGAACTTGAAGCACAATCGATC | [Ejaz et al., 2009] |
| | R: TGGTTTAGCATAGACGTGCACA | |
| GAPDH | F: ACAACTTTGGCATTGTGGAA | [Kanamoto, et al. 2011] |
| | R: GATGCAGGGATGATGTTCTG | |

**Cell culture and treatments**

Cell culture was performed as described in Section A. To search for the inhibitory effect of CAR and FKB on the intracellular lipid accumulation , post-confluent 3T3-L1 cells in the 96-well plates were first treated with CAR, FKB and ALP or DMSO alone as a vehicle control (a final concentration at 0.25%) for 3 days. sudan II staining were carried out at Days 0, 3, 6, 8, and 10. To investigate the molecular mechanism by which the CAR and FKB inhibited adipocyte differentiation, 3T3-L1 cells were plated on a 35-mm dish and were treated with each compound for 3 days. Then, cells were prepared for protein and mRNA analyses at the times indicated in each figure. For treatment with the ERK inhibitor, 3T3-L1 cells were plated in a 35-mm dish, and were simultaneously treated with the indicated chalcone plus 20 μM PD98059 during MDI-induced adipocyte differentiation.

**Sudan II staining**

Sudan II staining was performed as described in Section A.

**RNA isolation and real-time quantitative PCR (RT-PCR) analysis**

RNA isolation and RT-PCR analysis were performed as described in Chapter 1B.

**Western blotting analysis**

Preparation of the cell lysate and western blotting were performed according to the previous report [Furuyashiki *et al*., 2004]. Specific immune complexes were detected with the ATTO Light-Capture II Western Blotting Detection System. The density of specific bands was calculated using ImageJ image analysis software (NIH, Bethesda, MD, USA).

**Statistical analysis**

All data are expressed as the mean ± standard deviation (SD) of at least three independent determinations for each experiment. Statistical significance was analyzed using the Dunnett’s test, and probability levels of 0.05 and 0.01 were considered to indicate statistical significance.

## RESULTS

### CAR and FKB inhibited lipid accumulation during differentiation of 3T3-L1 adipocytes

Morphological analysis showed that treatment with 5 μM CAR and FKB significantly inhibited lipid accumulation, whereas ALP did not (Fig. 1C-1A). Sudan II staining at Days 0, 3, 6, 8, and 10, revealed that 5 μM CAR and FKB suppressed lipid accumulation in a time-dependent manner (Fig. 1C-1B). Lipid accumulation was significant suppressed from Day 6 onwards, and lipid accumulation at Day 10 had decreased to approximately 55% and 66% in cells treated with CAR and FKB, respectively. ALP did not significantly affect lipid accumulation under the same experimental conditions.

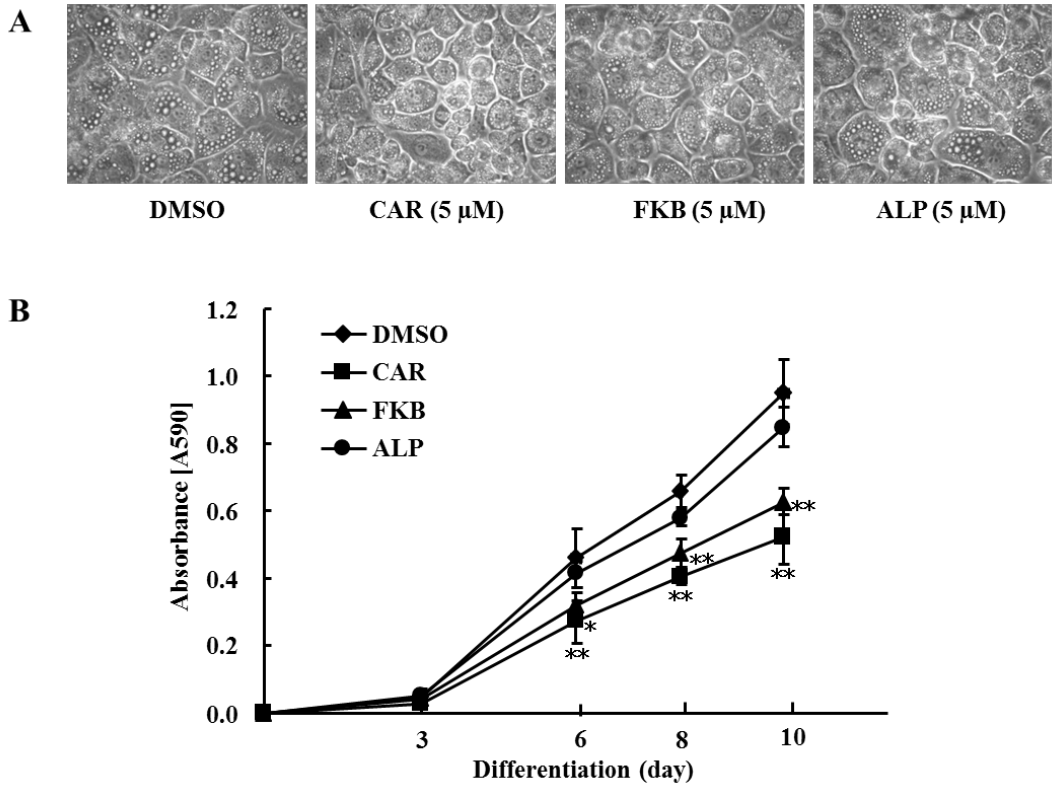


**Fig. 1C-1. Inhibitory effects of CAR and FKB on lipid accumulation in 3T3-L1 cells.** The cells were treated with 5 μM CAR and FKB, or ALP, or DMSO as a vehicle control, for first 3 days. (A) Intracellular lipid accumulation was determined by Sudan II straining at Day 8. The stained cells were photographed at a magnification of 200×. (B) Intracellular lipid accumulation was measured at Days 0, 3, 6, 8, and 10 after Sudan II straining. The results are presented as the mean ± SD of three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO alone at each time-point (Dunnett's test).

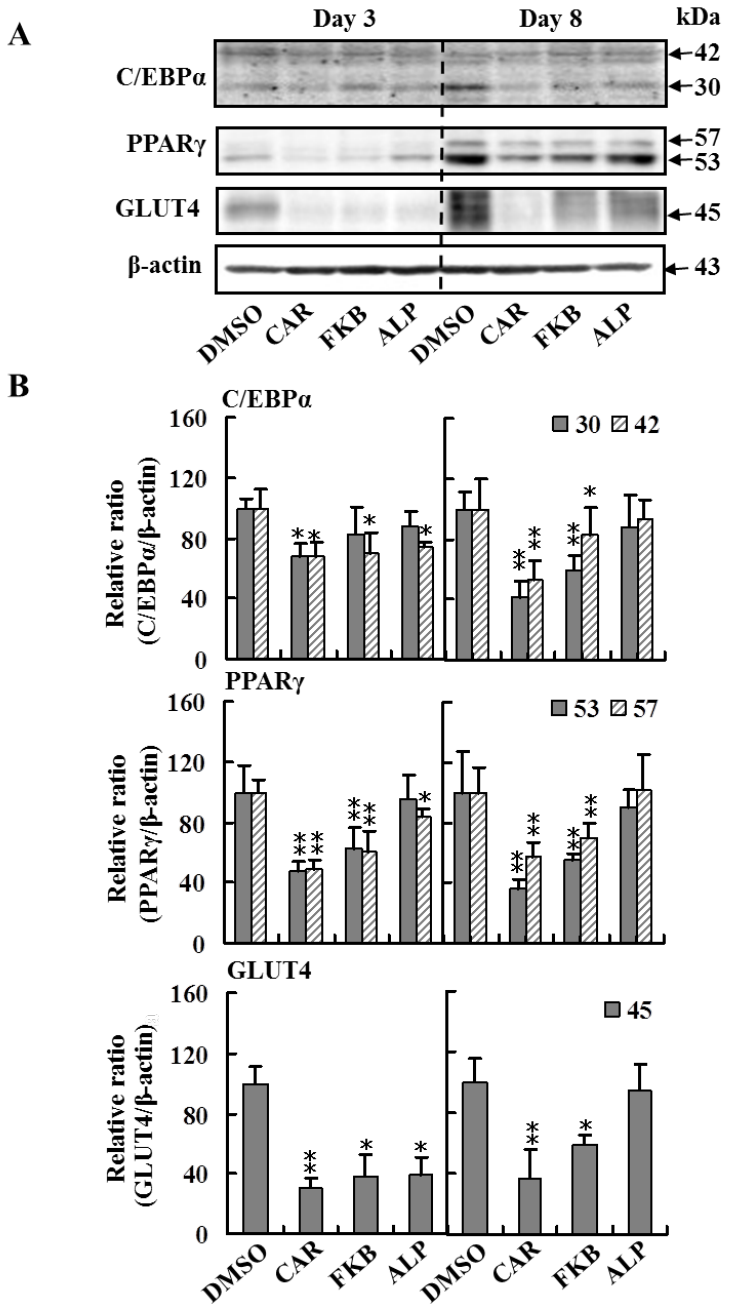


**Fig. 1C-2. CAR and FKB downregulated the protein expression of C/EBPα, PPARγ, and GLUT4 during 3T3-L1 adipocyte differentiation.** The cells were treated with 5 μM CAR, FKB and ALP or DMSO as a vehicle control, for the first 3 days. (A) The protein expression levels of C/EBPα, PPARγ, and GLUT4 were determined by western blotting. Typical results of three representative, independent experiments are shown. (B) Densitometric analysis of specific bands after normalization for β-actin. The results are presented as the mean ± SD of three independent experiments. **P*<0.05 and ***P*<.001 *vs*. DMSO alone at each time-point (Dunnett's test).

**Effect of CAR and FKB on the expression of adipocyte-specific transcription factors during 3T3-L1 adipocytes differentiation**

To determine whether the suppression of lipid accumulation was due to downregulation of adipocyte-specific differentiation markers, the quantitative RT-PCR and western blotting were conducted to determine the mRNA and protein expression levels of C/EBPα, PPARγ, and GLUT4. As shown in Figure 1C-2, treatment with 5 μM CAR and FKB decreased the expression of C/EBPα (42–83%) and PPARγ (36–70%) at Days 3 and 8, respectively, whereas

ALP did not significantly affect their expression levels. CAR and FKB also significantly downregulated the protein expression of GLUT4, a late-stage marker of differentiation, at Day 3 by 31% and 38%, respectively, and at Day 8 by 37% and 59%, respectively. ALP decreased GLUT4 expression by 39% at Day 3, but its effect was diminished at Day 8, at which time the expression level was 95% of that in the DMSO control group. CAR and FKB, but not ALP, significantly downregulated the mRNA levels of C/EBPα (CAR: 31%; FKB: 72%) and PPARγ (CAR: 51%; FKB: 77%) at Day 5 (Fig. 1C-3).

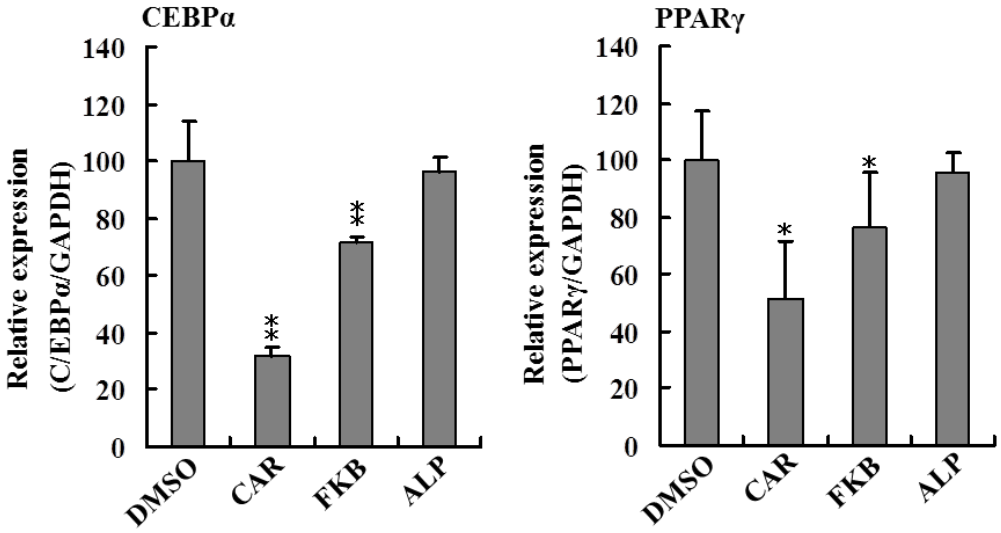


**Fig. 1C-3. CAR and FKB downregulated the mRNA expression levels of C/EBPα and PPARγ during 3T3-L1 adipocyte differentiation.** The cells were treated with 5 μM CAR, FKB and ALP, or DMSO as a vehicle control, for the first 3 days. The mRNA expression levels of C/EBPα and PPARγ were determined by quantitative RT-PCR at Day 3. The results are presented as the mean ± SD of three independent experiments. **P*<0.05 and ***P*<0.01 *vs.* DMSO-treated cells (Dunnett's test).

**Effects of CAR and FKB on ERK activation during adipocyte differentiation**

ERK activation is associated with a modulatory effect on obesity, including adipocyte differentiation [Sakaue *et al.*, 2004]. To investigate whether ERK activation was involved in the inhibitory effects of CAR and FKB on adipocyte differentiation, the phosphorylated level of ERK was determined by western blotting analysis. These chalcones increased the phosphorylation level of ERK: CAR increased ERK phosphorylation (1.28– to 1.82–fold) on both Days 1 and 3, and FKB (1.33– to 1.61–fold), respectively (Fig. 1C-4). Both chalcones increased ERK phosphorylation in a dose-dependent manner at Day 2 of adipocyte differentiation. By contrast, ALP did not significantly affect ERK phosphorylation.

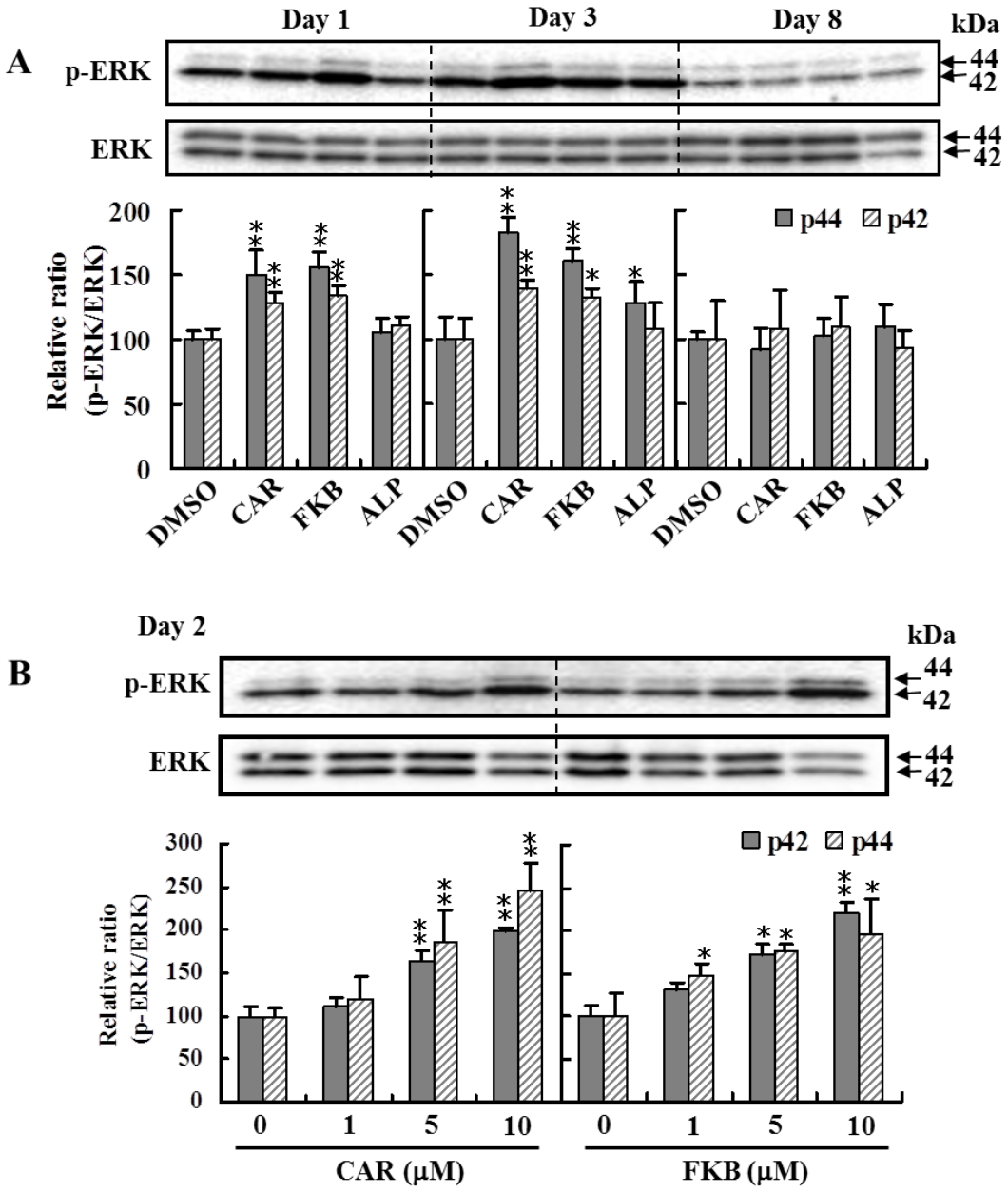


**Fig. 1C-4. Effects of CAR and FKB on ERK activation in 3T3-L1 cells.** (A) The cells were treated with 5 μM of CAR, FKB and ALP during MDI-induced adipocytes. ERK and p-ERK protein expression levels were determined by western blotting at Days 1, 3, and 8. (B) The dose-dependent effects of cardamonin and flavokawain B on ERK phosphorylation were estimated at Day 2. Typical results of three representative, independent experiments are shown. Densitometric analysis of p-ERK was conducted after normalization for ERK. The results are presented as the mean ± SD of three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO alone at each time-point (Dunnett's test).

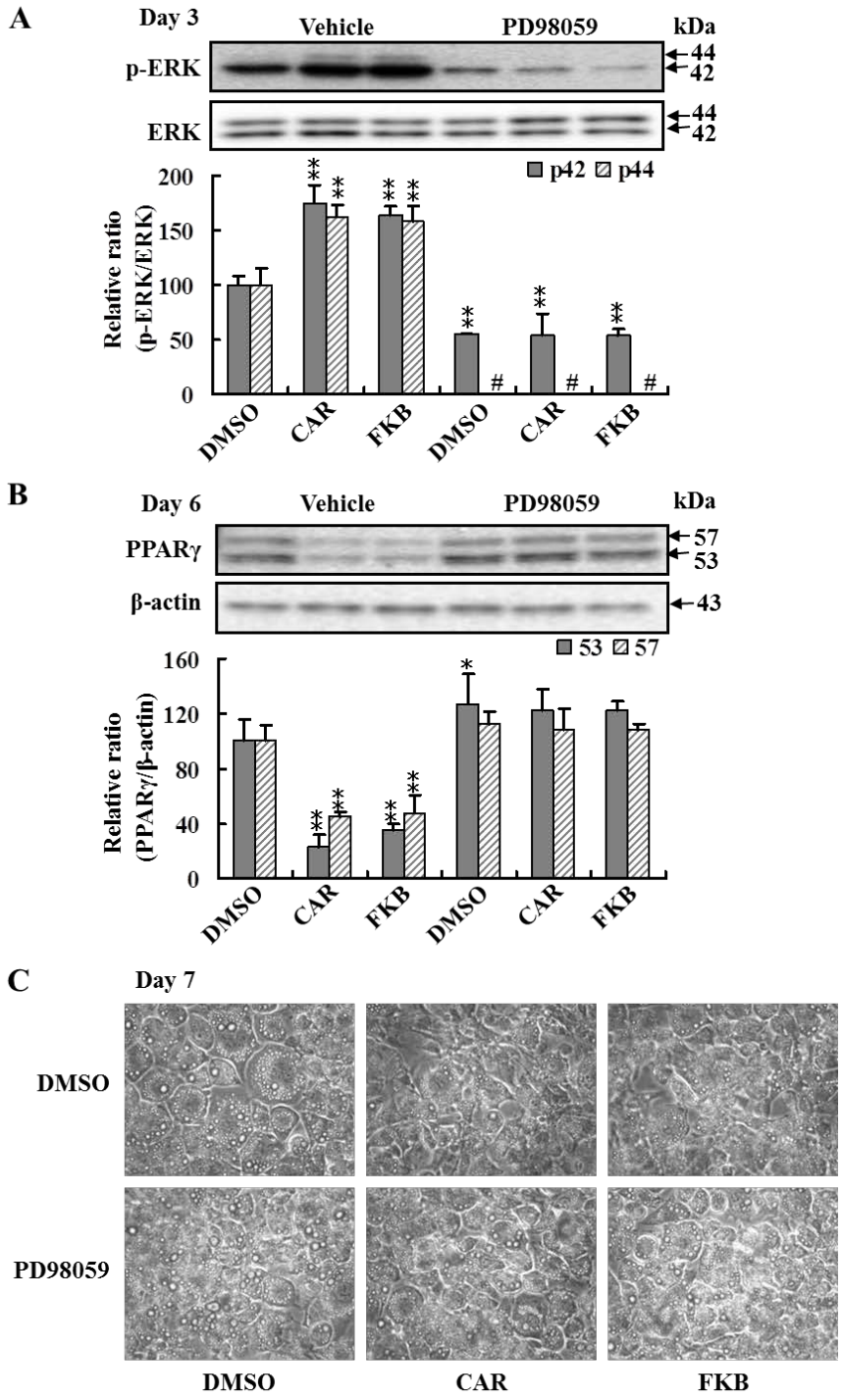


**Fig. 1C-5. Effects of the ERK inhibitor PD98059 on the suppressive effects of CAR and FKB on PPARγ expression and lipid accumulation.** 3T3-L1 cells were treated with 5 μM CAR and FKB in the presence of 20 μM PD98059 for first 3 days. (A) ERK and p-ERK protein expression levels were examined at Day 3. (B) The expression of PPARγ was determined at Day 6. (C) Lipid accumulation was assessed at Day 7. Typical results of three representative, independent experiments are shown. Densitometric analysis of p-ERK was conducted after normalization for ERK, while that of PPARγ was normalized for β-actin. The results are presented as the mean ± SD of three independent experiments. **P*<0.05 and ***P*<0.01 *vs.* DMSO alone at each time-point (Dunnett's test).

**The ERK inhibitor PD98059 restored CAR- and FKB-inhibiting adipocytes differentiation.**

To confirm that ERK activation was involved in the inhibitory effects of CAR and FKB on adipocyte differentiation, the author treated cells with 20 μM of PD98059, an ERK inhibitor. The author confirmed that PD09059 restored CAR- and FKB-induced ERK phspharylation at Day 3 (Fig. 1C-4A). PPARγ expression at Day 6 was downregulated by CAR (53 kDa: 23%; 57 kDa: 45%) and FKB (53 kDa: 35%; 57 kDa: 47%) compared with DMSO-treated cells. These effects were abolished by treatment with PD98059 (Fig. 1C-5B). PD98059 also abolished the inhibitory effects of CAR and FKB on lipid accumulation (Fig. 1C-5C). Treatment with PD98059 alone slightly increased PPARγ expression and lipid accumulation (Fig. 1C-5B,C). These results strongly indicated that CAR and FKB inhibit adipocyte differentiation by activating ERK.

**DISCUSSION**

In the Section A, the author found that CAR and FKB inhibited lipid accumulation in 3T3-L1 cells without cytotoxic effects when administered at a concentration of 5 μM. In this section, the author further analyses CAR and FKB, and found that their inhibitory effects on lipid accumulation in 3T3-L1 cells involved downregulation of the adipocyte-specific transcription factors PPARγ and C/EBPα (Fig. 1C-2 and 3) as a consequence of ERK activation (Fig. 1C-4 and 5).

CAR is one of the main constituents of the seeds of *Alpinia katsumadai* Hayata, which has been used as a traditional Chinese herbal antiemetic and gastric drug [Kuroyanagi *et al*., 2011]. ALP, a flavonoe also contained in *Alpinia katsumadai* Hayata, was also reported to have antibacterial, antitumor, and anti-inflammatory effects [Tang *et al*., 2012; He *et al*., 2005; Chen *et al*., 2013]. ALP and CAR have the same molecular weight but different molecular structures. In this section, ALP was used as a reference compound to investigate the effects of chalcones on the differentiation of 3T3-L1 cells. FKB, an active constituent in *Alpia pricei* Hayata and Kava, also has antioxidant, anti-inflammatory, and antidiabetic effects [Yamamoto *et al*., 2011; Zhou *et al*., 2009; Yu *et al*., 2009; Lin *et al*., 2009]. The author found that CAR and FKB, but not ALP, inhibited lipid accumulation in 3T3-L1 cells (Fig. 1C-1). These results showed that the α, β-unsaturated carbonyl moiety in CAR and FKB might play an important role in the inhibition of lipid accumulation.

It is well known that C/EBPα and PPARγ are master regulators of adipocyte differentiation. PPARγ forms a heterodimer with retinoic acid X-receptor [Tontonoz *et al.*, 1994], and regulates the transcription of adipocyte-specific genes [Wu, *et al.*, 1999]. C/EBPα is another key mediator of adipogenesis and is very abundant in mature adipocytes, where it plays a crucial role in regulating adipocyte differentiation and glucose uptake [Cao *et al.*, 1991]. In this adipogenic process, GLUT4, which is regulated by C/EBPα, is responsible for glucose uptake in adipocytes [Kaestner *et al.*, 1990]. Through the coordinated regulation of these adipocyte-specific transcription factors and GLUT4, adipocytes accumulate lipids during differentiation. This study demonstrated that CAR and FKB, but not ALP, significantly downregulated the expression of C/EBPα, PPARγ, and GLUT4 (Fig. 1C-2 and 3). These results indicate that downregulation of C/EBPα and PPARγ inhibits lipid accumulation in 3T3-L1 adipocytes. Thereafter, the author determined whether ERK activation was involved in the inhibitory effects of CAR and FKB on adipocyte differentiation. The author found that CAR and FKB, but not ALP, enhanced ERK phosphorylation during MDI-induced adipocyte differentiation (Fig. 1C-4), and this enhancement was canceled by the ERK inhibitor PD98059 (Fig. 1C-5). ERK is a member of the mitogen-activated protein kinase family and plays a pivotal role in many essential cellular processes [Sakaue *et al.*, 2004]. It was reported that ERK activation was necessary for adipocyte differentiation, and that a reduction in ERK expression in preadipocytes decreases adipocyte differentiation [Sale *et al.*, 1995]. However, ERK activation also attenuates differentiation by inducing PPARγ phosphorylation during adipocyte differentiation [Camp *et al.*, 1999; Hu *et al.*, 1996]. It seems that ERK activation is tightly controlled in a temporal manner, being activated in preadipocytes and deactivated during adipocyte differentiation to prevent PPARγ phosphorylation. Recent studies showed that ERK activation is required for cell mitosis in the early stage of adipogenesis, and that activated (phosphorylated) ERK needs to be dephosphorylated thereafter because ERK might enhance PPARγ efflux from nucleus, suppress PPARγ degradation, and inhibit adipocyte differentiation [Burgermeister *et al.*, 2007; Wang *et al.*, 2009; Kim *et al.*, 2009]. Moreover, diallyl trisulfide suppresses the adipogenesis of 3T3-L1 cells by activating ERK, and evodiamine improved diet-induced obesity by inhibiting adipocyte differentiation via ERK activation [Lii *et al.*, 2012; Wang *et al.*, 2009]. Therefore, the author's results showing that CAR and FKB inhibit adipocyte differentiation through ERK activation are consistent with those of prior studies.

In conclusion, the author found that CAR and FKB effectively suppressed lipid accumulation in 3T3-L1 cells. CAR and FKB inhibited the differentiation of preadipocytes into adipocytes. The inhibitory effects of these chalcones were at least partly regulated by ERK activation, which downregulated the expression of adipocyte-specific transcription factors. The author's findings suggest that CAR and FKB might be valuable for preventing obesity and obesity-related metabolic syndrome.

## CHAPTER DISCUSSION

The prevalence of obesity has increased dramatically in recent years. Obese individuals are more likely than lean individuals to develop type 2 diabetes mellitus, cardiovascular diseases, and some types of cancer [Kaul *et al.*, 2012; Vernooij *et al.*, 2012; Vucenik and Stains, 2012]. Obesity is recognized as a disease state that requires a range of interventions to treat and prevent the progression of obesity. Polyphenols are expected to be useful to treat and prevent obesity and other diseases [Williamson *et al.*, 2005; Rayalam *et al.*, 2008]. In this chapter, the author searched for a panel of 49 polyphenols capable of inhibiting lipid accumulation in 3T3-L1 cells. Six of these polyphenols, including four chalcones 4HD, XAG, CAR and FKB inhibited lipid accumulation in 3T3-L1 cells without cytotoxic effects when administered at a concentration of 5 μM (Section A). The author conducted further analyses in 4HD, XAG, CAR and FKB inhibiting adipocyte differentiation and attempted to clarify the underlying molecular mechanism in Section B and C.

During adipogenesis, fibroblast-like preadipocytes differentiate into adipocytes, the process occurs in several stages and involves a cascade of transcription factors. Among them, PPARγ and C/EBPs are considered to be the master regulators or the crucial determinants of adipocyte fate [Tontonoz *et al*., 1994; Lefterova *et al*., 2009]. AMPK is a key enzyme to maintain cellular as well as body energy balance. Intense research in recent years has revealed the critical roles that AMPK plays in modulating ever-expanding many biological pathways. The broad spectrum of activities of AMPK in lipid and glucose metabolism makes it a very attractive target for obesity, diabetes and metabolic syndrome [Zhang *et al*., 2009; Luo *et al*., 2005]. MAPKs are intracellular signaling pathways that play a pivotal role in many essential cellular processes including differentiation [Sakaue *et al*., 2004]. Most of the knowledge comes from the study of three groups of MAPKs: ERK, JNK and p38 MAPK [Bost *et al*., 2005]. In Section B, the author demonstrated the mechanism of Ashitaba chalcones 4HD and XAG on adipocyte differentiation which is deeply involed in the onset of obesity. The results showed that 4HD and XAG are able to inhibit adipocytes differentiation through down-regulating expression of C/EBPβ, C/EBPα and PPARγ in 3T3-L1 adipocytes. Moreover, the author found 4HD and XAG regulated adipocyte differentiation though activation of AMPK, ERK and JNK. These factors could be available as the molecular targets for anti-obesity therapeutics. In Section C, the author selected another two chalcones CAR and FKB, and a flavonone ALP as a reference compound to confirm the inhibitory effects of

chalcones on adipocyte differentiaton. The author found that CAR and FKB but not ALP effectively suppressed lipid accumulation in 3T3-L1 cells. CAR and FKB also inhibited the differentiation of preadipocytes into adipocytes. The inhibitory effects of these chalcones were at least partly regulated by ERK activation, which downregulated the expression of adipocyte-specific transcription factors.

In conclusion, the author found that chalcones 4HD, XAG, CAR and FKB effectively suppressed lipid accumulation in adipocytes. These chalcones are able to inhibit adipocyte differentiation through down-regulating expression of C/EBPs and PPARγ. Moreover, the author found chalcones regulated adipocyte differentiation though AMPK and MAPK pathways. These findings provide a possibility that chalcones are of great benefit to prevent obesity and obesity-related disorders.

## Chapter 2

## Chalcones suppress fatty acids-induced lipid accumulation through LKB1/AMPK signaling pathway in hepatocytes.

### INTRODUCTION

The liver is an important organ that maintains whole-body energy homeostasis though metabolizing fatty acids (FAs) and glucose [Cheung and sanyal, 2008]. Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide and characterized by hepatic lipid accumulation in the absence of significant ethanol consumption. It comprises of a broad spectrum of the liver diseases ranging from hepatic steatosis to steatohepatitis and latter progressing to cirrhosis [Matteoni *et al.* 1999]. Several studies suggest that excessive intake of calories, visceral obesity, and insulin resistance will burden liver function and be important risk factors for NAFLD development [Browning and Horton, 2004]. Over-accumulation of lipids in hepatocytes is currently recognized as the most common cause of chronic liver disease and an increase in the risk of other diseases [Targher *et al.* 2007; Ahmed *et al.* 2009].

Lipid accumulation in the liver is caused by enhancing *de novo* lipogenesis, and lowing of lipid catabolism. Studies have shown that sterol regulatory element binding protein-1 (SREBP-1) regulates lipid metabolism. SREBP-1 plays an essential role in the regulation of lipogenesis in FA and triglycerides synthesis [Brown and Goldstein, 1997; Sanyal, 2005]. It is also known that peroxisome proliferators-activated receptors (PPARs), which are ligand-activated nuclear receptors, mediate the critical transcriptional regulation of genes associated with lipid homeostasis [Lee *et al*., 2003]. Especially, PPARα is the most abundantly expressed in the liver and has the effect of diminishing circulating triglycerides and prevent hepatic steatosis involving in increasing hepatic FA oxidation [Braissant and Wahli, 1998]. A lot of evidences indicates that AMP-activated protein kinase (AMPK) is involved in regulating hepatic lipogenesis [Schimmack *et al*., 2006]. Activation of hepatic AMPK attenuates FA synthesis and promotes FA oxidation [Hardie, 2007; McGarry and Brown, 1997]. Recent studies revealed that liver kinase B1 (LKB1) is one of the important upstream kinases of AMPK [Hawley *et al*., 2003; Woods *et al*., 2003]. LKB1 can activate AMPK by phosphorylation at Thr172 in mammalian cells. The activation of AMPK leads to the phosphrylation and inhibition of acetyl-CoA carboxylase activity and leads to an increase in FA oxidation [Park *et al*., 2002]. It is also reported that activated AMPK interacted with

SREBP-1 and inhibited its transcriptional activity, leading to reduced lipogenesis and lipid accumulation [Li *et al*., 2011]. Thus, AMPK should be a therapeutic target for treating fatty liver diseases. The mitogen-activated protein kinases (MAPKs) signaling occurs in response to almost any change in the extracellular or intracellular milieu that affects the metabolism of the cell, organ or the entire organism [Gehart *et al*., 2010]. MAPKs play an important role in cellular metabolism including regulating in insulin signaling, adipocyte development as well as hepatic lipid metabolism [Gehart *et al*., 2010; Deak *et al*., 1998; Xiong *et al*., 2007; Malhi *et al*., 2006].

Chalcones are containing in various plants and have a variety of health-promoting effects. Several synthetic chalcones also showed biologically active [Dimmock *et al*., 1999; Bandgar *et al*., 2009]. 4HD and XAG are two major chalcones in *Angelica keiskei* Ashitaba, a Japanese herb, which has been used as a traditional medicine for long time. They have been reported to exert various biological effects such as anti-tumor [Kimura *et al*., 2004; Akihisa *et al*., 2011], anti-inflammatory [Ohkura *et al*., 2011] and anti-diabetes [Kawabata *et al*., 2011, Enoki *et al*., 2007] effects. CAR is one of the main constituents of the seeds of *Alpinia katsumadai* Hayata, which has been used as a traditional Chinese herbal antiemetic and stomachic drug [Kuroyanagi *et al*., 1983]. FKB, a 4'-*O*-methylated analog of CAR, is an active constituent in *Alpia pricei* Hayata and Kava. These two chalcones are also reported to possess antioxidant, antiinflammatory, antidiabetic and other important therapeutic activities of significant potency [Lee *et al*., 2003; Yu *et al*., 2009; Lin *et al*., 2009]. In Chapter 1, the author found that these chalcones also showed strong inhibitory effect on adipocytes differentiation. However, the inhibitory effects of these chalcones on hepatic steatosis are unknown yet.

In this chapter, the author first designed to define an experimental model of hepatic steatosis without cytotoxicity using unsaturated and saturated FAs in human HepG2 cells. Then, the author used this model to investigate the preventive effect of 4HD, XAG, CAR and FKB on FAs-induced lipid accumulation. To clarify the underlying molecular mechanisms, the author, further, investigated the effect of these chalcones on the expression of SREBP-1, PPARα and phosphorylation of LKB1, AMPK and MAPKs.

## MATERIALS METHODS

### Reagents

4HD and XAG were purified from "Ashitaba Chalcone Powder" as a commercial

product of Japan Bio Science Laboratory (Osaka, Japan). CAR was isolated from the seeds of *A. katsumadai Hayata*, and FKB was synthesized by direct aldol condensation of acetophenone with benzaldehyde, as previously described [Yamamoto *et al*., 2011]. The cell proliferation reagent WST-1 substrate was purchased from Roche Diagnostics (Mannheim, Germany). Antibodies for PPARα, horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG and anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), β-actin, p-AMPK, AMPK, p-LKB1, LKB, p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38 and p38 were from Cell Signaling Technology (Beverly, MA), and for SREBP-1 was from Abcam (Cambrige, MA). Palmitic acid, oleic acid, linoleic acid and linolenic acid were purchased from Wako (Osaka, Japan), and stearic acid was from sigma (St. Louis, MO). Bovine serum albumin (BSA) was from Nakarai Tesque (Kyoto, Japan).

**Cell culture**

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Sigma, USA), 4 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin under a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. The cells were grown to 80% confluence and incubated in serum free medium overnight before treatments. The cells were exposed to FAs and simultaneously treated with various concentrations of chalcones (4HD, XAG, CAR or FKB) or to vehicle (DMSO) for 24h. Stock solution of each FA was dissolved in 99% MetOH and diluted in culture medium containing 1% BSA to obtain the desired final concentrations.

**Cell viability assay**

The cell viability was determined by WST-1 assay. To detect the effects of FAs on HepG2 cells viability, the cells were treated with the each FA at 0.5 or 1 mM for 24h, respectively. To detect the effects of chalcones on HepG2 cells viability, the cells were treated with the each chalcones at 1, 5, 10 and 20 μM or DMSO alone as a vehicle control (final concentration, 0.25%) for 24h in culture medium. The WST-1 assay was performed as described in Section A of Chapter 1.

**Determination of intracellular lipid content**

To detect the effects of FAs on lipid accumulation in HepG2 cells, the cells were treated

with the each FA at 0.5 or 1 mM for 24h, respectively. To detect the effects of chalcones on FAs-induced lipid accumulation in HepG2 cells, the cells were exposed to a mixture of FAs (palmitic acid : oleic acid = 1:2 ratio), and simultaneously treated with the each chalcones with various concentrations as indicated in each figure or DMSO alone as a vehicle (final concentration, 0.25%) for 24h. Then Sudan II straining was carried out as described in Section A of Chapter 1.

**Western blotting analysis**

Preparation of the cell lysate and western blotting were performed according to previous report [Furuyashiki *et al*., 2004]. Specific immune complexes were detected with the ATTO Light-Capture II Western Blotting Detection System. The density of specific bands was calculated using ImageJ image analysis software.

**Statistical analysis**

All data are expressed as the mean ± standard deviation (SD) of at least three independent determinations for each experiment. Statistical significance was analyzed using the Dunnett's test, and probability levels of 0.05 and 0.01 were considered to indicate statistical significance.

**RESULTS**

**Efffects of FA on lipid accumulation and cells viability in HepG2 cells.**

The lipid accumulation (Fig. 2-1A) and the cell viability (Fig. 2-1B) were investigated after treatment with palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid at 0.5 or 1 mM to HepG2 cells for 24h. The author found that all FAs significantly increased lipid accumulation in HepG2 cells (Fig. 2-1A). Under the same conditions, linoleic acid and linolenic acid showed cytotoxity at both 0.5 and 1 mM (Fig. 2-1B). Palmitic acid and stearic acid also showed cytotoxicity at 1 mM. Palmitic and oleic acids are the most abundant FAs in liver triglycerides in both normal subjects and patients with NAFLD [Araya *et al*., 2004]. Therefore, HepG2 cells were incubated with a mixture of palmitic acid and oleic acid with different proportions to induce fat-overloading. Results from lipid accumulation (Fig. 2-1C) and cells viability (Fig. 2-1D) assays showed that palmitic acid was easy to increase lipid accumulation but exhibited cytotoxicity over 1.2 mM. Although lipid accumulation of oleic

acid was weaker than that of palmitic acid, oleic acid did not exhibit cytotoxicity. To achieve maximal fat overaccumulation without cytotoxicity, a FAs mixture consisting of a low proportion of palmitic acid (palmitic acid : oleic acid = 1:2 ratio) was selected for further experiments.

**4HD, XAG, CAR and FKB inhibited FAs-induced cellular lipid accumulation in HepG2 cells.**

The author first investigated that the effect of 4HD, XAG, CAR and FKB on cell viability of HepG2 cells by WST-1 assay and found that all chalcones did not show any cytotoxicity up to 20 μM (Fig. 2-2A). To investigate the inhibitory effect of chalcones on the lipid accumulation in HepG2 cells, 5 μM of each chalcone and various concentrations of FAs mixture were simultaneously treated to HepG2 cells for 24h and the lipid contents were determined by Sudan II staining. As showed in Fig. 2-2B, the FAs mixture dose-dependently increased lipid accumulation in HepG2 cells compared with DMSO-treated control cells. Simultaneous with 4HD, XAG, CAR and FKB at 5μM decreased the FAs mixture-induced lipid accumulation: CAR and FKB showed significant effect at 0.3, 0.6 and 1.2 mM, 4HD at 0.6 and 1.2 mM, and XAG at 1.2 mM. Thus, the author selected 1.2 mM FAs mixture and investigated the dose-dependent effect of chalcones on FAs mixture-induced lipid accumulation. As showed in Fig. 2-2C, these chalcones decreased lipid accumulation in a dose-dependent manner. All chalcones tested significantly inhibited the FAs mixture-induced lipid accumulation at 5, 10 and 20 μM.

**4HD, XAG, CAR and FKB down-regulated SREBP-1 and up-regulated PPARα expression in HepG2 cells**

To determine the mechanism by which 4HD, XAG, CAR and FKB decreased the FAs-induced lipid accumulation in HepG2 cells, western blotting analysis was performed to evaluate the expression levels of important factors SREBP-1 and PPARα. These proteins are involved in hepatic lipogenesis and FA oxidation, respectively [Brown and Goldstein, 1997; Sanyal, 2005; Braissant and Wahli, 1998]. As shown in Fig. 2-3, the author confirmed that the FAs mixture enhanced expression of SREBP-1 but did not affect expression of PPARα. 4HD and XAG dose dependently decreased expression of SREBP-1, while they increased expression of PPARα. Significant differences were observed over 5 μM (Fig. 2-3A). The

same trends were observed by treatment with CAR and FKB; ie, they dose dependently decreased expression of SREBP-1 while increased that of PPARα (Fig. 2-3B).

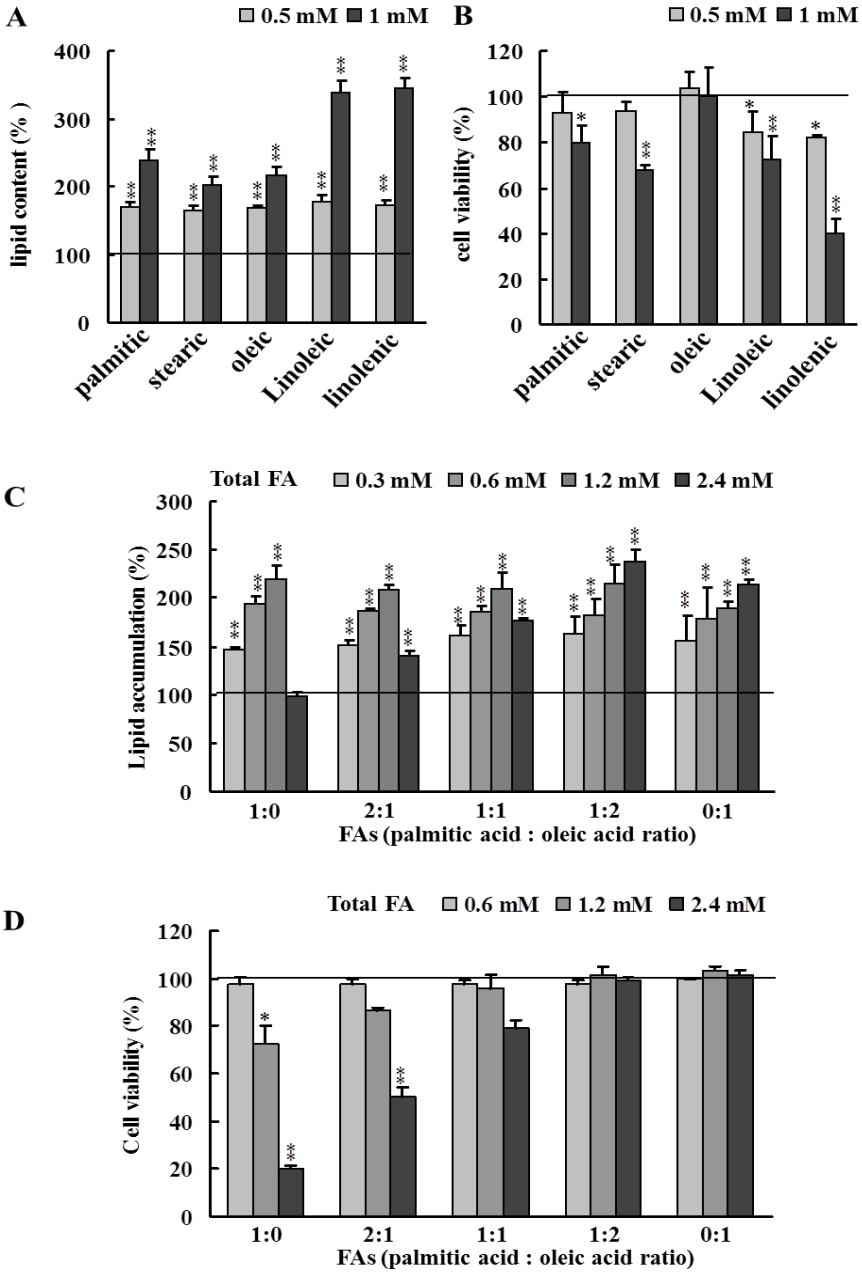


**Fig. 2-1. Effects of FA on lipid accumulation and cells viability in HepG2 cells.** (A, B) Effects FA on lipid accumulation and cytotoxicity in HepG2 cells. The cells were incubated with each FA at 0.5 or 1 mM for 24h. MetOH was used as a vehicle control. (C, D) Effects of different ratio of palmitic acid and oleic acid on lipid accumulation and cytotoxicity in HepG2 cells. The cells were incubated with palmitic acid and oleic acid mixture with different ratios (1:0, 2:1, 1:1, 1:2 and 0:1) for 24h. Intracellular lipid accumulation was determined by Sudan II staining (A, C). Cell viability was measured by WST-1 assays (B, D). The results are presented as the mean ± SD from three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. MetOH-treated control cells (Dunnett's test).

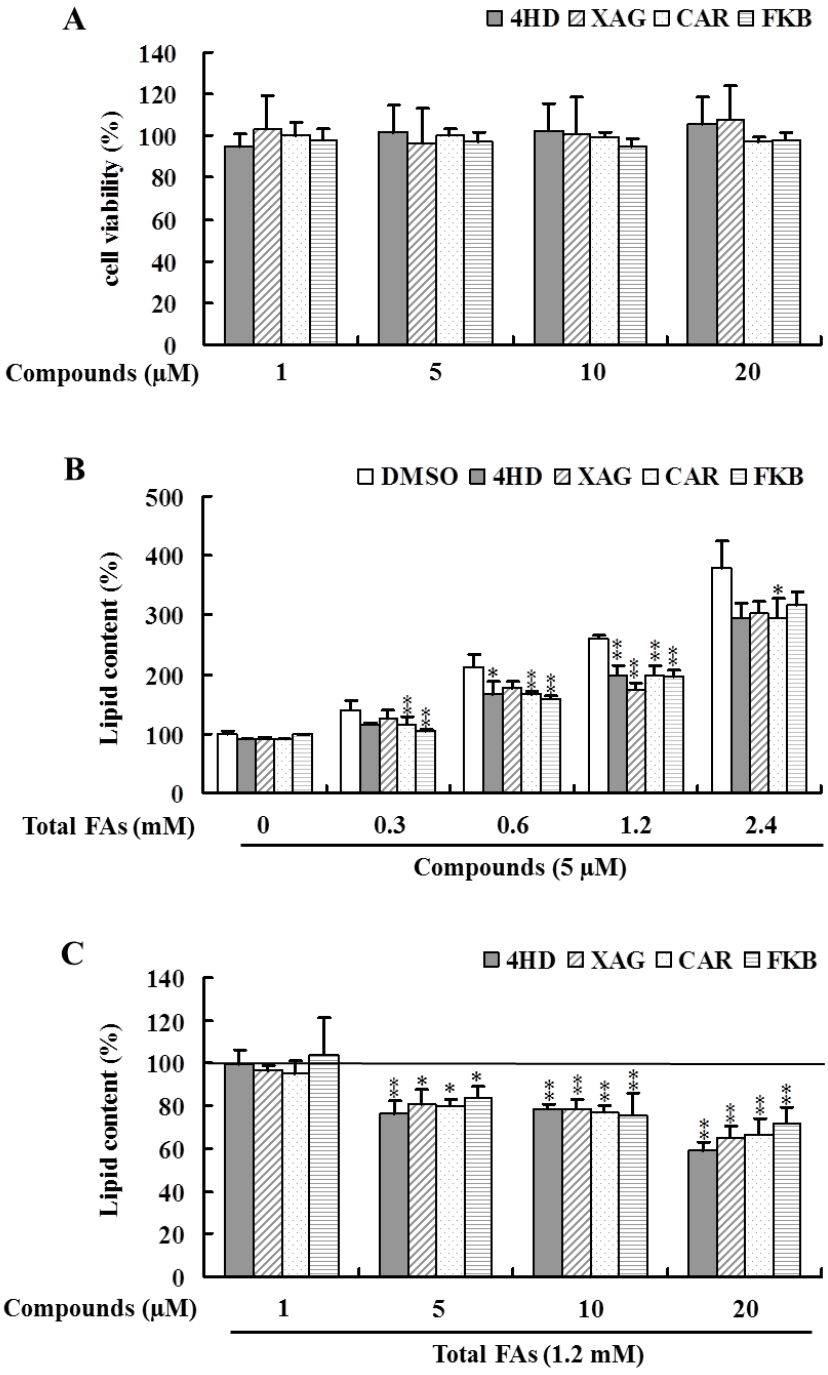


**Fig. 2-2. 4HD, XAG, CAR and FKB suppressed a FAs mixture-induced lipid accumulation in HepG2 cells.** (A) The cells were treated with each chalcone at concentrations of 1, 5, 10, or 20 μM for 24h. Cell viability was measured by WST-1 assays. (B) The cells were exposed to 0, 0.3, 0.6, 1.2 and 2.4 mM FAs mixture (palmitic acid : oleic acid =1:2 ratio) with or without 5 μM each chalcone for 24h. (C) The cells were exposed to 1.2 mM FAs mixture consisting of 0.4 mM palmitic acid and 0.8 mM oleic acid with or without each chalcone at 1, 5, 10, or 20 μM for 24h. In these experiments, DMSO was used as a vehicle control. Intracellular lipid content was determined by Sudan II staining. The results are presented as the mean ± SD from three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO-treated control cells at each dose of FA (Dunnett's test).A

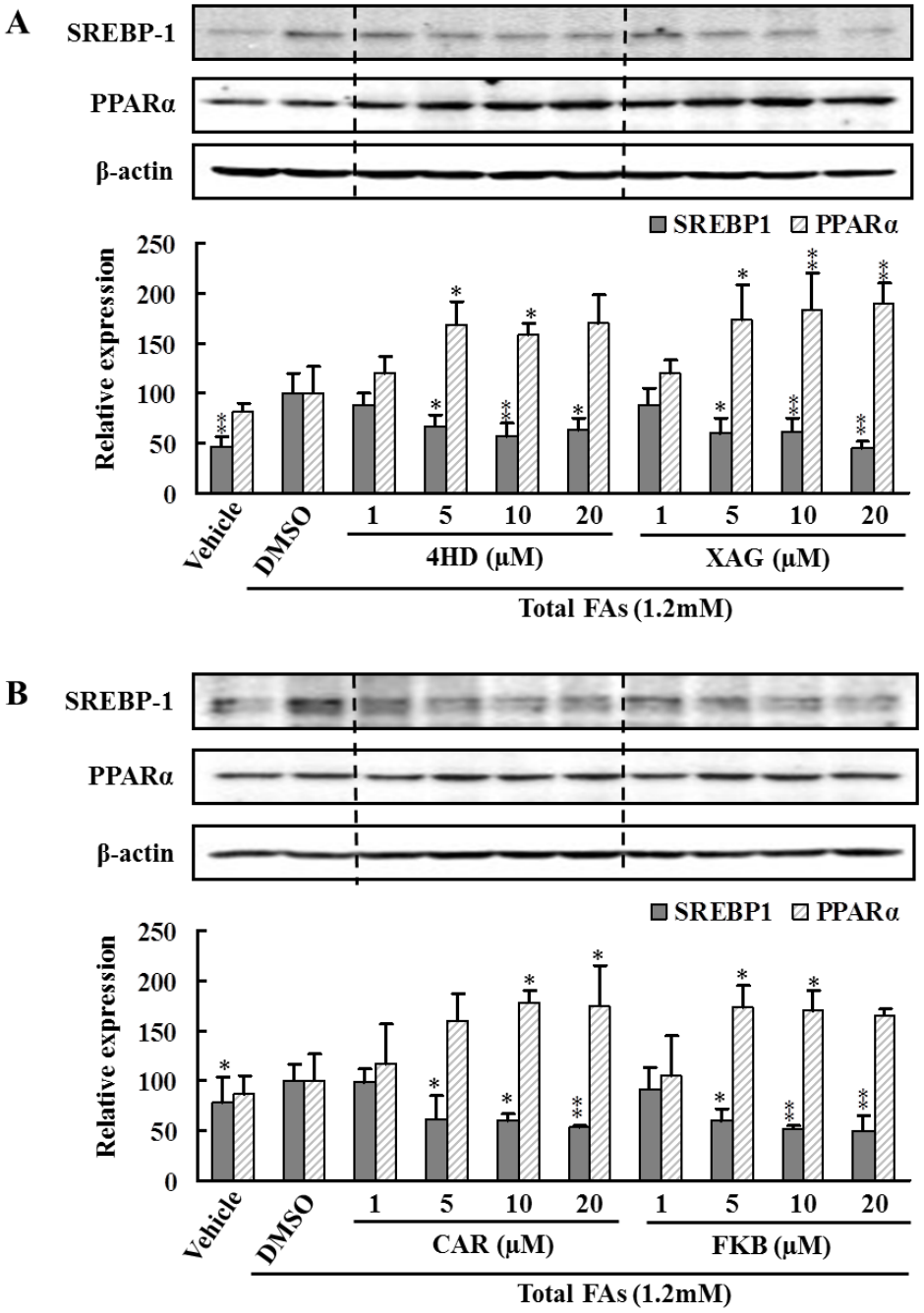


**Fig. 2-3. 4HD, XAG, CAR and FKB downregulated SREBP-1 expression and upregulated PPARα xpression in HepG2.** The cells were exposed to 1.2 mM FAs mixture consisting of 0.4 mM palmitic acid and 0.8 mM oleic acid with or without (A) 4HD or XAG, and (B) CAR or FKB at 5 μM for 24h. DMSO was used as a vehicle control. The protein expression levels of SREBP-1 and PPARα were determined by western blotting. Typical representative result was shown from three independent experiments. Densitometric analysis of specific bands was also shown after normalization by β-actin expression. The results are presented as the mean ± SD from three independent experiments. **P*<0.05 and ***P*<0.01 *vs*. DMSO-treated cells (Dunnett's test).

**Effects of 4HD, XAG, CAR and FKB on LKB1/AMPK and MAPKs pathways in HepG2 cells**

AMPK is a key regulator of lipogenesis and FA oxidation in metabolic tissues including the liver. The change of AMPK activity in HepG2 cells is strongly associated with intracellular lipid metabolism [Schimmack *et al*., 2006]. To investigate the effects of chalcones on the phosphrylation of AMPK and its upstream kinase LKB1, HepG2 cells were treated with 1.2 mM FAs mixture and 5 μM chalcone for 24 h. As shown in Fig. 2-4 A and B, all chalcones significantly simulated phosphorylation of AMPK (4HD, 2.18-fold; XAG, 2.08-fold; CAR, 1.74-fold; and FKB, 1.85-fold) and that of LKB1 (4HD, 1.58-fold; XAG, 1.52-fold; CAR, 1.72-fold; and FKB, 1.64-fold). To confirm that AMPK activation was involved in the inhibitory effects of chalcones on the FAs mixture-induced lipid accumulation, an AMPK inhibitor, compound C was introduced (Fig. 2-5 A and B). Compound C decreased chalcones-caused AMPK activation. 4HD, XAG (Fig. 2-5A) and CAR, FKB (Fig. 2-5B) down-regulated expression of SREBP-1 (4HD, 67%; XAG 67%; CAR, 70%; and FKB, 74%) and up-regulated PPARα (4HD, 1.80-fold; XAG, 1.69-fold; CAR, 1.56-fold; and FKB, 1.54-fold) as the same manner as the results in Fig. 2-3. In the same series of cultures, these effects were abolished by treatment with compound C. These results indicated that the activation of LKB1/AMPK pathway by chalcones is involved in the FAs mixture-induced lipid accumulation.

MAPKs play an important role in various cellular metabolism including regulation of hepatic lipid metabolism [Malhi *et al*., 2006]. Thus, the author investigated whether chalcones active MAPKs. However, 4HD, XAG, CAR and FKB did not alter the MAPKs activation under the present experimental conditions (Fig. 2-6).

**Discussion**

In this Chapter, the author found that the treatment with a FAs mixture consisting of palmitic acid and oleic acid (1:2 ratio) to HepG2 cells is suitable *in vitro* model for hepatic steatosis (Fig. 2-1). Using this model, the author found that chalcones 4HD, XAG, CAR and FKB significantly decreased the FA mixture-induced lipid accumulation in HepG2 cells without cytotoxicity (Fig. 2-2). These results indicated that 4HD, XAG, CAR and FKB will be effective compounds to prevent hepatic steatosis. The author conducted further experiments and found that their inhibitory effect on the FAs mixture-induced lipid

accumulation is involved in down-regulation of the SREBP-1 and up-regulation of PPARα (Fig. 2-3) as a consequence of LKB1/AMPK activation (Figs. 2-4 and 5) but not MAPKs (Fig. 2-6).

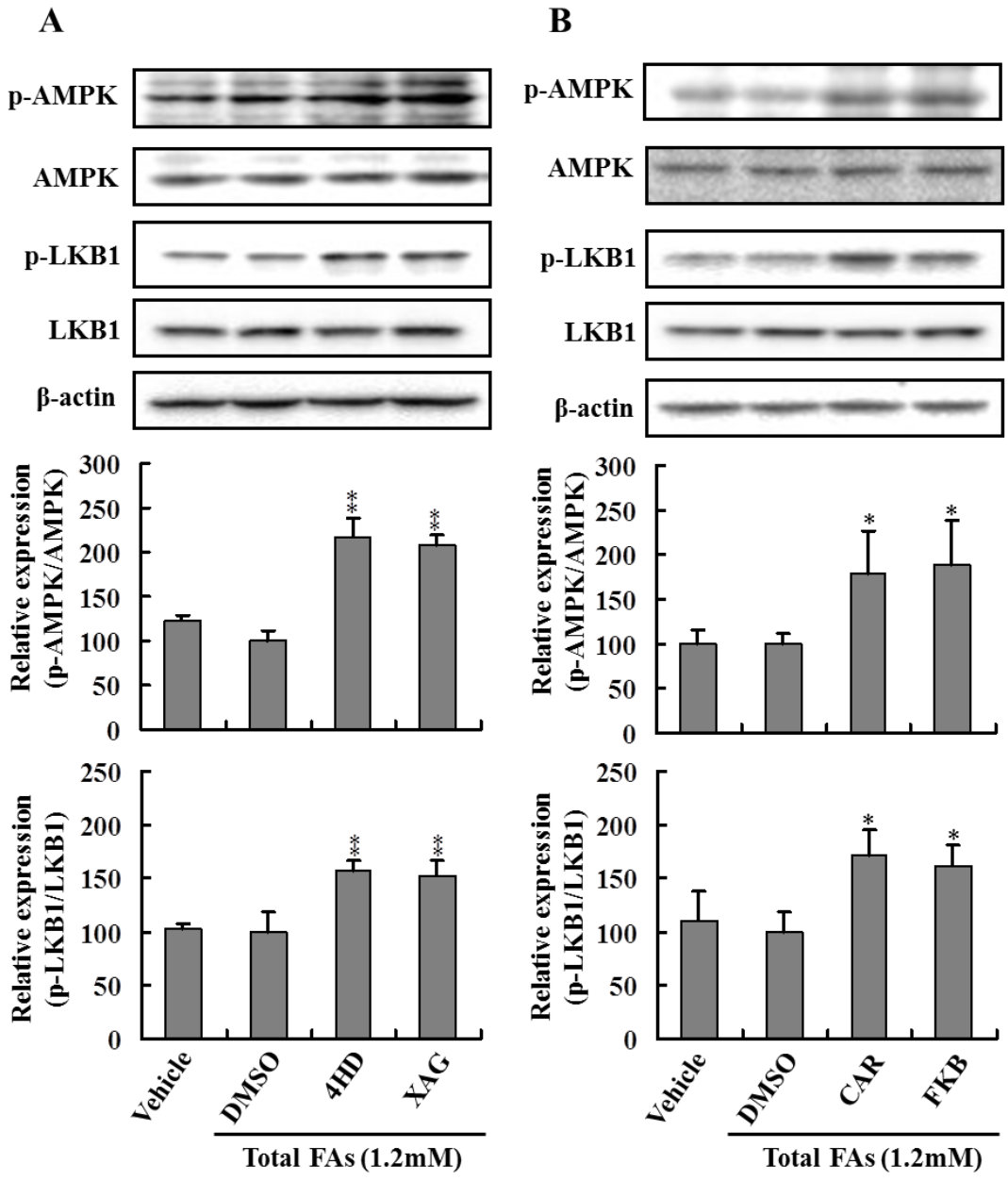


**Fig. 2-4. 4HD, XAG, CAR and FKB simulated the phosphorylation of AMPK and LKB1 in HepG2 cells.** The cells were exposed to 1.2 mM FAs mixture consisting of 0.4 mM palmitic acid and 0.8 mM oleic acid with or without (A) 4HD or XAG, and (B) CAR or FKB at 5 μM for 24h. DMSO was used as a vehicle control. The phosphorylation levels of AMPK and LKB1, and the expression levels of AMPK, LKB1 and β-actin were determined by western blotting. Typical representative result was shown from three independent experiments. Densitometric analysis of specific bands for pAMPK and pLKB1 was also shown after normalization by corresponding protein expression. The results are presented as the mean ± SD from three independent experiments. *$P$<0.05 and **$P$<0.01 *vs*. DMSO-treated cells (Dunnett's test).

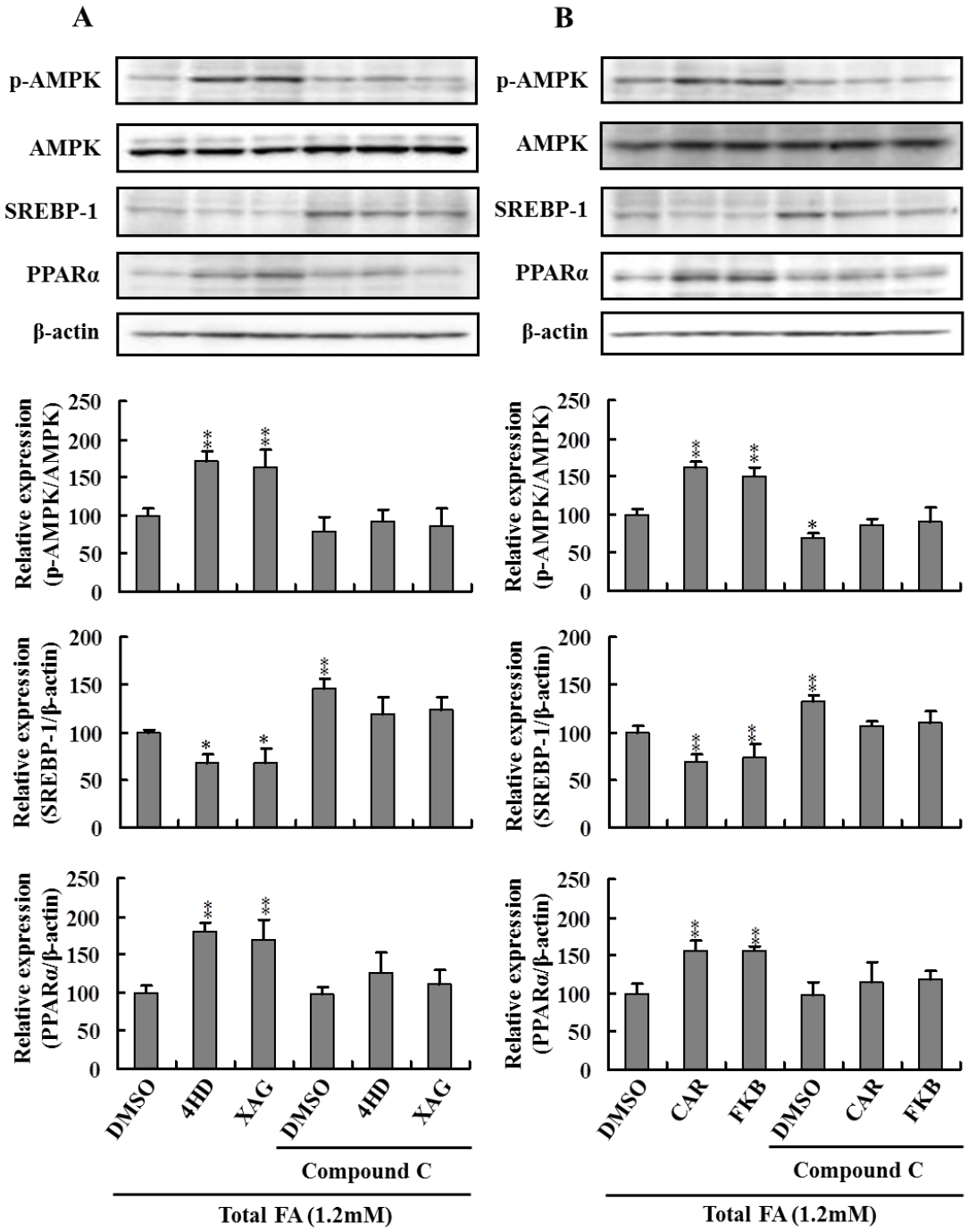


**Fig. 2-5. The AMPK inhibitor compound C restored the effects of 4HD, XAG, CAR and FKB on SREBP-1 and PPARα expression.** The cells were exposed to 1.2 mM FAs mixture consisting of 0.4 mM palmitic acid and 0.8 mM oleic acid with or without (A) 4HD or XAG, and (B) CAR or FKB at 5 μM for 24h. Compound C at 20 μM was simultaneously treated to the cells. DMSO was used as a vehicle control. The phosphorylation levels of AMPK, and the expression levels of AMPK, SREBP-1, PPARα and β-actin were determined by western blotting. Typical representative result was shown from three independent experiments. Densitometric analysis of specific bands for pAMPK was shown after after normalization by AMPK expression, and that of SREBP-1 and PPARα was also shown after normalization by β-actin expression. The results are presented as the mean ± SD from three independent experiments. **P*<0.05 and ***P*<0.01 *vs.* DMSO-treated cells (Dunnett's test).

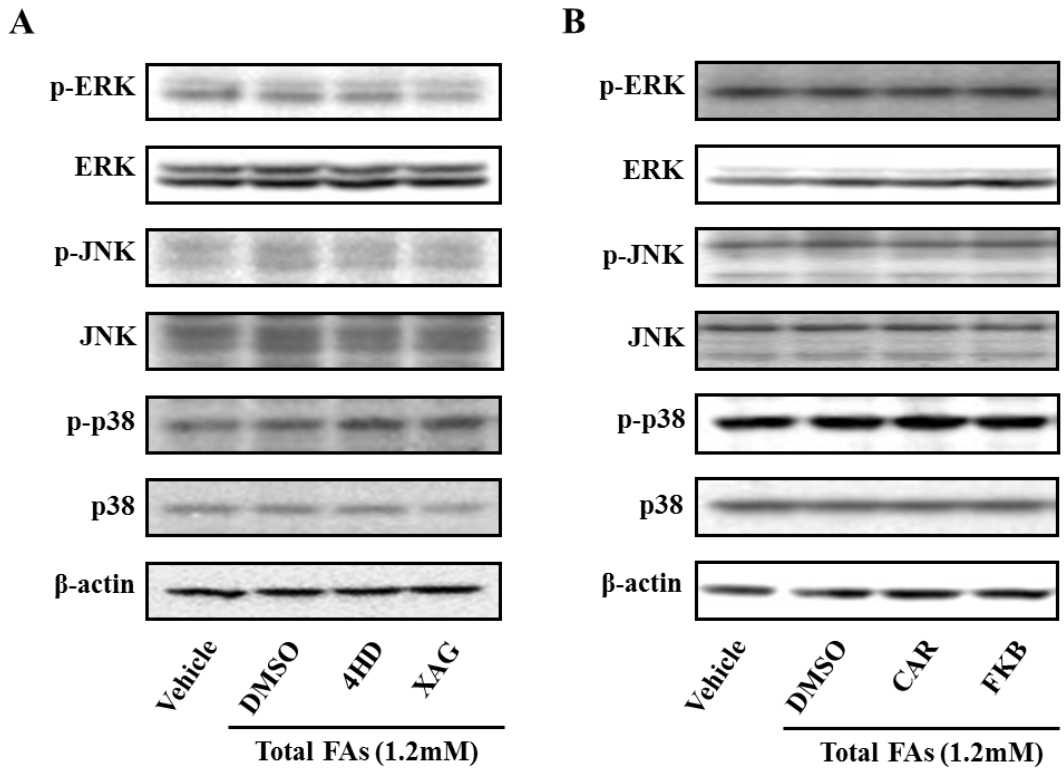


**Fig. 2-6. Effects of 4HD, XAG, CAR and FKB on the phosphorylation of MAPKs in HepG2 cells.** The cells were exposed to 1.2 mM FAs mixture consisting of 0.4 mM palmitic acid and 0.8 mM oleic acid with or without (A) 4HD or XAG, and (B) CAR or FKB at 5 μM for 24h. DMSO was used as a vehicle control. The phosphorylation and total levels of ERK, JNK and p38 were determined by western blotting. Typical representative result was shown from three independent experiments.

Intracellular lipid accumulatin is the main pathological characteristic of a human liver with NAFLD [Wu *et al*., 2008]. FAs are the main substrates for synthesis of triglycerides in hepatocytes. It is known that twenty five percent of liver triglycerides is derived from increased *de novo* lipogenesis [Donnelly *et al*., 2005]. *De novo* lipogenesis is mediated by SREBP-1 that is a key lipogenic transcription factor and nutritionally regulated by glucose and insulin [Goldstein *et al*., 2008; Zhou *et al*., 2001]. SREBP-1 preferentially regulates the lipogenic process by activating genes involved in FA and triglyceride synthesis which contribute to hepatic steatosis [Sanyal, 2005]. SREBP-1 plays a considerable role in the pathogenesis of NAFLD [Ahmed and Byrne, 2007]. Increased SREBP-1 levels have been found in patients with histologically diagnosed NAFLD [Kohjima *et al*., 2008], and in the fatty livers of obese (ob/ob) mice [Ahmed and Byrne, 2007] and obese rats induced by fat-diet feeding [Madsen *et al*., 2003]. The author found that 4HD, XAG, CAR and FKB decreased

the FAs mixture-induced SREBP-1 expression in HepG2 cells (Fig.2-3). The results in this chapter indicate that 4HD, XAG, CAR and FKB prevent hepatic steatosis by down-expression of SREBP-1. Moreover, the author also found that 4HD, XAG, CAR and FKB increased expression of PPARα in HepG2 cells. PPARα is expressed at high levels in tissues with high rates of FA oxidation, such as brown fat, liver and heart [Braissant and Wahli, 1998]. PPARα-mediated responses have been well studied in the liver. It has been reported PPARα agonist normalized fatty livers in fat-fed rats [Braissant and Wahli, 1998] and markedly improved lipid accumulation in the liver of rats [Ye *et al*., 2003]. Curcumin also decreases oleic acid-induced lipid accumulation through enhancing the expression of PPARα [Kang *et al*., 2013]. The author's findings and these previous results indicate that 4HD, XAG, CAR and FKB prevent hepatic steatosis by up-expression of PPARα, which involved in FA oxidation. Collectively, 4HD, XAG, CAR and FKB reduces the FAs mixture-induced hepatic lipid accumulation by down regulating *de novo* lipogenesis and up regulating FA oxidation.

To determine the signaling mechanism underlying down-regulation of SREBP-1 and up-regulation of PPARα by 4HD, XAG, CAR and FKB, the author investigated the effects of these chalcones on LKB1/AMPK activation in HepG2 hepatocytes. It is known that AMPK plays a key role in regulating carbohydrate and fat metabolism, serving as a metabolic master switch response to alterations in cellular energy charge [Schimmack *et al*., 2006; Winder and Hardie, 1999]. AMPK is expected to be a therapeutic target for treating fatty liver disease. AMPK is involved in regulating hepatic lipogenesis [Schimmack *et al*., 2006]. Activation of hepatic AMPK turn off a switch for FA synthesis via decreasing expression of SREBP-1 which regulating genes involved in *de novo* lipogenesis [Hardie, 2007]. The author's founding showed that 4HD, XAG, CAR and FKB increased AMPK activation (Fig. 2-4). When an AMPK inhibitor compound C was treated to the cells, the expression of SREBP-1 was increased and the inhibitory effect of chalcones on SREBP-1 expression was canceled (Fig. 2-6). In addition, AMPK also simulates FA oxidation by up-regulating expression of proteins involved in FAs oxidation, including PPARα [Lee *et al*., 2006;]. The author's founding showed that 4HD, XAG, CAR and FKB increased expression of PPARα (Fig. 2-3) and phophorylation of AMPK (Fig. 2-4) simultaneously. AMPK inhibitor compound C partly canceled the increase in the expression of PPARα by chalcones (Fig. 2-5). These results indicate that 4HD, XAG, CAR and FKB have an ability to activate AMPK then reduce SREBP-1 expression and enhance PPARα expression, finally leading to inhibit hepatic

steatosis. AMPK activity is regulated by phosphorylation at Thr172 by the upstream serine/threonine kinase LKB1 [Hawley *et al*., 2003; Shaw *et al*., 2004; Woods *et al*., 2003]. Consistent with phosphorylation of AMPK α at Thr172, 4HD, XAG, CAR and FKB phosphorylated LKB1 at Ser428 in HepG2 cells (Fig. 2-4). These results indicate that 4HD, XAG, CAR and FKB inhibit the FAs mixture-induced lipid accumulation by the activation of LKB1/AMPK pathway. MAPKs are involved in regulating lipid metabolism [Gehart *et al*., 2010]. It was reported that SREBP-1 expression is linked to the MAPK cascade [Kotzka *et al*., 2000]. However, the author found that 4HD, XAG, CAR and FKB did not altered the phosphorylation levels of ERK, JNK and p38 under this experimental condition (Fig. 2-6).

In conclusion, the author established an *in vitro* model of hepatic steatosis using a mixture of palmitic acid oleic acid mixtures. Using this model, the author found that chalcones 4HD, XAG, CAR and FKB inhibit the FAs mixture-induced lipid accumulation in HepG2 cells. The inhibitory mechanism is, at least in part, dependent on down-regulation of SREBP-1 and up-regulation of PPARα expressions though the activation of LKB1/AMPK signaling pathway. The author's findings suggest that 4HD, XAG, CAR and FKB might be valuable for preventing NAFLD and NAFLD-related metabolic syndrome.

## Chapter 3

## Ashitaba (*Angelica keiskei*) extract prevent obesity and insulin resistance in high-fat diet-fed C57BL/6 mice.

### INTRODUCTION

The rates of obesity have increased dramatically in recent years [Friedman, 2000]. Obesity usually results from an energy imbalance [Takahashi *et al.*, 2000]: Excessive energy storage and insufficient energy expenditure induced lipid accumulation in both adipose tissue and liver leading to type II diabetes, cardiovascular disease, nonalcoholic fatty liver disease (NALFD) and other metabolic disorders [Lois *et al*., 2008; Despres *et al*., 2008; Friedman, 2000]. Dietary fat is considered to be one of the most important factors in the pathophysiology of obesity. C57/BL/6 mice are obesity-prone and develop obesity, hyperglycemia, and hyperlipidemia when fed on a high-fat (HF) diet. C57/BL/6 mice are, therefore, commonly used for obesity and obesity-related diseases research [Nishikawa *et al*., 2007; Surwit *et al*., 1995].

Lipid accumulation and energy metabolism are tightly controlled in the adipose tissue and liver. Adipogenesis is a differentiation process by which undifferentiated preadipocytes are converted to fully differentiated adipocytes. This process is regulated by a highly organized cascade of transcription factors such as members of the peroxisome proliferator-activated receptor gamma (PPARγ), the CCAAT/enhancer-binding proteins (C/EBPs), and sterol regulatory element binding protein-1 (SREBP-1) [Lefterova and Lazar, 2009]. Differentiation of adipocytes stores FAs in the cytoplasm, leading to visceral fat accumulation. There is a metabolic rationale linking the expanded abdominal to hyperglycemia, hyperlipidemia and insulin resistance, which are often accompanied with impaired metabolic regulation in adipose tissue, leading to an overproduction of free FAs [Despres, 2006]. Such flux of free FAs toward the liver results in an increase of triglyceride deposition and secretion of triglyceride-rich lipoproteins, which in turn affect the lipo-lipase activity and the distribution of lipoprotein subtypes [Peng *et al*., 2011]. During this process, SREBP-1 plays an essential role in the regulating genes in liver, including FA synthase (FAS) as an enzyme for lipogenesis, in the liver [Brown and Goldstein, 1997; Sanyal, 2005]. On the other hand, the expressions of carnitine palmitoyltransferase-1 (CPT-1), acy-CoA oxidase (ACO) and peroxisome proliferator-activated receptor gamma (PPARα) were critically associated with the process of FAs oxidation. [Bonnefont *et al*., 2004; Miyazawa *et al*., 1987;

Wanders *et al*., 2001; Braissant and Wahli, 1998]. In addition, uncoupling proteins (UCPs) have attracted considerable interest in the context of energy expenditure. UCP-2 is one of the members of UCP family, and involved in energy metabolism in the adipose tissue and liver [Boss et al., 1998; Ricquier, 2000]. AMPK is a key modulator to maintain the cellular as well as whole-body energy balance. There increased an interest in developing AMPK activators as potential therapies for obesity, diabetes and hepatic steatosis [Carling *et al*., 2012, O'Neill *et al*., 2012].

A Japanese herb Ashitaba, an umbelliferous perennial plant found along the Pacific coast from the Bousou peninsula to the Kii peninsula and on the Izu islands of Japan, is drunk as a tea and used as a vegetable as well as the folk medicine as a diuretic, laxative, analeptic and galactagogue [Kim *et al*., 1992]. Several of the compounds in this plant have been identified including coumarins, flavanones and chalcones. Among them, 4HD and XAG are considered to be major active compounds [Kozawa *et al.*, 1977]. In the previous chapters, the author found that 4HD and XAG inhibited the differentiation of preadipocytes into adipocytes via the activation of AMPK, ERK and JNK signal pathways leading to down-regulating expression of C/EBPβ, C/EBPα and PPARγ (Chapter 1). The author also found that 4HD and XAG also suppressed FAs mixture-induced lipid accumulation through LKB1-AMPK signaling pathway in HepG2 cells (Chapter 2). However, the effects of AE on the HF diet-induced obesity, hepatic steatosis and insulin resistance are still lack information.

In this chapter, AE was given to C57BL/6 mice fed a control or HF diet for 16 weeks to examine whether AE could prevent HF diet-induced obesity and insulin resistance. The author further examined its effects on lipid metabolism in adipose and liver tissues to identify the underlying molecular mechanisms.

## MATERIAL AND METHODS

### Materials

AE was prepared from Ashitaba Chalcone Powder supplied by Japan Bio Science Laboratory (Osaka, Japan), as previously described [Kawabata, K., *et al*., 2011]. The powder (10 g) was treated with ethyl acetate (100 mL × 3 times) at room temperature, and the solution was evaporated. The yield of AE is 17%. Antibodies for PPARγ, C/EBPα, PPARα, ACC horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG and anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), β-actin, p-AMPK, AMPK and

p-ACC were from Cell Signaling Technology (Beverly, MA). SREBP-1 CPT-1A and ACOX1 were from Abcam (Cambrige, MA) and UCP-2 was from BioLegend Inc. (San Diego, CA). All other reagents used were of the highest grade available from a commercial source.

**Measurement of 4HD and XAG in AE by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)**

Detection and quantification of 4HD and XAG were performed with LC-MS/MS (4000 Q TRAP, AB Sciex, Foster City, CA, USA) using electrospray ionization. HPLC separation was done with a gradient system using solvent A (0.1% formic acid) and solvent B (acetonitrile) using a L-column-2 ODS (2.1×150 mm) column (Chemicals Evaluation and Research Institute, Tokyo, Japan) at a flow rate of 0.2 mL/min. The column oven was maintained at 40 °C. The gradient program was: 0-2 min, 45% A; 2-7 min, linear gradient to 0% A; 7-8 min, 0% A hold; 8-8.1 min, linear gradient to 45% A; and 8.1-15 min, 45% A hold. The chalcones were detected by multiple reaction monitoring as follows: 4HD 339.2/163.1 $[M + H]^+$, xanthoangelol 393.2/131.0 $[M + H]^+$, chalcone 209.1/131.0 $[M + H]^+$. For quantification, standard curves of Ashitaba chalcones from 0.05 to 500 fmol/5 μL injection were generated as described in previously study [Nakamura *et al.*, 2012]. Concentrations of 4HD and XAG were corrected by the comparison between a peak area of these compounds and that of an internal standard. Concentrations of 4HD and XAG in AE were showed in Table 3-1.

**Table 3-1. 4HD and XAG in an extract of Ashitaba (AE) identified by LC-MS/MS**

| Name | Concentration in AE (mg/g) |
|---|---|
| 4HD | 64.89 |
| XAG | 84.86 |

**Animal treatment**

All animal experiments were approved by the Institutional Animal Care and Use Committee (Permission #25-04-02) and were carried out according to the guidelines for Animal Experiments at Kobe University. Male C57BL/6 mice (5 weeks old, n = 36) were

obtained from Japan SLC (Shizuoka, Japan) and maintained in a temperature-controlled room (22°C) as described above. The mice had free access to tap water and an AIM-93M laboratory purified diet (Oriental Yeast, Tokyo, Japan), and were acclimatized for 7 days before the experiments. They were then randomly divided into six groups of six mice each and fed a control (AIN-93M) or high-fat diet containing 30% (w/w) lard for 16 weeks. The compositions of the diets and energy densities are shown in Table 3-2. The diets were supplemented with 0% (C-0 and HF-0 groups), 0.01% (C-0.01 and HF-0.01 groups) or 0.1% (C-0.1 and HF-0.1 groups) AE. Food and water intake were measured, and the diets replaced every 2 days. Body weight was measured weekly. After 16 weeks of feeding, the mice were fasted for 18 h and sacrificed under anesthesia with sodium pentobarbital, blood was collected from cardiac puncture using a heparinized syringe. The liver, white adipose tissue (subcutaneous, epididymal, mesenteric and retroperitoneal adipose tissues) and brown adipose tissue were also collected. Plasma and tissue samples were washed with 1.15% (w/v) KCl, weighed, immediately frozen using liquid nitrogen, and kept at −80°C until use.

**Table 3-2. Composition of the control and high-fat diets**

| | Control | High-fat |
|---|---|---|
| Ingredients | (g/100 g diet) | |
| Casein | 14 | 14 |
| L-Cystin | 0.2 | 0.2 |
| Cornstarch | 46.6 | 16.6 |
| Dextrin | 15.5 | 15.5 |
| Sucrose | 10 | 10 |
| Soybean oil | 4 | 4 |
| Cellulose | 5 | 5 |
| Mineral mixture | 3.5 | 3.5 |
| Vitamin mixture | 1 | 1 |
| Choline bitartate | 0.3 | 0.3 |
| Tertiary butyl hydroxyl quinone | 0.0008 | 0.0014 |
| Lard | 0 | 30 |
| | (kcal/100 g diet) | |
| Energy density | 348 | 518 |

**Measurement of plasma parameters related to lipid and glucose metabolism**

Plasma triglyceride, total cholesterol, non-esterified fatty acid (NEFA) and glucose levels were measured using the commercial assay kits s according to the instructions (Triglyceride-E test, Cholesterol-E test, NEFA-C test, and Glucose CII-test, respectively, Wako Pure Chemical). Plasma insulin and adiponectin levels were measured by the commercial ELISA assay kits according to the manufacturer's instructions (mouse insulin ELISA kit, Shibayagi, Gunma, Japan; and mouse/rat adiponectin ELISA kit, Otsuka Pharmaceutical, Tokyo, Japan, respectively). The index of the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the relationships between the plasma glucose and insulin levels, according to the following formula [Trout *et al.*, 2007]: 14 HOMA-IR = fasting glucose (mg per 100 mL) × fasting insulin (μU per mL)/405.

**Measurement of hepatic lipid levels**

An aliquot of 100 mg of liver was homogenized with 0.35 mL of distilled water, and the homogenate was extracted three times with 0.7 mL of chloroform–methanol (2 : 1, *v/v*) solution. The chloroform layer was collected by centrifugation at 1800 × *g* for 10 min, and washed with a 1/4 volume of 0.88% (*w/v*) KCl. The obtained chloroform layer was evaporated, and measured the weight of the residue as total lipids. The residue was dissolved in isopropanol containing 10% (*v/v*) Triton-X, and triglyceride and cholesterol levels were measured using the commercial kits as described above.

**Western blotting**

Preparation of the cell lysate was performed according to the previous study [Nishiumi and Ashida, 2007]. Proteins in the cell lysate fractions of adipose tissue and liver were separated by SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking with commercial Blocking One solution (Nacalai Tesque), The membrane was incubated with primary antibodies for PPARγ (1:20000), C/EBPα (1:10000), SREBP-1 (1:10000), PPARα (1:20000), CPT-1A (1:5000), ACOX1 (1:20000), UCP-2 (1:20000), β-actin (1:20000), AMPK (1:10000), p-AMPK 1:5000), ACC (1:10000) and p-ACC (1:5000) overnight at 4 °C, followed by the corresponding HRP-conjugated secondary antibody for 1 h at room temperature. Specific immune complexes were detected with the ATTO Light-Capture II Western Blotting Detection System. The density of specific bands

was calculated using ImageJ image analysis software.

### Statistical analysis

Data are represented as the means ± SD. The statistical significance of experimental observations was determined using the Tukey-Kramer multiple comparison test. The level of significance was set at $p < 0.05$.

## RESULTS

### Effect of AE on body weight and adipose tissue weight

During the feeding period, body weight was significantly lowered in the 0.01%AE and 0.1%AE groups compared with the HF-0%AE group from week 10 to 16, respectively (Fig. 3-1). At the end of experiment (week 16), the author confirmed that the body weight of the mice was significantly higher in the HF-0 group than in C-0%AE group, and 0.01%AE and 0.1%AE significantly lowered body weight compared with the HF-0 group (Table 3-3). The weight of white adipose tissue (epididymal, mesenteric, retroperitoneal, and subcutaneous adipose tissue) was greater in the HF-0%AE group than in C-0%AE group. AE suppressed an increase in adipose tissue weight compared with the HF-0%AE group. In the groups given the control diet, AE did not affect body weight or adipose tissue weights (Table 3-2). AE has the potential to reduce body weight and white adipose weight gain induced by the HF diet.

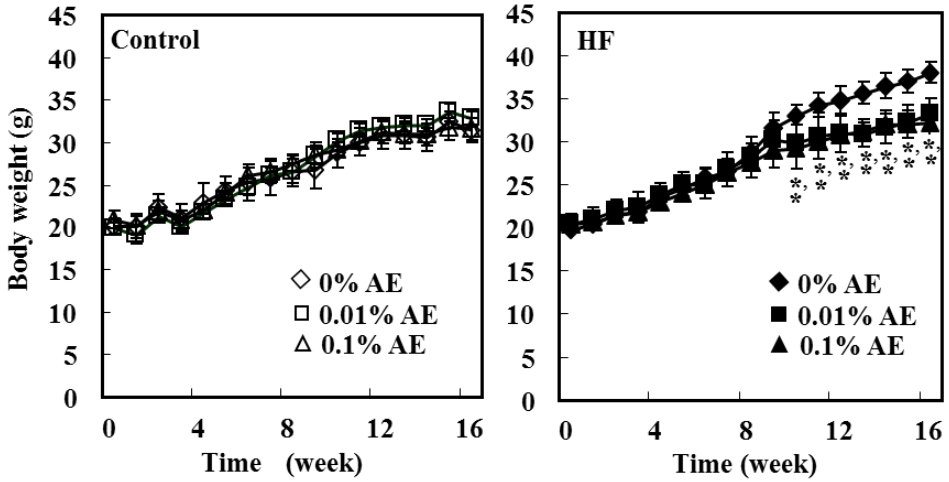


**Fig. 3-1. Changes in body weight of mice fed the control and HF diets containing AE for 16 weeks.** Open symbols represent control diet-fed groups, while closed symbols HF diet-fed groups. Values are the mean ± SD (n=6). Significant differences between the 0%AE group (Diamonds) and 0.01%AE (spuares) or 0.1%AE (triangles) group are shown ($p<0.05$, Tukey-Kramer multiple comparison test).

Table 3-3. Effects of AE on body weight, and adipose tissue weights of mice fed control and high fat for 16 weeks

| | Group | | | | | |
|---|---|---|---|---|---|---|
| | Control | | | HF | | |
| Ashitaba extract(%) | 0 | 0.01 | 0.1 | 0 | 0.01 | 0.1 |
| Final body weight (g) | 30.8±2.2[a] | 32.8±1.1 [ab] | 29.2±2.21 [a] | 35.9±3.34 [b] | 31.5±2.11[a] | 31.5±1.64[a] |
| Tissue weight (g per 100 g body weight) | | | | | | |
| Liver | 3.79±0.36 [a] | 3.71±0.33 [a] | 3.88±0.47 [a] | 3.74±0.27 [a] | 3.91±0.32 [a] | 3.62±0.56 [a] |
| White adipose tissue weight | | | | | | |
| Total | 10.91±4.06 [a] | 13.98±1.18[a] | 9.75±2.60 [a] | 19.90±4.79[b] | 11.99±2.8[a] | 12.25±1.48[a] |
| Epididymal | 3.22±0.65[a] | 4.31±0.49[ab] | 2.94±0.74[a] | 5.50±1.26[b] | 3.60±1.26[a] | 3.85±0.56[a] |
| Mesenteric | 1.47±0.45[ab] | 2.10±0.32[ab] | 1.36±0.24[ac] | 2.40±0.30[b] | 1.71±1.03[ab] | 1.31±0.21[ac] |
| Retroperitoneal | 1.62±0.61[a] | 2.22±0.32[ab] | 1.59±0.42 [a] | 3.10±1.00[b] | 1.72±0.69[a] | 2.10±0.73[ab] |
| Subcutaneous | 4.59±2.47[a] | 5.53±0.53[ab] | 3.85±1.61[a] | 8.90±3.37[b] | 4.97±1.71[a] | 4.98±0.87[a] |
| Brown adipose tissue | 0.73±0.16 [a] | 0.72±0.09 [a] | 0.55±0.12 [a] | 0.47±0.11 [a] | 0.56±0.20 [a] | 0.70±0.19 [a] |

Mice were fed the control or high-fat diet containing AE for 16 weeks. At the end of the experiment, body weight and adipose tissue weights were measured after 18 hours fasting. Values are the mean ± SD (n=6). Values without a common letter in a row differ significantly among groups ($p$<0.05) by the Tukey-Kramer multiple comparison test.

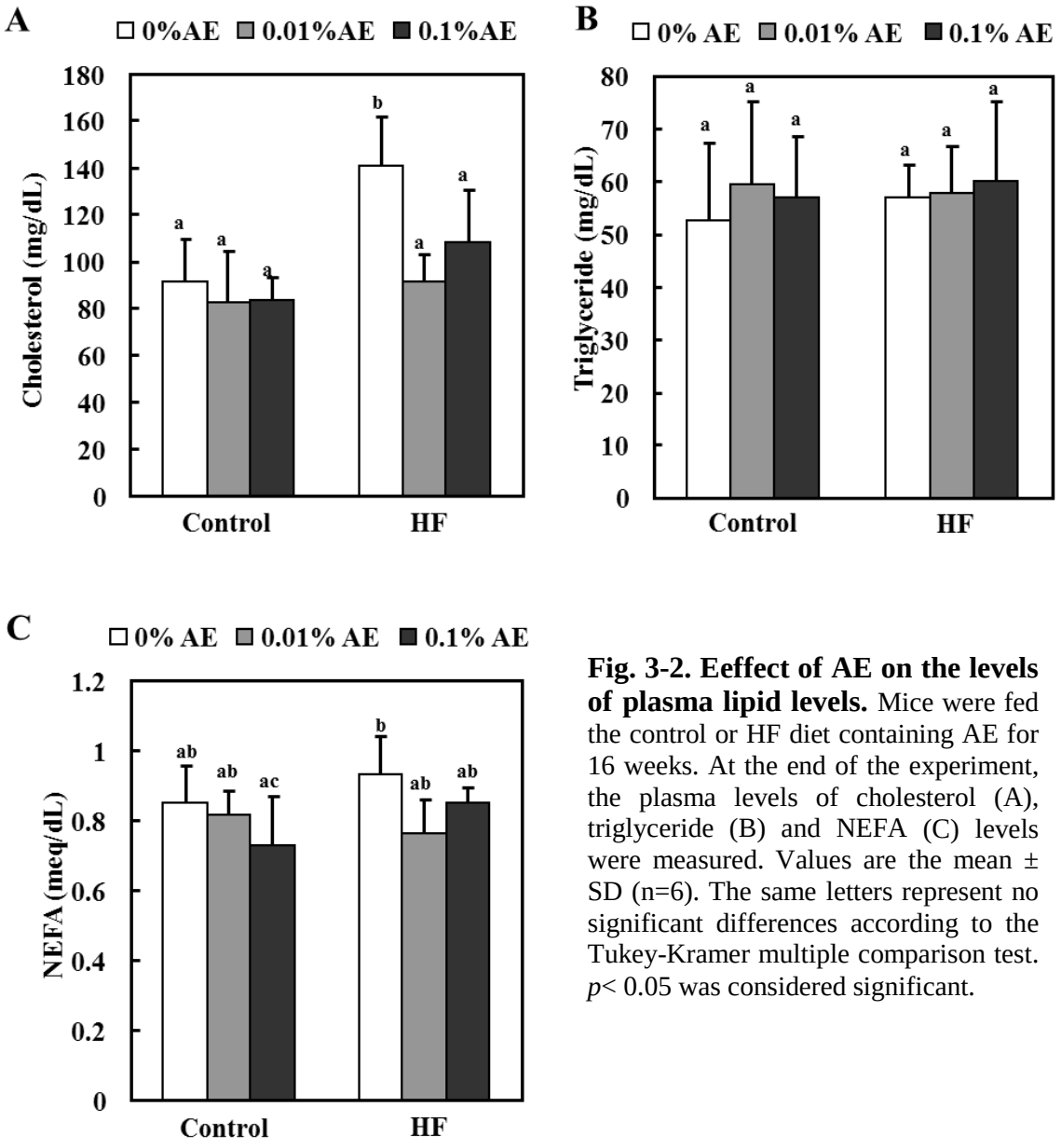


**Fig. 3-2. Eeffect of AE on the levels of plasma lipid levels.** Mice were fed the control or HF diet containing AE for 16 weeks. At the end of the experiment, the plasma levels of cholesterol (A), triglyceride (B) and NEFA (C) levels were measured. Values are the mean ± SD (n=6). The same letters represent no significant differences according to the Tukey-Kramer multiple comparison test. $p < 0.05$ was considered significant.

**Effects of AE on lipid metabolism**

Plasma levels of cholesterol, triglyceride, and NEFA were shown in Fig.3-2. Total plasma cholesterol level was significantly increased in the HF-0%AE group compared with the C-0%AE group. HF-0.01%AE and -0.1%AE group significantly lowered plasma cholesterol level to the almost same level as the control diet-fed groups. On the other hand, AE did not affect plasma triglyceride and NEFA levels in both the control and high-fat diet-fed groups. Intake of a HF diet is reported to induce hepatic lipid accumulation, which are involved in systemic insulin resistance [Korenblat *et al.*, 2008; Hwang *et al.*, 2007]. As

shown in Fig. 3-3, the hepatic total lipids, triglyceride and cholesterol levels in HF-0%AE group were significantly higher than those in the C-0%AE group. The hepatic total lipid, triglyceride and cholesterol levels were significantly lower in HF-0.01% AE and HF-0.1%AE groups than in the HF-0%AE group. In the groups given the control diet, AE did not affect hepatic total lipid triglyceride and cholesterol levels. These results indicate that AE has the ability to prevent HF diet-induced hepatic lipid accumulation.

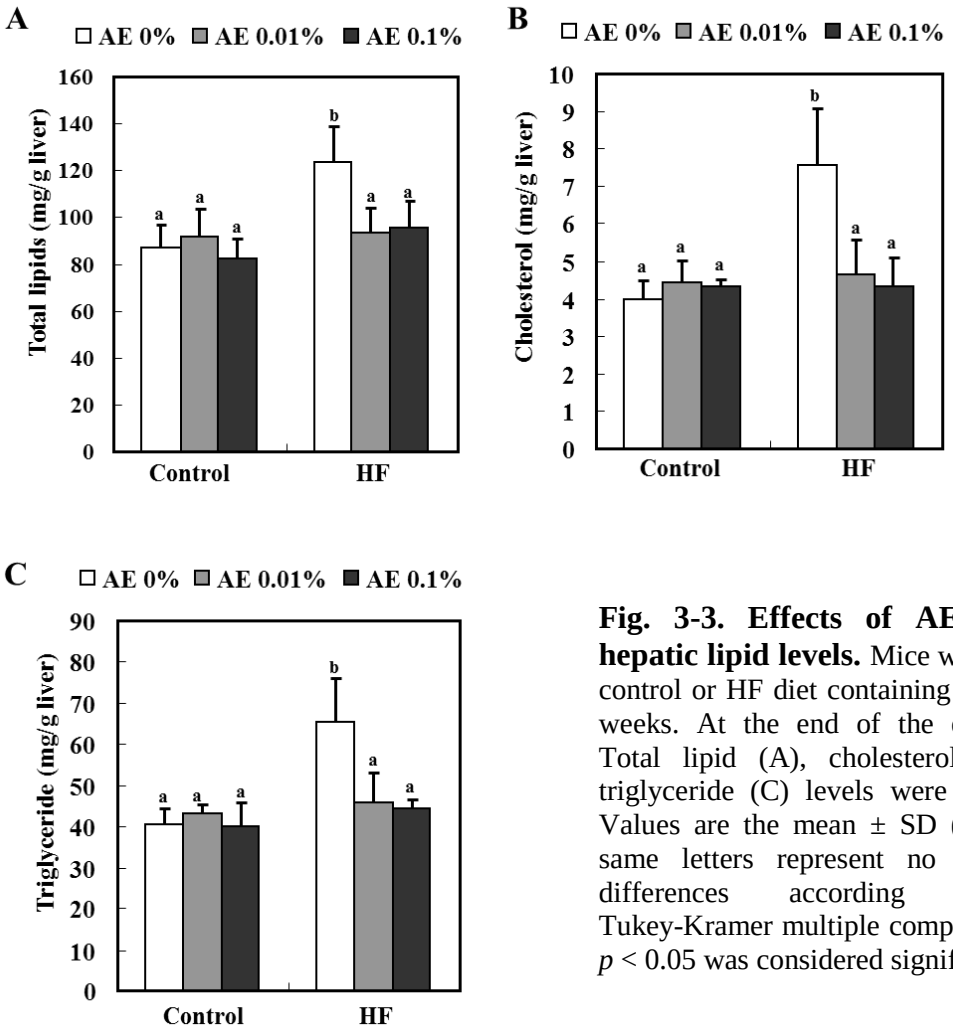


**Fig. 3-3. Effects of AE on the hepatic lipid levels.** Mice were fed the control or HF diet containing AE for 16 weeks. At the end of the experiment Total lipid (A), cholesterol (B) and triglyceride (C) levels were measured. Values are the mean ± SD (n=6). The same letters represent no significant differences according to the Tukey-Kramer multiple comparison test. $p < 0.05$ was considered significant.

**Effect of AE on plasma glucose, insulin and adiponectin levels.**

The plasma glucose level at the end of the experiment was significantly higher in the HF-0%AE group, compared with the C-0%AE group (Fig. 3-4A). Supplementation of the HF diet with 0.01% and 0.1% of AE reduced the plasma glucose levels significantly. The plasma insulin level in the HF-0%AE group was also higher than that in the C-0%AE group. Supplementation of the HF diet with 0.01% and 0.1% of AE also reduced the insulin levels

compare with HF-0%AE group. The insulin levels in the HF-0.01%AE and HF-0.1%AE groups were normalized, the level in the control diet group (Fig. 3-4B). Neither glucose nor insulin levels significantly changed in the control diet groups. HOMA-IR is a good predictor of total insulin sensitivity, and was significantly higher in the HF-0%AE group than that in the C-0%AE group (Fig. 3-4C). Supplementation of the HF diet with 0.01% and 0.1% AE significantly attenuated the HF diet-induced increase in HOMA-IR.

White adipose tissue is a major endocrine tissue that releases various adipocytokines into the bloodstream. Because adiponectin is one of the major adipocytokines associated with maintaining glucose homeostasis [Tilg and Moschen, 2008], the author measured the plasma level of adiponectin (Fig. 3-4D). The plasma adiponectin level was lower in the HF-0%AE than that in control diet groups, AE dose-dependently increased: 0.1%AE group significantly increased adiponectin level compared with HF-0%AE and recovered the same level in the control diet groups.

**Effect of AE on expression of adipogenesis- and energy metablism-proteins in the adipose tissue**

Since PPARγ, C/EBPα, and SREBP-1 are associated with adipogenesis [Lefterova and Lazar, 2009], the expression of these proteins levels in white adipose tissue were determined by western blotting. As shown in Fig. 3-5, the expression of PPARγ, C/EBPα, and SREBP-1 were significantly increased in HF-0%AE group compared with those in the control diet groups. Both 0.01%AE and 0.1%AE significantly decreased HF diet evoked these adipogenesis-related protein expression. In the groups given the control diet, AE did not affect the expression of PPARγ, C/EBPα, and SREBP-1. AE also did not affect the expression UCP-2 in both control diet and HF diet groups. It has been known that HF diet suppresses AMPK activation [Pang *et al*., 2008; Kim *et al.*, 2008]. Interestingly, the author found AE supplentation restored AMPK phosphorylation in HF diet mice (Fig. 3-5).

**Effect of AE on expression of adipogenesis- and energy metabolism-proteins in the liver**

To investigate whether the reduction of fat mass in AE-dosed mice is accompanied by changes in lipogenesis in the liver, western blotting was performed. As shown in Fig. 3-6, HF diet significantly increased the expression of SREBP-1 and FAS and AE was significantly decreased HF-diet induced expression of these proteins in HF diet groups. Then, the author

investigated the expression of protein responsible for FA oxidation and thermogenesis in the liver. HF diet tended to decrease the expression of CPT1, ACOX1 and PPARα and AE was significantly increased HF diet depressed expression of these proteins in HF diet groups. AE did not alter the expression of these proteins in the control diet groups. Similar to the adipose tissue, AE also did not alter UCP-2 expression in the liver. Interestingly, HF-0.01%AE and HF-0.1%AE groups also increased in phosphorylation of AMPK and ACC compared with HF-0%AE group.

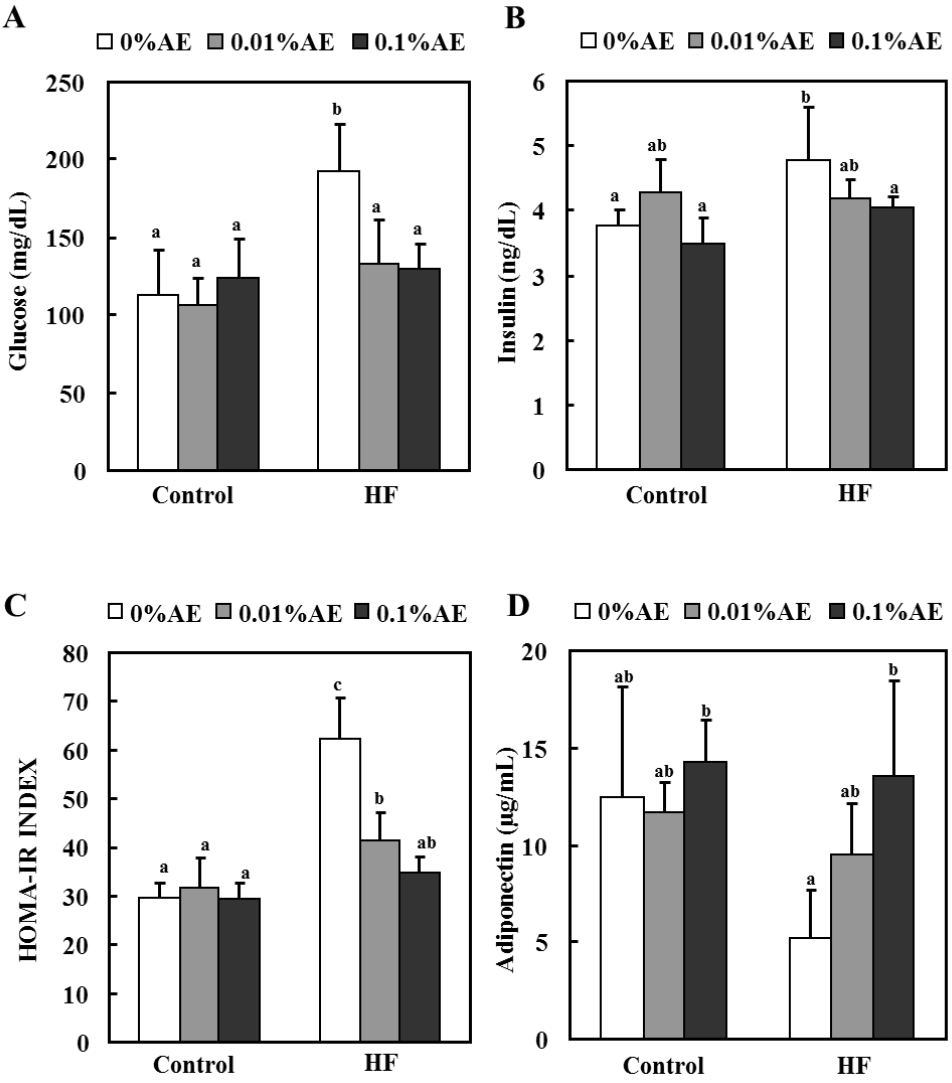


**Fig. 3-4. Effects of AE on plasma glucose, insulin and adiponecitn levels.** Mice were fed the control or HF diet containing AE for 16 weeks. At the end of the experiment on the levels of glucose (A) and insulin (B) levels were measured and the homeostasis model assessment of insulin resistance index (HOMA-IR) was calculated (C). The plasma adiponectin was also measured (D). Values are the mean ± SD (n=6). The same letters represent no significant differences according to the Tukey-Kramer multiple comparison test. $p< 0.05$ was considered significant.

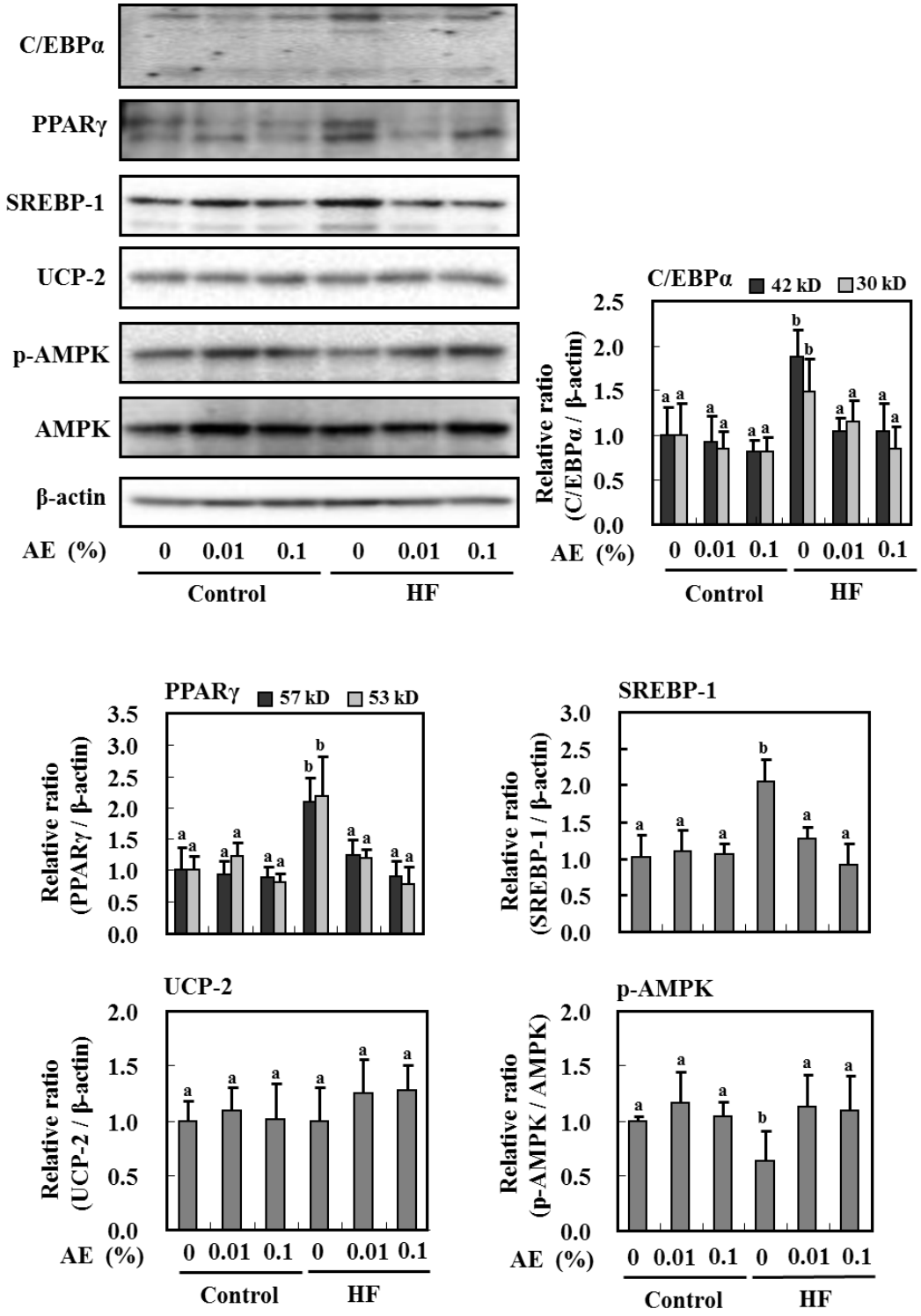


**Fig. 3-5. Effects of AE on expression of adipogenesis- and energy metabolism-proteins in adipose tissue.** Mice were fed the control or HF diet containing AE for 16 weeks. The expression of C/EBPα, PPARγ, SREBP-1, UCP2, AMPK and p-AMPK in white adipose tissue was evaluated by western blotting. Densitometric analysis of specific bands for C/EBPα, PPARγ, SREBP-1 and UCP-2 was shown after normalization by β-actin expression, p-AMPK was also shown after after normalization by AMPK expression. Values are the mean ± SD (n=6). The same letters represent no significant differences according to the Tukey-Kramer multiple comparison test. $p < 0.05$ was considered significant.

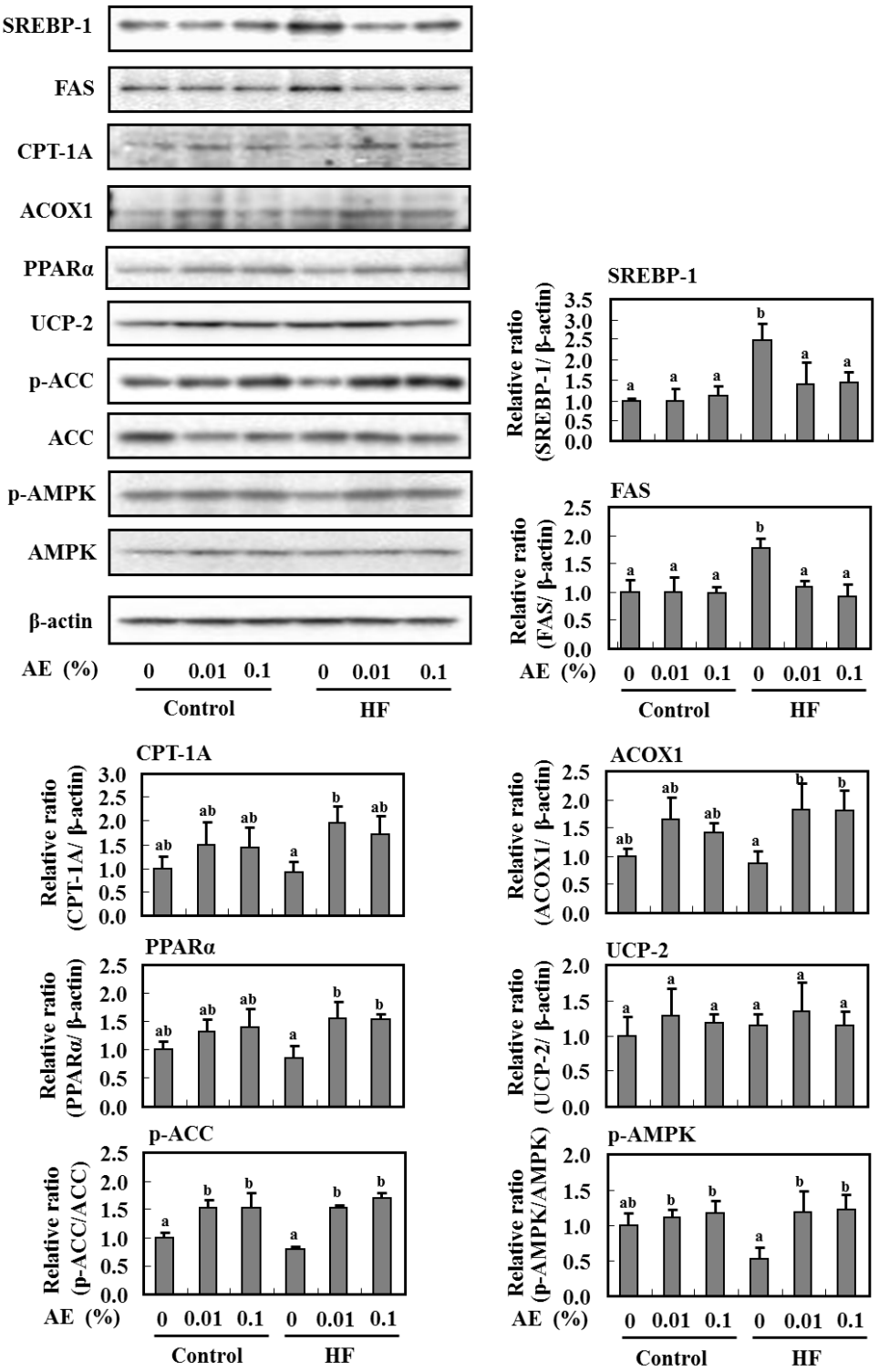


**Fig. 3-6. Effects of AE on expression of adipogenesis- and energy metabolism-proteins in liver tissue.** Mice were fed the control or HF diet containing AE for 16 weeks. The expression of SREBP-1, FAS, CPT-1A, ACOX1, PPARα, UCP-2, ACC, p-ACC, AMPK and p-AMPK in liver tissue was evaluated by western blotting. Densitometric analysis of specific bands for SREBP-1, FAS, CPT-1A, ACOX1, PPARα and UCP-2 was shown after normalization by β-actin expression, p-AMPK, p-ACC was also shown after normalization by AMPK and ACC expression, respectively. Values are the mean ± SD (n=6). The same letters represent no significant differences according to the Tukey-Kramer multiple comparison test. $p < 0.05$ was considered significant.

## DISCUSSION

In this chapter, AE showed their good ability to lowering body weight and body fat (Table 3-1 and Fig. 3-1), accompanied with the prevention of hyperglycemia, and hyperlipidemia effects, as estimated by reducing serum levels of cholesterol, glucose, insulin and enhancing adiponectin (Figs. 3-2 and 3-4). AE decreased the hepatic contents of triglyceride and cholesterol AE also protected the liver from HF diet-induced dysfunctions (Fig. 3-3). AE decreased expression of PPARγ, C/EBPα and SREBP-1, which were involved in adipogenesis in the adipose tissue (Fig. 3-5). AE also decreased the expression of SREBP-1 and FAS, and increased the hepatic expression of CPT-1, ACOX1 and PPARα (Fig. 3-6). It was noteworthy that AE restored HF-diet-induced inactivation of AMPK in both adipose tissue and liver (Figs. 3-5 and 3-6).

AE significantly suppressed lipid accumulation in the white adipose tissue, including visceral adipose tissue. As decreased in the previous chapters, PPARγ and C/EBPα are considered to be the master regulators or the crucial determinants of adipocyte fate and play an important role in adipogenesis [Tontonoz, *et al*., 1994; Lefterova *et al*., 2009]. For instance, immortalized fibroblasts lacking PPARγ lose the potential for differentiation to mature adipocytes [Rosen *et al*., 2002]. C/EBPα functions as a principal player in adipogenesis also resulted from gain-of-function studies in cultured cells [Freytag *et al*., 1994] as well as establishment of appropriate knockout mice [Wang *et al*., 1995]. Whole-body C/EBPα-knock mice, which die shortly after birth because of liver defects and hypoglycemia, fail to accumulate lipid in white or brown adipocytes [Wang *et al*., 1995]. SREBP-1 regulate lipid metabolism and play an essential role in the regulation of lipogenesis in FA and triglyceride synthesis [Brown and Goldstein, 1997]. This study found that AE effectively decreased the expression of C/EBPα, PPARγ and SREBP-1 in HF-diet group (Fig. 3-5). The results in Chapter 1 showed that 4HD and XAG, which are two major components in AE, inhibited adipocytes differentiation by down-regulating C/EBPs and PPARγ expression. These results indicated that AE suppressed the lipid accumulation in the white adipose tissue by decreasing adipocyte differentiation and lipogenesis.

AE normalized hepatic lipid content in the HF-diet groups, suggesting that it could prevent HF diet-induced fatty liver. Lipid accumulation in the liver is caused by enhancing *de novo* lipogenesis, and lowing of lipid catabolism. It is known that 25% of liver triglyceride is derived from increased *de novo* lipogenesis [Donnelly *et al*., 2005]. *De novo* lipogenesis is

mediated by SREBP-1 that is a key lipogenic transcription factor and nutritionally regulated by glucose and insulin [Goldstein *et al*., 2008; Zhou *et al*., 2001]. SREBP-1 preferentially regulates the lipogenic process by activating genes including FAS, involved in FA and triglyceride synthesis, which contribute to hepatic steatosis. The author found that AE significantly decreased SREBP-1 and FAS expression in HF-diet treated mice (Fig. 3-6). Moreover AE also increased CPT-1A, ACOX1 and PPARα expression in HF-diet treated mice. CPT-1, ACO X1 and PPAR α were critically associated with the process of FAs oxidation. [Bonnefont *et al*., 2004; Miyazawa *et al*., 1987; Wanders *et al*., 2001; Braissant and Wahli, 1998]. The oxidation of intra-hepatocellular FAs occurs mainly in mitochondria, and to a much lesser extent in peroxisomes and microsomes. CPT regulates the transport of FAs from the cytoplasm to the mitochondrial matrix across the membrane [Bonnefont *et al*., 2004], while ACO is the initial enzyme in the peroxisomal β-oxidation system [Miyazawa *et al*., 1987; Wanders *et al*., 2001]. PPARα-mediated responses have been well studied in the liver. It has been reported PPARα agonist normalized fatty livers in fat-fed rats [Braissant and Wahli, 1998] and markedly improved lipid accumulation in the liver of rats [Ye *et al*., 2003]. The author's results in Chapter 2 showed that 4HD and XAG, the major components in AE, inhibited the FAs-mixture induced lipid accumulation by down-regulating SREBP-1 and up-regulating PPARα expression. These results indicated that AE suppressed the lipid accumulation of liver tissue by decreasing lipogenesis and increasing FA oxidation.

AMPK is a key modulator to maintain the cellular as well as whole-body energy balance. AMPK is activated in response to an increase in the AMP: ATP ratio within the cell and therefore acts as a sensor for cellular energy regulation. Binding of AMP with AMPK allosterically phosphorylates and activates AMPK [Ruderman *et al*., 1999], which in turn shuts down anabolic pathways and supports catabolic pathways through regulating the expression of several key enzymes of energy metabolism. The activation of AMPK interacted with SREBP-1 and inhibited SREBP-1 target including FAS expression, leading to reduced lipogenesis and lipid accumulation [Brown and Goldstein, 1997; Sanyal, 2005]. Moreover, the activation of AMPK also leads to the phosphrylation and inhibition of ACC activity and an increase in FA oxidation. Phosphorylation of AMPK reversibly and inactivates ACC through phosphorylation of this enzyme [Ejaz *et al*., 2009]. Inactivation of ACC reduces the synthesis of malonyl-CoA, which in turn de-represses CPT-1A and ACOX1, and activates FA oxidation [McGarry and Brown, 1997]. In addition, AMPK also simulates FA oxidation by

up-regulating expression of PPARα [Lee, 2006]. It is also reported that the activation of AMPK also inhibites the differentiation of adipocytes by down-regulating the expression of C/EBPα and PPARγ [Gao *et al*., 2008]. It has been known that HF diet suppresses AMPK activation [Pang *et al*., 2008; Kim *et al*., 2008]. Noticeably, AE treatment restored AMPK phosphorylation in HF diet mice in both adipose and liver tissues (Fig. 3-5 and 3-6). These results indicated that AE regulated lipid metabolism in adipose and liver through activation of AMPK.

Visceral adipose tissue is an important predictor of insulin resistance, hyperglycemia and other metabolic risk factors [Gastaldelli *et al*., 2002; Després *et al*., 2008]. Increased adipose tissue weights are accompanied by the induction of inflammatory cytokines involved in insulin resistance [Hotamisligil, 2006; You *et al*., 2005]. In addition, visceral adipose tissue has been correlated with intrahepatic triglyceride content, and an increase in intrahepatic triglycerides is associated with the metabolic abnormalities [Korenblat *et al*., 2008; Hwang, Korenblat *et al*., 2007; Fabbrini *et al*., 2009]. The inhibition of fat accumulation in the white adipose and liver tissue by AE may, therefore, also contribute to its prevention of hyperglycemia and insulin resistance. The author found that AE decreased plasma glucose and insulin levels and increasing adiponectin level (Fig. 3-4). Adiponectin is one of the major adipocytokines associated with maintaining glucose homeostasis [Tilg and Moschen, 2008]. The previous study also showed that AE suppressed acute hyperglycemia in oral glucose tolerance test of mice, and 4HD and XAG, major polyphenols in AE stimulate glucose uptake in skeletal muscles cells [Kawabata *et al.*, 2011]. These results indicate that AE should be effective material for inhibition of hyperglycemia and insulin resistance.

In conclusion, the author found that administration of AE to mice with HF-diet-induced obesity reduced body weight gain, adipose tissue weight, serum levels of cholesterol, glucose, insulin and enhanced the level of adiponectin, thereby inhibiting lipogenesis both in the adipose tissue and liver. In addition, AE promote FA oxidation in the liver. These effects are involved in AMPK activation. Together, these findings demonstrate that AE should be of benefit to improve HF diet-induced obesity and insulin resistance.

## Conclusion Remarks

Obesity has increased at an alarming rate in recent years and is now a worldwide public health problem [Friedman, 2000]. Obesity is defined as an abnormal increase in fat, even without necessarily in body weight gain. Obesity is a significant risk factor for certain metabolic disorder diseases, including type 2 diabetes, cardiovascular diseases and the certain forms of cancers [Kaul *et al*., 2012; Vernooij *et al*., 2012; Vucenik and Stains, 2012; Ligibel, 2011]. Thus, weight loss has been recognized to a major health beneficial way for overweight people and also increases life expectancy in people having obesity-related complications. The diets as well as energy intake and nutritional balance are major causes for obesity and insulin resistance [Eaton, 2006]. Certain food components are used as the ingredient of medicine, which have been established for over 4000 years by a natural or traditional herbal medicine. Thus, extensive researches have been carried out to clarify the cellular and molecular mechanism of functional foods that have the prevention and/or amelioration effects on obesity and insulin resistance [Pinent, 2004 & 2006; Hanhineva, 2010; Holt, 2012; Montagut, 2010].

Chalcones are containing in various plants and have a variety of health-promoting effects. Several synthetic chalcones also showed biologically active [Dimmock *et al*., 1999; Bandgar *et al*., 2009]. In this dissertation, the author clarified the mechanisms of 4HD, XAG, CAR and FKB on adipocytes differentiation (Chapter 1) and FAs-mixture-induced steatosis in HepG2 cells (Chapter 2). Moreover, the author demonstrated the preventive effects of Ashitaba (*Angelica keiskei*) extract, which contains two main phytochemicals 4HD and XAG, on high-fat diet-induced obesity and insulin resistance in C57BL/6 (Chapter 3).

Adipocytes differentiation is deeply involved in the onset of obesity. In this dissertation, the author found that chalcones 4HD, XAG, CAR and FKB effectively suppressed lipid accumulation in adipocytes. These chalcones are able to inhibit adipocyte differentiation through down-regulating expression of CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor gamma (PPARγ) (Chapter 1). The Ashitaba extract, which contains two main chalcones 4HD and XAG, also inhibited high-fat diet-induced C/EBPa and PPARγ expression in white adipose tissue of C57BL/6 (Chapter 3). C/EBPs and PPARγ are master regulators during the adipocyte differentiation. PPARγ forms a heterodimer with retinoic acid X-receptor (RXR) [Kliewer *et al*., 1992], and regulates the transcription of

adipocyte-specific genes [Tontonoz *et al*., 1994]. C/EBPα functions as another principal player in adipogenesis and is most abundant in mature adipocytes, where plays a crucial role in insulin-dependent glucose uptake [Wu *et al*., 1999]. At the early stage of adipocyte differentiation, activations of C/EBPβ and C/EBPδ are the initial events that subsequently lead to increase expression of C/EBPα, PPARγ and probably other adipogenic factors [Cao *et al*., 1991; Yeh *et al*., 1995]. Previous study also showed that tea catechin [Furuyashiki *et al.*, 2004] and crucumim [Ejaz *et al*., 2009] also inhibited adipocyte differentiation and obesity in mice by down-regulating of C/EBPs and PPARγ. These results indicated that chalcones should be an effective compounds for prevent obesity and obesity-related disorders. Moreover, the author found chalcones regulated adipocyte differentiation though AMP-activated protein kinase (AMPK) activation (Section B of Chapter 1). AMPK is a key enzyme to maintain cellular as well as body energy balance. Intense research in recent years has revealed the critical roles that AMPK plays in modulating ever-expanding many biological pathways [Zhang *et al*., 2009; Luo *et al*., 2005]. The author's results showed that an inhibitor of AMPK, compound C enhanced the expression of C/EBPs and PPARγ, leading to increasing lipid accumulation in adipocytes (Section B of Chapter 1). Previous study showed that the activation of AMPK also inhibited the differentiation of 3T3-L1 cells by down-regulating the expression of C/EBPβ, δ, α, and PPARγ [Gao *et al*., 2008]. These results indicated that should be a molecular target for anti-obesity therapeutics. In Chapter 1, the author found that chalcones regulated adipocyte differentiation by extracellular signal-regulated kinase (ERK) and c-Jun amino-terminal kinases (JNK) activation. It was reported that ERK activation was necessary for adipocyte differentiation, and that a reduction in ERK expression in preadipocytes decreases adipocyte differentiation [Sale, *et al.*, 1995]. However, ERK activation also attenuates differentiation by inducing PPARγ phosphorylation during adipocyte differentiation [Camp, *et al.*, 1999; Hu, *et al.*, 1996]. It seems that ERK activation is tightly controlled in a temporal manner, being activated in preadipocytes and deactivated during adipocyte differentiation to prevent PPARγ phosphorylation. Recent studies showed that ERK activation is required for cell mitosis in the early stage of adipogenesis, and that activated (phosphorylated) ERK needs to be dephosphorylated thereafter because ERK might enhance PPARγ efflux from nucleus, suppress PPARγ degradation, and inhibit adipocyte differentiation [Burgermeister, *et al.*, 2007; Wang, *et al.*, 2009; Kim, *et al.*, 2009]. Moreover, diallyl trisulfide suppresses the adipogenesis of 3T3-L1 cells by activating ERK, and

evodiamine improved diet-induced obesity by inhibiting adipocyte differentiation via ERK activation [Lii, *et al.*, 2012; Wang, *et al.*, 2009]. Therefore, our results, showing that chalcones inhibit adipocyte differentiation through ERK activation, are consistent with those of prior studies. It has been known that JNK is involved in insulin signaling pathway [Hirosumi *et al*., 2002; Sabio *et al*., 2009]. However, the effect of JNK on adipocytes differentiation was still not clear. In the present study, we attempted to clarify this effect using a JNK inhibitor SP600125 and found phosphorylation of JNK inhibited adipocytes differentiation. In addition, we also found that SP600125 abolished the inhibitory effects of 4HD and XAG on lipid accumulation and on the expression of C/EBPα and PPARγ but not C/EBPβ (Chapter 1). Previous studies reported that activation of JNK inhibits adipocyte differentiation by enhancing phosphorylation of PPARγ and negatively regulating its transcriptional activity [Ohkura *et al*., 2011; Hu *et al*., 1996]. These results indicate that the inhibitory effects of chalcones on adipocytes differentiation is also depend on enhancing phosphorylation of JNK and decreasing expression of C/EBPα and PPARγ. It is interesting that the author found 4HD and XAG regulated adipocytes differentiation through activation of AMPK, ERK and JNK multiple signaling pathways. These factors could be available as the molecular targets for anti-obesity therapeutics. However, the mechanisms of between these factors for modulating adipocytes differentiation did not elucidate in present study. It should be an issue for the future study.

Hepatic steatosis is caused by enhancing *de novo* lipogenesis, and lowing of lipid catabolism. The author found the expression of (sterol regulatory element binding protein-1) SREBP-1 was attenuated while peroxisome proliferator-activated receptor alpha (PPARα) was increased in these chalcones-treated cells (Chapter 2). The author also found that AE efficiently inhibited hepatic lipid lipogenesis via suppressing expression of SREBP-1 and its target enzyme Fatty acid synthase (FAS), and increased fatty acid (FA) oxidation via promoting expression of carnitine palmitoyltransferase-1(CPT-1A), acy-CoA oxidase (ACO) and peroxisome proliferator-activated receptor alpha (PPARα) in the liver (Chapter 3). *De novo* lipogenesis is mediated by SREBP-1 that is a key lipogenic transcription factor and nutritionally regulated by glucose and insulin. SREBP-1 preferentially regulates the lipogenic process by activating genes including FAS, involved in FA and triglyceride synthesis, which contribute to hepatic steatosis [Goldstein *et al*., 2008; Zhou *et al*., 2001]. On the other hand, CPT-1, ACOX1 and PPAR α were critically associated with the process of FAs oxidation.

[Bonnefont *et al*., 2004; Miyazawa *et al*., 1987; Wanders *et al*., 2001; Braissant and Wahli, 1998]. The oxidation of intra-hepatocellular FAs occurs mainly in mitochondria, and to a much lesser extent in peroxisomes and microsomes. CPT-1 regulates the transport of FAs from the cytoplasm to the mitochondrial matrix across the membrane [Bonnefont *et al*., 2004], while ACO is the initial enzyme in the peroxisomal β-oxidation system [Miyazawa *et al*., 1987; Wanders *et al*., 2001]. PPARα-mediated responses have been well studied in the liver. It has been reported PPARα agonist normalized fatty livers in fat-fed rats [Braissant and Wahli, 1998] and markedly improved lipid accumulation in the liver of rats [Ye *et al*., 2003]. It is noteworthy that chalcones not only inhibited FAs mixture-induced lipid accumulation in the HepG2 cells (Chapter 2), but also HF diet-induced lipid accumulation in liver of mice (Chapter 3) through decreasing *de novo* lipogenesis while increasing FA oxidation. These results indicate that chalcones should be the effective materials for prevention of hepatic steatosis.

The author also found that AE significantly lowered plasma glucose and insulin levels, and increased adiponectin level compared to the HFD group (Chapter 3). Visceral adipose tissue is an important predictor of insulin resistance, hyperglycemia and other metabolic risk factors [Gastaldelli *et al*., 2002; Després *et al*., 2008]. Increased adipose tissue weights are accompanied by the induction of inflammatory cytokines involved in insulin resistance [Hotamisligil, 2006; You *et al*., 2005]. In addition, visceral adipose tissue has been correlated with intrahepatic triglyceride content, and an increase in intrahepatic triglycerides is associated with the metabolic abnormalities [Korenblat *et al*., 2008; Hwang, Korenblat *et al*., 2007; Fabbrini *et al*., 2009]. The inhibition of fat accumulation in the white adipose tissue and liver by AE may, therefore, also contribute to its prevention of hyperglycemia and insulin resistance. The author found that AE decreased plasma glucose and insulin levels and increasing adiponectin level. Adiponectin is one of the major adipocytokines associated with maintaining glucose homeostasis [Tilg and Moschen, 2008]. The previous study also showed that AE suppressed acute hyperglycemia in oral glucose tolerance test of mice, and 4HD and XAG, major polyphenols in AE, stimulate glucose uptake in skeletal muscles cells [Kawabata *et al*., 2011]. These results indicate that chalcones-rich AE should be effective material for inhibition of hyperglycemia and insulin resistance.

It is noteworthy that chalcones regulate lipid metabolism though increasing phosphorylation of AMPK in adipocytes (Chapter 1), HepG2 cells (Chapter 2) and C57BL/6

mice (Chapter 3). AMPK is a key modulator to maintain the cellular as well as whole-body energy balance. AMPK is activated in response to an increase in the AMP: ATP ratio within the cell and therefore acts as a sensor for cellular energy regulation. Binding of AMP with AMPK allosterically phosphorylates and activates AMPK [Ruderman *et al*., 1999], which in turn shuts down anabolic pathways and supports catabolic pathways through regulating the expression of several key enzymes of energy metabolism. The activation of AMPK interacted with SREBP-1 and inhibited SREBP-1 target including FAS expression, leading to reduced lipogenesis and lipid accumulation [Brown and Goldstein, 1997; Sanyal, 2005]. Moreover, the activation of AMPK also leads to the phosphrylation and inhibition of ACC activity and an increase in FA oxidation. Phosphorylation of AMPK reversibly and inactivates ACC through phosphorylation of this enzyme [Ejaz *et al*., 2009]. Inactivation of ACC reduces the synthesis of malonyl-CoA, which in turn derepresses CPT-1A and ACOX1, and activates FA oxidation [McGarry and Brown, 1997]. In addition, AMPK also simulates FA oxidation by upregulating expression of PPARα [Lee, 2006]. It is also reported that the activation of AMPK also inhibites the differentiation of adipocytes by down-regulating the expression of C/EBPα and PPARγ [Gao *et al*., 2008]. These results indicated that AMPK should be the core molecular target for chalcones prevents obesity and insulin resistance. Recent studies revealed that liver kinase B1 (LKB1) is one of the important upstream kinases of AMPK [Hawley *et al*., 2003; Woods *et al*., 2003]. LKB1 can active AMPK by phosphorylation at Thr172 in mammalian cells. chalcones phosphorylated LKB1 at Ser428 in HepG2 cells (Chapter 2). Ca(2+)/CaM-dependent protein kinase kinase β, is another upstream kinase of AMPK, has a profound impact on feeding, body weight, glucose homeostasis and insulin sensitivity in rodents and humans [Lage *et al*., 2008]. However, in this dissertation, the author did not pay attention to this kinase for chalcones preventing obesity and insulin resistance. It should be an interest issue for the future study.

In Chapter 3, the author found HF diet supplementary of 0.01%AE and 0.1%AE significantly promoted HF diet-induced obesity and insulin resistance. To understand the beneficial effects of functional compounds, it is essential to determine their bioavailability and metabolism *in vivo*. The previous study [Nkamura *et al*., 2012] demonstrated that 4HD and XAG are rapidly absorbed into plasma and are distributed to various tissues in mice orally administered with Ashitaba extract. Similar to other polyphenols [Mullen *et al*., 2006; Kawai, *et al*., 2008; Zhang *et al*., 1999], it is thought that Ashitaba chalcones are metabolized

by phase 2 drug-metabolizing enzymes. Although we could not identified exact chemical structure of metabolite, previous reports demonstrated the structure of metabolite. For example, in the case of naringenin chalcone, which is one of the well-researched chalcones containing tomato, it was reported that glucuronide conjugated C-2′ hydroxyl group was major metabolites in the plasma and urine in rat [Yoshimura *et al*., 2009]. As expected, 4-HD and XAG were absorbed and conjugated after oral administration of Ashitaba extract. Thereafter, the aglycones and metabolites of these compounds were distributed to many tissues including the liver, kidney, spleen, muscle, perirenal fat and epididymal fat. It is interesting that prenylated chalcones, particularly 4-HD, mainly exist as aglycones, rather than metabolites, in plasma and tissues, indicating that these compounds contribute to the beneficial effects of Ashitaba extract [Nakamura *et al*., 2012]. These studies provide strong evidences that chalcones 4-HD and XAG should be the active compounds in AE for prevent obesity and insulin resistance.

In conclusion, 4HD, XAG, CAR and FKB suppressed 3T3-L1 adipocytes differentiation though AMPK and MAPK pathways, resulting in the down-expression of adipocyte-specific transcription factors. These chalcones also decreased FAs-induced lipid accumulation through LKB1/AMPK signaling pathway accompanying with decreasing down-expression of SREBP1 and up-expression of PPARα in HepG2 cells. Moreover, the author found that administration of AE to mice with HF diet-induced obesity reduced body weight gain, adipose tissue weight, serum levels of cholesterol, glucose, insulin and enhanced the level of adiponectin, thereby inhibiting lipogenesis both in adipose and liver tissue. In addition, AE also promote FA oxidation in liver. These effects are involved in AMPK activation. The hypothetical chalcones in the prevention of obesity and hyperglycemia is shown in Fig. Concl. Together, these results indicate that chalcones should be of great benefit to prevent obesity and insulin resistance.

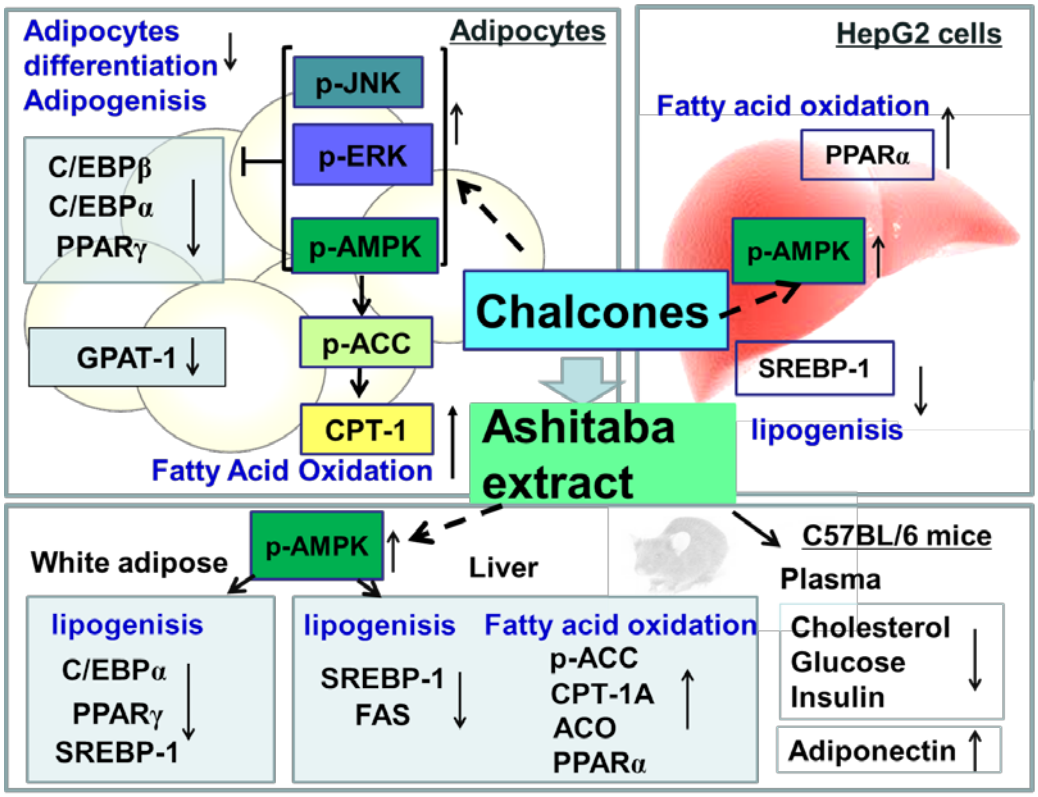


**Fig. Concl. The mechanism of chalcones in the prevention of obesity and insulin resistance.**

**Future Research**

In this dissertation, the author demonstrated that chalcones are attractive food components for prevention of obesity and insulin resistanc through modulation of the central and peripheral functions. To elucidate the functions of food components, clarification of bioavailability for the target compound is important. Regarding bioavailability of chalcones, extent of absorption and metabolism are controversial. It was reported that Ashitaba chalcones are rapidly absorbed into plasma and are distributed to various tissues in mice orally administered with Ashitaba extract [Nakamura *et al*., 2012], but absorption and metabolism of Ashitaba chalcones is still not clear under the long time feeding at low concentration. Further study is needed to clarify this important issue. In addition, in the Chapter 1, the author found that chalcones modulated adipocyte differentiation through multiple signaling pathways. However, the mechanisms of between these factors for modulating adipocytes differentiation still not clear. It should be another issue for the future study.

In generally, it is common that the patients with insulin resistance, NAFLD and extreme extent of obesity are treated with medicine. However, the pharmaceutically treatments have certain risk of adverse effects. In addition, it is impossible for normal people to use pharmaceutically treatments to maintain and improve their health. Recently, much attention has paid to functional foods from the view point of preventive medicine, because of lower adverse effects with less cost. Dietary phytochemicals may be another cure for weight control and management of metabolic syndrome. The findings of this dissertation are little evidences for prevention of obesity and insulin resistance by chalcones. More attention should be pay to the scientific evidences for understanding the functions and safety of foods. Therefore, the author expects to continue the studies on the function of chalcones to improve human health.

## Appendix

**Preventive effects of black soybean seed coat polyphenols on DNA damage.**

### APPENDIX INTRODUCTION

DNA damage can lead to degenerative diseases including cancer, diabetes and cardiovascular disease [Ames, 1989; Reardon *et al*., 1992; Lee and Blair, 2001]. Environmental pollution and some dietary habits have been associated with genetic damage [Ames and Gold, 1998]. Several studies have reported that certain extracts of fruits and vegetables modify DNA damage [Godits *et al*, 1985; Freedman *et al*., 2008].

Black soybean has been a functional food for many years in East Asia [Inagaki *et al*., 2005]. Recently, many studies have focused on the bioactivities of the color coats, which are rich in various polyphenols, such as catechins, anthocyanins, procyanidins and other flavonoids. Constituents in black soybean seed coat have been reported to possess various physiological functions, such as antioxidant activity *in vitro* [Ignasius *et al*., 2009], inhibition of inflammatory [Kim *et al*., 2008] and anti-obesity and anti-diabetic activity in mice [Kanamoto *et a*l., 2011]. However, information concerning the preventative effect of compounds from black soybean seed coat extract against DNA damage is still lacking, though it has been reported that black soybean components have prevented genetic damage induced by chemical mutagens in mice [Azevedo *et al*., 2003].

The appendix was divided into 3 parts. In appendix A, Ames test were carried out to investigate the safety of black soybean seed coat extract (BE). Then, the author investigated contribution of the antimutagenic compounds from BE in *Salmonella tyhimurium* TA 98 and TA 100. In appendix B, the author investigated the protective effects of BE and its components on benzo[a]pyrene [B(a)P]-induced DNA damage in HepG2 cells and ICR mice. Moreover, the author clarified the underlying protective mechanism of BE on B(a)P-induced DNA damage by examining drug metabolism enzymes system. In appendix C, the author, further, investigated the protective effect of BE and its polyphenols on oxidative DNA damage by estimating the levels of 8-OHdG in human hepatoma HepG2 cells.

**Appendix A**

**Preventive effects of black soybean seed coat polyphenols against mutagens-induced DNA damage in *Salmonella typhimurium.***

## INTRODUCTION

Among the methods for evaluating DNA damage, the Ames test serves as a quick assay to estimate the mutagenic and anti-mutagenic potential of compounds (Mortelmans and Zeiger, 2000). B(a)P is a polycyclic aromatic hydrocarbon and is widespread in the environment (Hattemer-Frey and Travis, 1991). It is an indirect mutagen that exhibits biological activity by P450-dependent monooxygenase enzymes (Schoket *et al*., 2001). B(a)P metabolites, such as epoxides, can bind to DNA to form B(a)P-DNA adducts that can interfere with or alter DNA replication, increasing the risk of several cancers (Rodriguez *et al*., 1999). 4-Nitroquinoline-1-oxide (4NQO) is a direct mutagen and binds covalently to DNA. 4NQO also plays a role as an oxidative mutagen that generates superoxide radicals and reactive oxygen species by redox recycling (Nunoshiba and Demple, 1993).

BE is rich in various polyphenols, such as catechins, anthocyanins and procyanidins. In appendix A, mutagenic and cytotoxicity assays were performed to investigate the safety of BE. Then, the author investigated the contribution of the anti-mutagenic compounds from BE in *Salmonella typhimurium* stains TA 98 and TA 100.

## MATERIAL AND METHODS

### Materials

Strains of *S. typhimurium* (TA 98 and TA 100) were used for mutagenic and anti-mutagenic assays. S9 mix (S9 fraction of liver homogenate with cofactor I) was purchased from Oriental Yeast Co. Ltd (Tokyo, Japan). 2-Aminoanthracene (2-AA), 2-(2-furyl-3-(5-nitro-2-furyl) acrylamide (AF-2), 4-nitroquinoline-1-oxide (4NQO), and benzo[a]pyrene [B(a)P] were purchased from Sigma (St. Louis, MD).

**Table appendix A-1. Composition of polyphenols in BE and PC-rich BE.**

| Compounds | BE | PC-rich BE |
|---|---|---|
| | % (w/w) | |
| Total polyphenols | 67 | 85.4 |
| Total flavanols | 45.9 | 80.6 |
| Total isoflavones | 0.96 | 0.69 |
| | | |
| Cyanidin-3-glucoside | 9.2 | 0.9 |
| Epicatechin | 6.2 | 13.8 |
| Procyanidin | 39.7 | 66.8 |
| Dimer | 6.1 | 11.8 |
| Trimer | 3.4 | 7.8 |
| Tetramer | 0.5 | 3.1 |

Black soybean seed coat extract (BE) and procyanidin-rich BE (PC-rich BE) were gifts from Fujico Co. Ltd (Kobe, Japan); their compositions are shown in Table appendix A-1. Cyanidin 3-glucoside (C3G), procyanidin B1 (PCB1) and procyanidin B2 (PCB2) were purchased from Funakoshi (Tokyo, Japan). Procyanidin C (PCC), EC-(4β→6)-EC-(4β→8)-EC-(4β→8)-EC (PC4-1) and cinnamtannin A2 (PC4-2) were obtained from Fujico Co. Ltd. (+)-Catechin and (-)-epicatechin (EC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The chemical structures of the major polyphenols are shown in Fig. appendix A-1. All other reagents used were of the highest grade available from a commercial source.

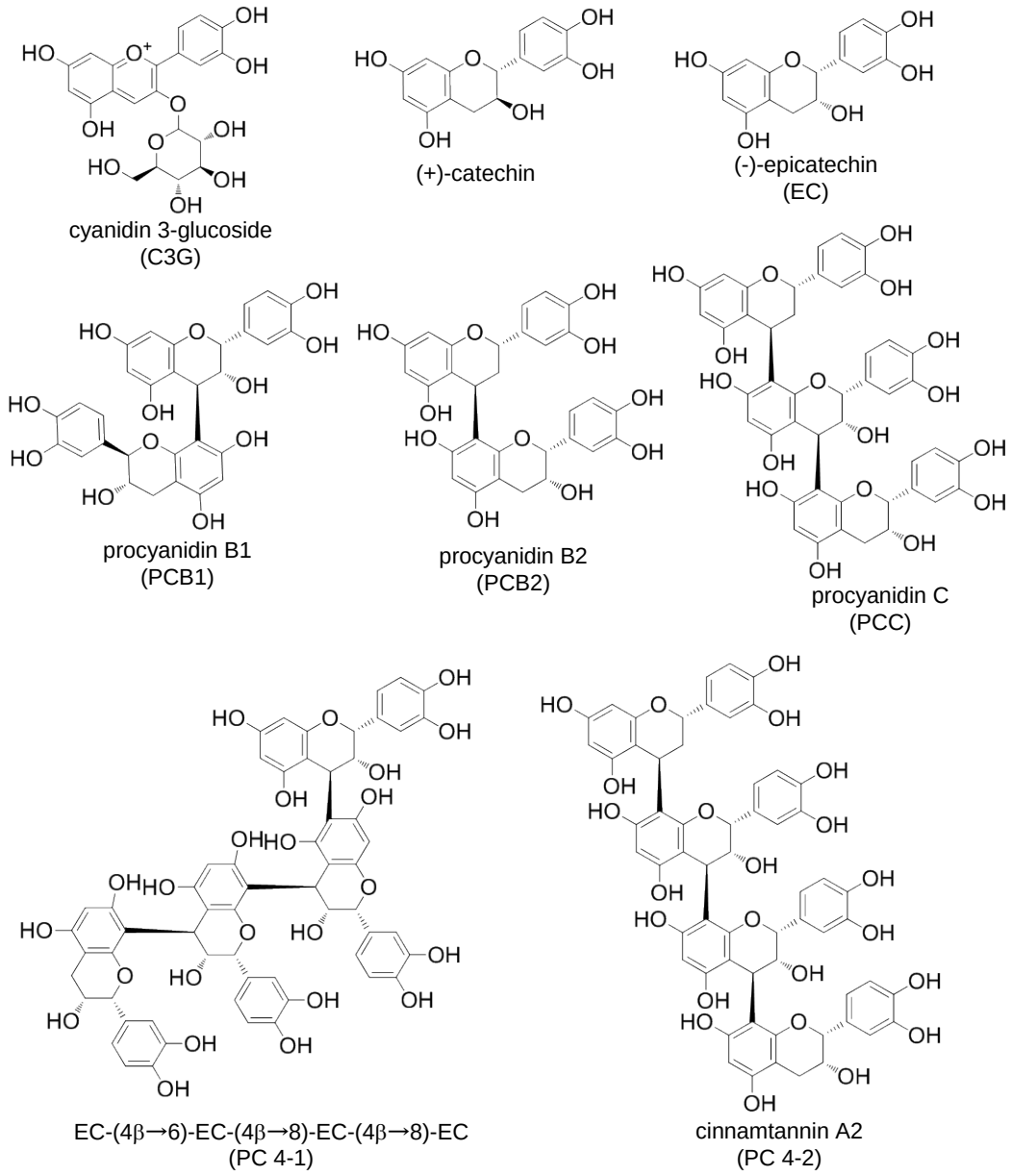


**Fig. appendix A-1, Chemical structures of major polyphenols in BE.**

**Mutagenic assay**

The mutagenicity of BE was assayed according to the method of Ames [Maron and Ames, 1983] using *S. typhimurium* strains TA 98 and TA 100. A solution of 0.1 mL of BE at various concentrations (final 5, 50, 500 and 5000 μg/plate), DMSO as vehicle control, or mutagen was added to the mixture of 0.5 mL of S9 mix (S9+) or 0.1 M phosphate buffer (S9-), and 0.1 mL of *S. typhimurium* TA 98 or TA 100 overnight culture. As the mutagens, 2-AA (0.1 μg/plate in TA 98 and 0.01 μg/plate in TA 100) and AF-2 (0.5 μg/plate in TA 98 and 1 μg/plate in TA 100) were used. After pre-incubation at 37℃ for 30 min, 2 mL of molten top agar supplemented with l-histidine and (+)-biotin at 55◦C was added to the mixture. The solution was then gently mixed and poured onto minimal glucose agar plates. The plates were turned upside down and incubated at 37◦C for 48 h, and colony counts were determined. Three independent experiments were performed using triplicate plates in each experiment.

**Cytotoxicity assay**

The cytotoxicity of BE was also determined using *S. typhimurium* strains TA 98 and TA 100 as described by Ames *et al.* [Maron and Ames, 1983] with slight modification. Cells of *Salmonella* strains were cultured overnight at 37℃. Aliquots of cell suspensions (0.4 mL each) were supplemented with 0.4 mL of BE at 5000 μg/plate or DMSO as vehicle control, with or without S9 mix, respectively. The mixtures were incubated at 37◦C for 30 min. The cells were washed 3 times with saline by centrifugation at 3000×g for 20 min and resuspended in 0.4 mL of 0.1 M phosphate buffer (pH 7.4). A 0.1 mL aliquot of the cell suspension was added to 2 mL of molten soft agar and poured onto minimal glucose agar medium. In parallel, a portion of the same volume of cell suspension was diluted 106-fold with saline and 0.1 mL aliquots were added to 2 mL of molten soft agar containing 5 mM L-histidine and poured onto minimal glucose agar medium. After incubation at 37◦C for 48 h, the revertant and surviving colonies were counted. Three independent experiments were performed using triplicate plates in each experiment.

**Anti-mutagenic assay**

The anti-mutagenicity of BE and its polyphenols was investigated using the Ames test as described previously [Maron and Ames, 1983]. B(a)P was used as an indirect mutagen, which required the S9 mix for metabolic activation, at 5 μg/plate for both TA 98 and TA 100, while

4NQO was used as a direct mutagen at 1 μg/plate for TA 98 and 0.5 μg/plate for TA 100. In the assay, 0.1 mL of BE, PC-rich BE, or C3G at different concentrations was added to 0.02 mL of mutagen solution and 0.5 mL of S9 mix (S9+) or 0.1 M phosphate buffer (S9-). Subsequently, 0.1 mL of bacterial cells was added to the mixture and incubated at 37°C for 30 min. The following procedure was carried out according to that for the mutagenic assay as described above. Data are presented as the mean of three independent experiments of triplicate plates.

For the anti-mutagenic assay, the inhibition of mutagenicity was calculated by using the following equation (A: number of revertants/plate induced by mutagen alone, B: number of spontaneous revertants, C: number of revertants/plate induced by the compound plus mutagen) Inhibition (%) = [(A-C)/ (A-B)] × 100.

**Statistical analysis**

The data are expressed as the mean ± SE. Analysis of variance (ANOVA) was conducted. Tukey's multiple-range test was used to estimate the significance of differences among groups. The level of statistical significance was set at $p<0.05$.

## RESULTS

**Mutagenic assay of BE**

The author first investigated BE mutagenicity by performing the Ames test with *S. typhimurium* strains TA 98 and TA 100. As shown in Table appendix A-2, in the presence of

**Table appendix A-2. Mutagenicity of BE**

| | | Number of revertant colonies | | | |
|---|---|---|---|---|---|
| | | TA 98 | TA 98 | TA100 | TA100 |
| Compounds | | S9+ | S9- | S9+ | S9- |
| 2-AA | | 63±12* | - | 476±9* | - |
| AF-2 | | - | 419±7* | - | 580±13* |
| BE | 5000 | 31±2 | 15±2 | 210±14 | 268±18 |
| (μg/plate) | 500 | 26±3 | 17±2 | 202±5 | 217±7 |
| | 50 | 24±2 | 19±3 | 172±17 | 202±5 |
| | 5 | 25±2 | 19±5 | 170±10 | 202±6 |
| Vehicle | | 22±3 | 18±2 | 177±7 | 210±5 |

Vehicle: DMSO (100 μL/plate); 2-AA: 2-aminoanthracene (0.1 μg/plate in TA 98 and 0.01 μg/plate in TA 100); AF-2: 2-(2-furyl-3-(5-nitro-2-furyl) acrylamide (0.5 μg/plate in TA 98 and 1 μg/plate in TA 100). *The number of revertant colonies was more than twice that of the vehicle. Values are expressed as mean ± SE (n=3).

S9 mix (S9+), the positive control 2-AA showed mutagenic activity compared with the DMSO control, which had 22 ± 3 and 177 ± 7 spontaneous revertants per plate in strains TA 98 and TA 100, respectively. In the absence of S9 mix (S9-), the positive control AF-2 also showed mutagenic activity compared with the DMSO control, which had 18 ± 2 and 210 ± 5 spontaneous revertants per plate in strains TA 98 and TA 100, respectively. However, these spontaneous mutation frequencies did not change under treatment with BE, even at the highest concentration of 5000 μg/plate.

**Cytotoxicity of BE**

BE cytotoxicity was assessed by the Ames test using *S. typhimurium* strains TA 98 and TA 100. As shown in Table appendix A-3, in the absence of L-histidine, BE at 5000 μg/plate did not change spontaneous revertants with or without S9 mix in both TA 98 and TA 100. In the presence of L-histidine, BE at 5000 μg/plate also did not change spontaneous surviving colonies, with or without S9 mix, in both strains.

**Table appendix A-3. Cytotoxicity of BE**

| Compound (μg/plate) | | Number of revertant colonies | | | |
|---|---|---|---|---|---|
| | | TA98 | | TA100 | |
| | | S9+ | S9- | S9+ | S9- |
| His+ | Vehicle | 638±23 | 47±4 | 534±13 | 717±22 |
| | +BE 5000 | 689±8 | 51±5 | 603±35 | 712±43 |
| His- | Vehicle | 20±2 | 21±3 | 169±19 | 195±18 |
| | +BE 5000 | 18±5 | 21±1 | 170±17 | 221±4 |

Vehicle: DMSO; (100 μL/plate). Values are expressed as mean ± SE (n=3).

**Anti-mutagenicity of BE and its polyphenols against B(a)P- and 4NQO-induced mutagenesis in *S. typhimurium* TA 98 and TA 100.**

The anti-mutagenic activities of BE, PC-rich BE and C3G on the indirect mutagen B(a)P (5 μg/plate) was evaluated. As shown in Fig. appendix A-2A, BE, PC-rich BE and C3G dose-dependently inhibited B(a)P-induced mutagenesis in the presence of S9 mix. In addition, they also exhibited strong inhibitory effect on 4NQO (1 μg/plate for TA 98 and 0.5 μg/plate for TA 100)-induced mutagenesis in the absence of S9 mix (Fig. appendix A-2B). Anti-mutagenic activity was in the rank order: C3G>BE>PC-rich BE. These results indicate that C3G is one of the active compounds in BE that prevents B(a)P- and 4NQO-induced mutagenesis. To clarify the antimutagenic effects of other polyphenols present in BE, the

anti-mutagenic effect of each compound at 5 μM (concentration in pre-incubated solution, 720 μL) was determined against the mutagenicity of B(a)P and 4NQO. In TA 98, the inhibition of C3G, catechin, epicatechin, PCB1, PCB2, PCC, PC4-1 and PC4-2 on B(a)P-induced mutagenesis was 40%, 16%, 20%, 34%, 33%, 41%, 59% and 52%, respectively, and the inhibition against 4NQO-induced mutagenesis was 37%, 23%, 23%, 29%, 26%, 37% 44% and 39%, respectively. In TA 100, the inhibition of these compounds on B(a)P-induced mutagenesis was 43%, 17%, 25%, 30%, 28%, 48%, 57% and 59%, respectively, and the inhibition on 4NQO-induced mutagenesis was 36%, 20%, 18%, 28%, 28%, 38%, 56% and 54%, respectively (Fig. appendix A-3). From these results, procyanidins, in addition to C3G, showed anti-mutagenic activity. In particular, the procyanidin trimer and tetramer showed strong anti-mutagenic activity, while catechin and epicatechin showed weak activity.

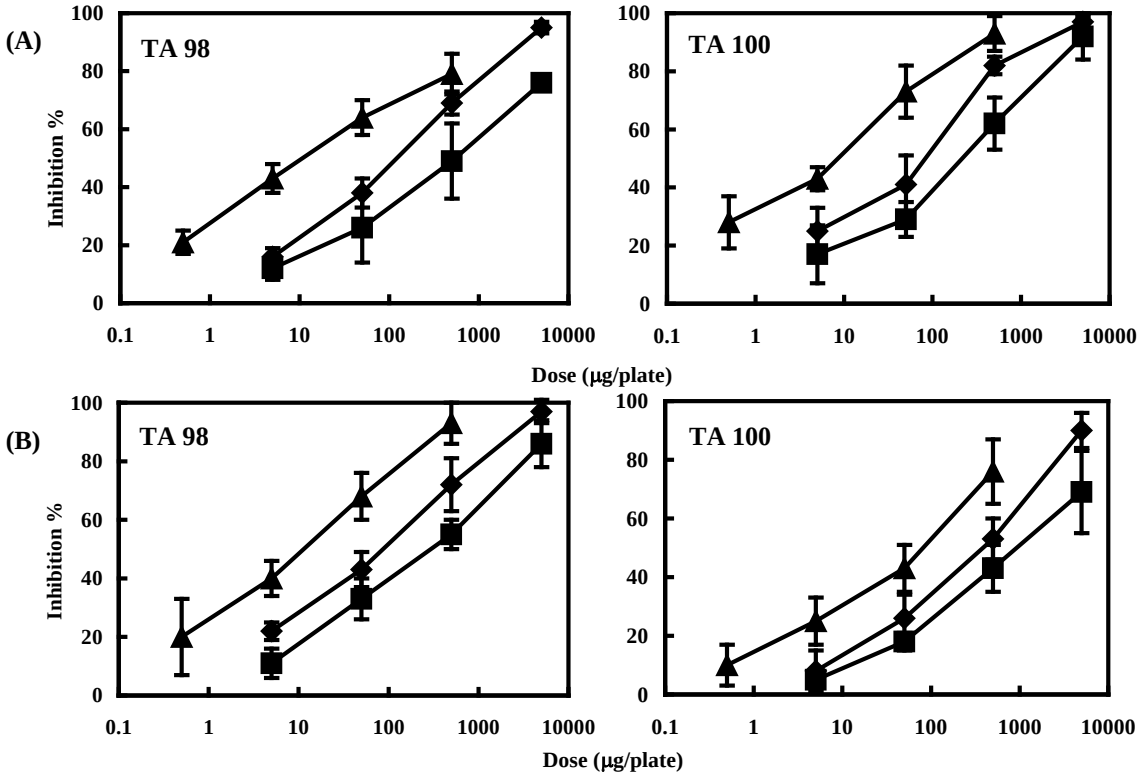


**Fig. appendix A-2. Anti-mutagenicity of BE, PC-rich BE and C3G against (A) B(a)P- and (B) 4NQO-induced mutagenesis in *Salmonella typhimurium* TA 98 and TA 100.** B(a)P was used at 5 μg/plate while 4NQO was used at 1 μg/plate for TA 98 and 0.5 μg/plate for TA 100. Diamonds, BE; squares, PC-rich BE; triangles, C3G. Results are expressed as the mean ± SE (n=3).

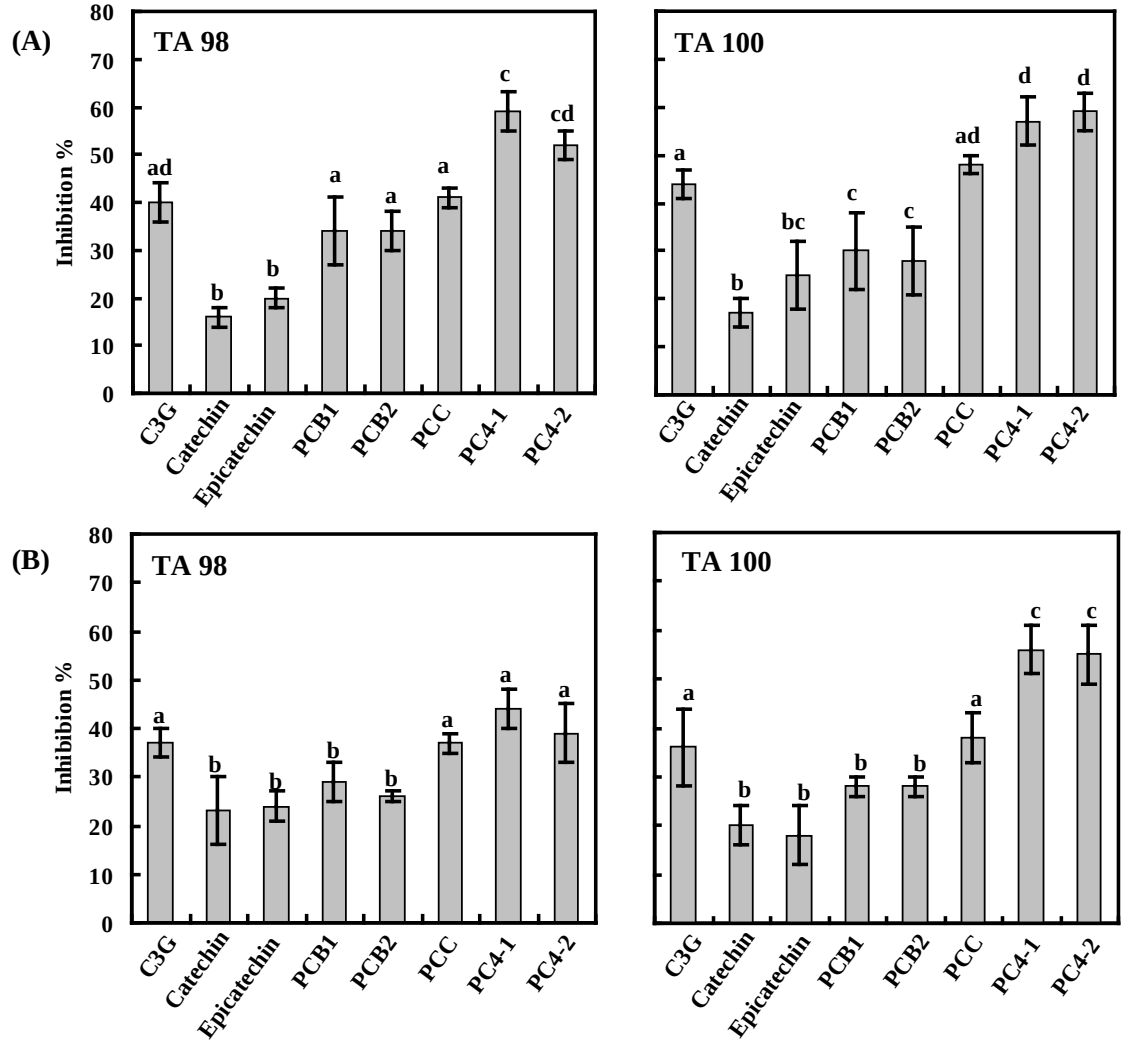


**Fig. appendix A-3. Anti-mutagenicity of C3G, (+)-catechin, (-)-epicatechin and procyanidins against (A) B(a)P- and (B) 4NQO-induced mutagenesis in *Salmonella typhimurium* TA 98 and TA 100.** Each polyphenol was added to the pre-incubation solution at 5 μM. B(a)P was used at 5 μg/plate while 4NQO was used at 1 μg/plate in TA 98 and 0.5 μg/plate in TA 100. Results are expressed as the mean ± SE (n=3). Means with different letters differ significantly ($p$<0.05), as determined by Tukey's multiple-range test.

## DISCUSSION

In the present study, the author demonstrated that BE exhibited neither mutagenicity nor cytotoxicity toward *S. typhimurium* TA 98 and TA 100 even at the highest concentration (5000 μg/plate) used in this study to (Table appendix A-2 and 3). In previous acute and chronic oral toxicity studies, the author also found that BE did not result in mortality or poisoning in rats and mice [Fukuda *et al.*, 2011]. These results indicate that BE is safe for consumption or for use as a food material. With respect to anti-mutagenicity, BE, C3G and PC-rich BE exhibited

a dose-dependent anti-mutagenic activity with or without the S9 mix, in TA 98 (frame-shift mutation) and TA 100 (single base-substitution mutation) (Fig. appendix A-2). The author's results indicate that C3G is one of the active compounds in BE that prevents mutagenesis. Moreover, procyanidins especially the dimer, trimer and tetramer, but not catechin and epicatechin (monomer), contributed to the anti-mutgaenic activity of BE (Fig. appendix A-3). Anti-mutagenic activity was in the rank order: tetramer>trimer>dimmer>monomer. The previous study showed that procyanidins prevented B(a)P-induced DNA damage via decreasing the expression of CYP1A1 and increasing the expression of glutathione S-transferases, depending on the degree of procyanidin polymerization [Zhang *et al*., 2013]. This result indicates that polymers are more potent than monomers in modulating the drug-metabolizing enzyme system, suggesting that polymers have strong effects on the prevention of chemically -induced mutagenesis and/or carcinogenesis, although the bioavailability of polymers is lower than that of monomers. From these results, C3G and procyanidins, the main components in BE, have the potential to protect against mutagen-induced DNA damage.

The mutagens used in this study were the indirect mutagen B(a)P and the direct mutagen 4NQO. B(a)P is a five-ring polycyclic aromatic hydrocarbon whose metabolites are mutagenic and highly carcinogenic. Numerous studies have documented B(a)P involvement in increased cancer risk [Xiong *et al*., 2001; Sinha *et al*., 2005]. It is known that B(a)P leads to cancer through several enzymatic reactions [Jiang and Whitlock, 2007]. The most important enzyme is cytochrome P4501A1 (CYP1A1), and B(a)P induces CYP1A1 through an aryl hydrocarbon receptor mediated pathway [James *et al*., 1999]. Induced CYP1A1 plays a role in metabolic activation of B(a)P to form its epoxides. The formed epoxides generate radicals and DNA -adducts [Lodovici *et al*., 2004]. Recent study showed that procyanidins and C3G from black soybean seed coat, prevented B(a)P-induced DNA damage via modulation of drug-metabolizing enzymes in HepG2 cells and ICR mice [Zhang *et al*., 2013]. Previous studies have shown that procyanidin -rich cacao polyphenol extract suppresses 3-methylcholanthrene-induced CYP1A1 expression in C57BL/6 mice [Mukai *et al*., 2008]. Moreover, anthocyanin -rich pomegranate juice prevented procarcinogen activation through the inhibition of CYP activity [Faria *et al*., 2007]. Besides, it was reported that certain polyphenols can form complexes with B(a)P and reduce its mutagenicity [Gupta *et al*., 2002; Mejia *et al*., 1999]. Thus, the anti-mutagenic activity of C3G and procyanidins of BE

may involve modifying the expression of cytochrome P4501A1 or forming complexes with B(a)P thereby inhibiting the metabolic activation of B(a)P and formation of DNA -adducts. Another mutagen, 4NQO, is a quinoline derivative and a tumorigenic compound. It is well-known that the mutagenic action of 4NQO is responsible for the generation of DNA -adducts [Galiegue-Zouitina *et al.*, 1986] and intracellular oxidative stress that undergoes redox recycling [Nunoshiba and Demple, 1993]. From the author's results, the antimutagenic activity of C3G and procyanidins of BE may be attributed to protection against the production of DNA -adducts or scavenging of superoxide radicals and other reactive oxygen species induced by 4NQO. Further study is required to clarify this issue.

In conclusion, BE exhibited neither mutagenicity nor cytotoxicity against *S. typhimurium*. In contrast, C3G and procyanidins, the main BE polyphenols, contributed to strong anti-mutagenicity against B(a)P- and 4NQO-induced DNA damage. Therefore, BE is a safe and effective material for use in functional foods and medicines to prevent or treat chemically-induced carcinogenesis.

**Appendix B**

**Black soybean seed coat polyphenols prevent B(a)P-induced DNA damage through modulating drug-metabolizing enzymes in HepG2 cells and ICR mice**

**INTRODUCTION**

B(a)P is a polycyclic aromatic hydrocarbon and widespread in the environment [Hattemer-Frey and Travis, 1991]. It is well documented that B(a)P requires metabolic activation to generate an electrophilic intermediate by CYP1A1-dependent monooxygenase enzymes [Gelboin, 1980; Schoket *et al*., 2001]. Through metabolic activation, B(a)P converts to its ultimate carcinogens, (+)-*anti*-7,8-dihydroxy-9,10-tetrahydrobenzo[a]pyrene [(+)-*anti*-BaPDE] [Gelboin, 1980; Buening *et al*., 1978; Slaga *et al*., 1979], which covalently binds to DNA to form B(a)P-DNA adducts; this is a critical event in B(a)P-induced DNA damage. Although, the constitutive level of CYP1A1 in mammal liver is low, B(a)P induces CYP1A1 expression through the action of an aryl hydrocarbon receptor (AhR) [Whitlock, 1999; Drahushuk *et al*., 1998]. After binding the ligands to AhR, the receptor protein translocates into the nucleus and forms a heterodimer with AhR nuclear translocator (Arnt) [Denison *et al*., 1986; Elferink *et al*., 1990]. This AhR/Arnt heterodimer functions as a transcription factor. The heterodimer binds to the dioxin responsive element (DRE) and induces the expression of various proteins including CYP1A1 [Whitlock, 1999]. Thus, the reduction of CYP1A1 expression should be a feasible way to prevent B(a)P-induced DNA damage.

(+)-*anti*-BaPDE, the final carcinogenic metabolite of B(a)P [Gelboin, 1980], has been known to induce mutagenicity *in vitro* and carcinogenicity *in vivo* [Buening *et al*., 1978; Slaga *et al*., 1979; Celotti *et al*., 1993]. To date, several mechanisms exist that can convert (+)-*anti*-BaPDE into less harmful compounds to protect against DNA damage [Singh *et al*., 1998; Robertson *et al*., 1986; Hu *et al*., 1996; Harvey, 2011]. Among them, the most important mechanism of (+)-*anti*-BaPDE inactivation seems to be its conjugation with glutathione, a reaction catalyzed by the glutathione S-transferase (GST) family. GSTs are one of the most important detoxifying enzymes related to the detoxification system. It is known that GSTs catalyze the conjugation of a wide variety of xenobiotics through conjugation with glutathione thereby reducing their gene toxicity and protection against carcinogen-induced DNA damage [Frova, 2006]. GST expression is regulated by multiple factors including nuclear factor-erythroid 2-related factor 2 (Nrf2) that binds to antioxidant response elements

(ARE), which are specific nucleotide sequences present in the promoter region of the gene encoding for GSTs [Zhu *et al*., 2005]. Accordingly, an increase in the expression of GSTs should be another possible mechanism to prevent B(a)P-induced DNA damage.

In appendix A, the author had showed that BE polyphenols showed strong anti-mutagenicity against B(a)P- and 4NQO-induced DNA damage in *Salmonella typhimurium* stains. In appendix B, the micronucleus (MN) assay was performed to investigate the protective effects of BE and its components on B(a)P-induced DNA damage in HepG2 cells. To clarify the underlying protective mechanism, the author examined the effects of BE and its components on B(a)P-induced CYP1A1 expression and the specific binding between AhR and DRE in HepG2 cells and ICR mice. The author further examined the effects of BE and its components on GSTα, μ and π expression and the specific binding between Nrf2 and ARE.

## MATERIAL AND METHODS

### Materials

Cytochalasin B, Giemsa solution were purchased from Sigma (St. Louis, MO, USA). Anti-CYP1A1 (Daiichi Pure Chemicals Co. LTD Tokyo, Japan), anti-GSTα (Alpha Diagnostic International, Texas, USA), anti-GSTμ (Proteintech Group, Inc., Chicago, IL, USA) and anti-GSTπ (Assay Designs, Michigan, USA) were used in this study. For the electrophoretic mobility shift assay (EMSA), oligonucleotide probes were synthesized as follows: DRE: 5’-GAT CCG GAG TTG CGT GAG AAG AGC CA-3’ (coding) and 5’-GAT CTG GCT CTT CTC ACG CAA CAC CG-3’ (non-coding) and ARE: 5’-TCT AGA GTC ACA GTG ACT TGG CAA AAT CTG A-3’ (coding) and 5’-TGA GAT TTT GCC AAG TCA CTG TGA CTC TAG A-3’ (non-coding). All other reagents used were of the highest grade available from a commercial source.

### HepG2 cells culture and treatment

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Sigma, USA), 4 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin under a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. After seeding in 6 cm dishes, the cells (90% confluent) were treated with BE (4.85 μg/mL equivalent to 10 μM of C3G) or its components

(10 μM) for 1 h. These cells were exposed to B(a)P (5 μM) or vehicle dimethyl sulfoxide (DMSO) for another 24 h. Thereafter, a post-nuclear fraction was extracted for western blotting analysis, whereas nuclear protein was prepared to measure the DNA-binding activity. Preparation of these fractions was performed according to the previous report [Nishiumi *et al*, 2008].

**Animal treatment**

Animal treatment in this study conformed to the "Guidelines for Care and Use of Experimental Animals", Rokkodai Campus, Kobe University (Permission # 24-11-01). Male ICR mice (6-weeks-old) were purchased from Japan SLC, Shizuoka and maintained in a temperature-controlled room (23 ± 2 °C) with a 12:12-h light/dark cycle (lights on at 9:00 am). For the first experiment, twelve male ICR mice (20-25 g) were divided at random into three groups of four each, i.e., the BE, PC-rich BE and water groups. All mice were orally administered BE and PC-rich BE (1 g/kg of body weight) suspension in deionized water or the vehicle alone (10 mL/kg of body weight) as a control. After 24 h, the liver was removed and used for experiments. For the second experiment, 24 male ICR mice (6 weeks old) were randomly divided into three groups of eight each for BE, PC-rich BE and water groups. All mice were orally administered BE and PC-rich BE (1 g/kg of body weight) suspension in deionized water or vehicle alone (10 mL/kg of body weight) as a control for 1 day or 3 consecutive days. These mice were further divided into two subgroups of 4 each for BE, PC-rich BE and water groups. One subgroup was given B(a)P (10 mg/kg of body weight) in corn oil through an intraperitoneal injection 1 h after the last administration of BE, PC-rich BE or water, whereas the other subgroup was given corn oil (5 mL/kg of body weight) as a vehicle control. After 25 h, the mice were sacrificed, and livers were removed for use in further experiments. Hepatic nuclear and post-nuclear fractions were prepared according to a previous report [Nishiumi *et al*., 2008] and subjected to western blotting analysis and EMSA as follows.

**MN assay**

The MN assays were performed according to the previously described method [Darroudi and Natarajan, 1993]. Briefly, HepG2 cells were first grown for 24 h and then washed twice with PBS. To estimate genotoxicity, the cells were exposed to BE at concentrations of 0, 6.25,

12.5 and 25 μg/mL for another 24 h. As a positive control, B(a)P at 2 μM was used. In the case of the anti-genotoxicity test, the cells were pre-treated with BE at concentrations of 0, 1, 5, 10 and 20 μg/mL for 24 h then exposed to B(a)P at 40 μM for another 2 h. After washing the cells, cytochalasin B (final concentration 3.0 μg/mL) was added to the medium for 24 h. For fixation, the cells were trypsinized and treated with cold hypotonic KCl solution (5.6 g/L) and subsequently air-dried preparations were made. To detect MN in the binucleated cells, the fixed cells were strained with 5.0% aqueous Giemsa solution. For each experiment, the percentage of MN frequency was determined in at least 1000 binucleated cells from triplicate independent cultures. To estimate cell division, the percentage of binucleated cells was also determined in the same culture dishes [Ehrlich *et al*., 2002]

**Western blotting analysis**

The post-nuclear fraction from HepG2 cells and the cytosolic fraction from ICR mice liver were used for the detection of CYP1A1 and GSTs by 10 and 12% of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. The proteins were transferred onto a PVDF membrane (GE Healthcare Bio-Science Co., Piscataway, NJ, USA). The membrane was treated with commercial blocking solution (Blocking One, Nacalai Tesque) for 30 min at room temperature. The membrane was incubated with primary antibodies for CYP1A1 (1:20000), GSTα (1:50000), GSTβ (1:20000) and GSTπ (1:20000) overnight at 4 °C, followed by the corresponding HRP-conjugated secondary antibody for 1 h at room temperature. The blots were developed using ImmunoStar® LD (Wako) Western Blotting Substrate and detected with Light-Capture II (ATTO, Tokyo, Japan). The densities of specific bands were determined using Image J image analysis software.

**EMSA**

EMSA was performed to determine the DNA-binding activity of DER and ARE. Briefly, nuclear extract (10 μg of protein) from the HepG2 cells or liver from the ICR mice was incubated with 500 ng of poly[dI-dC] and a $^{32}$P-labeled DRE or $^{32}$P-labeled ARE probe (30 kcpm, 10 fmols) in HEDG buffer for 15 min. The mixture was then loaded onto a 4% nonstacking polyacrylamide gel in TBE buffer (25 mM Tris, 22.5 mM borate and 0.25 mM EDTA). After electrophoresis, the AhR/DRE and Nrf2/ARE complexes were visualized by autoradiography and quantitatively analyzed by Gel-Pro Analyzer (Media Cybernetics,

Bethesda, MD, USA).

**Statistical analysis**

The data are expressed as the mean ± SE of at least three independent determinations for each experiment. Dunnett's test was used to determine the significance of differences between the treated and control groups. The level of statistical significance was set to $p<0.05$.

**RESULTS**

**Genotoxic and anti-genotoxic activities of BE in HepG2 cells**

Genotoxicity of BE was evaluated by MN assay. As shown in Fig. appendix B-1A, none of the tested concentrations led to increased MN frequencies compared with the background level 19 MN/1000 BNC. In addition, BE at concentrations up to 25 μg/mL did not significantly alter the number of binucleated cells (Fig. appendix B-1B). The effects of BE on B(a)P-induced MN are shown in Fig. appendix B-2A. It was found that BE at 5, 10 and 20 μg/mL significantly decreased the level of B(a)P-induced MN in HepG2 cells. To clarify the main active compounds in BE, the author next evaluated the effects of BE and its polyphenols on B(a)P-induced MN frequencies. Fig. appendix B-2B showed that treatment with BE and PC-rich BE (4.85 μg/mL equivalent to 10 μM C3G), C3G, EC and other PCs (10 μM each) significantly decreased MN frequencies induced by B(a)P.

**BE inhibits B(a)P-induced CYP1A1 expression and AhR transformation in HepG2 cells and livers of ICR mice**

To clarify the mechanism of the inhibitory effects of BE and its polyphenols on B(a)P-induced MN frequencies, the author investigated the effects of BE and its polyphenols on the expression of CYP1A1 and the upstream event AhR transformation *in vitro* and *in vivo*. For the *in vitro* experiment, the author isolated the post-nuclear fractions from the HepG2 cells that were treated with 5 μM B(a)P for 24 h as well as the vehicle-treated control cells. Results from western blotting analysis showed that CYP1A1 expression was dramatically induced following B(a)P treatment (Fig. appendix B-3A). The author found that B(a)P-induced CYP1A1 expression was significantly suppressed by BE and PC-rich BE at 4.85 μg/mL by 43 and 53%, respectively. Moreover, the author confirmed that BE and PC-rich BE, themselves did induce any changes to CYP1A1 expression. When AhR

transformation was evaluated by EMSA, B(a)P significantly promoted the transformation as expected (Fig appendix B-3B). BE and PC-rich BE at 4.85 μg/mL suppressed the B(a)P-induced transformation by 48 and 45%, respectively. To make clear the effective components, the author determined the effects of BE components C3G, catechin, EC and procyanidins (PCB1, PCB2, PCC, PCD1 and PCD2) on B(a)P-induced CYP1A1 expression in HepG2 cells. As shown in Fig. appendix B-3C, C3G, PCB1, PCB2, PCC, PCD1 and PCD2 down-regulated CYP1A1 expression by 54, 36, 47, 39, 33 and 32%, respectively, compared with the control group, whereas catechin and EC did not show significant inhibitory effect.

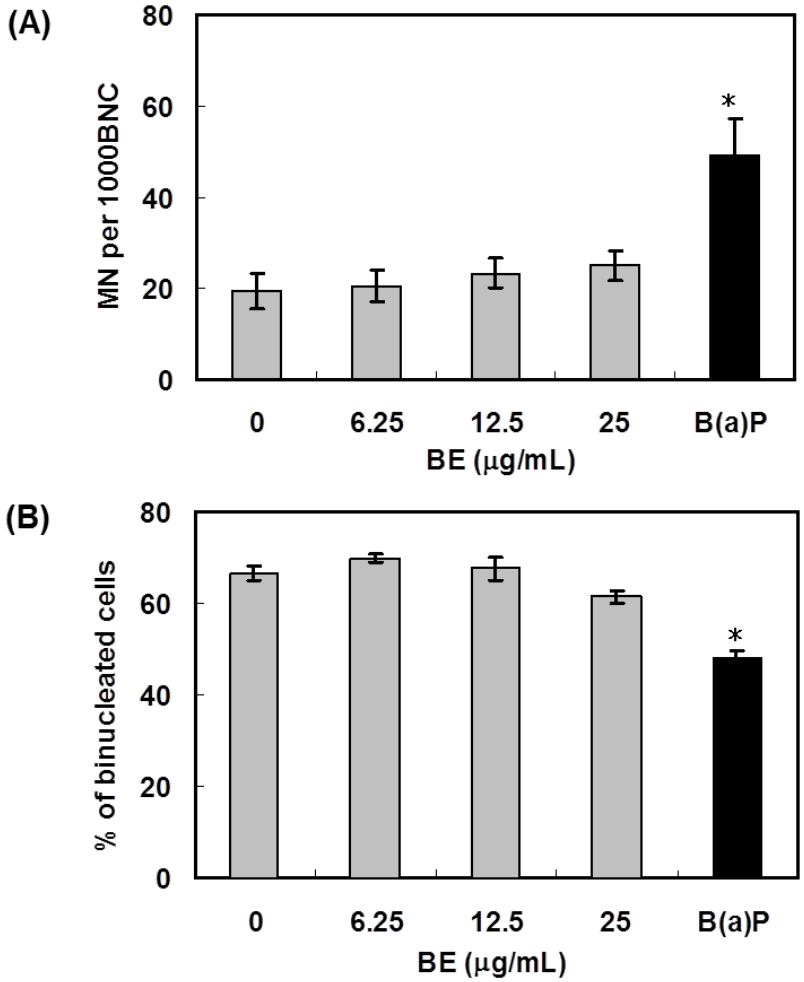


**Fig. appendix B-1. Effects of BE on MN formation (A) and cell division (B) in HepG2 cells.** The cells were exposed for 24 h to BE or B(a)P (2 μM) as a positive control followed by subcultivation for 24 h in the presence of cytochalasin B (3 μg/mL). (A) Bars represent MN numbers found in three cultures (in each culture, 1000 binucleated cells were evaluated for MN induction). (B) Number of binucleated cells (%) relative to the number of mono-, tri- and tetranucleated cells. The results are represented as the mean ± SE (n=3). *Indicates significant difference from the corresponding control by Dunnett's test ($p$<0.05).

To confirm the *in vitro* results, the author also performed *in vivo* experiments. After the mice were orally administered BE and PC-rich BE at 1 g/kg of body weight followed by an intraperitoneal injection of B(a)P at 10 mg/kg of body weight, both CYP1A1 expression and AhR transformation in mice liver were significantly decreased by approximately 40%, which was evaluated by western blotting and EMSA, respectively (Fig. appendix B-4).

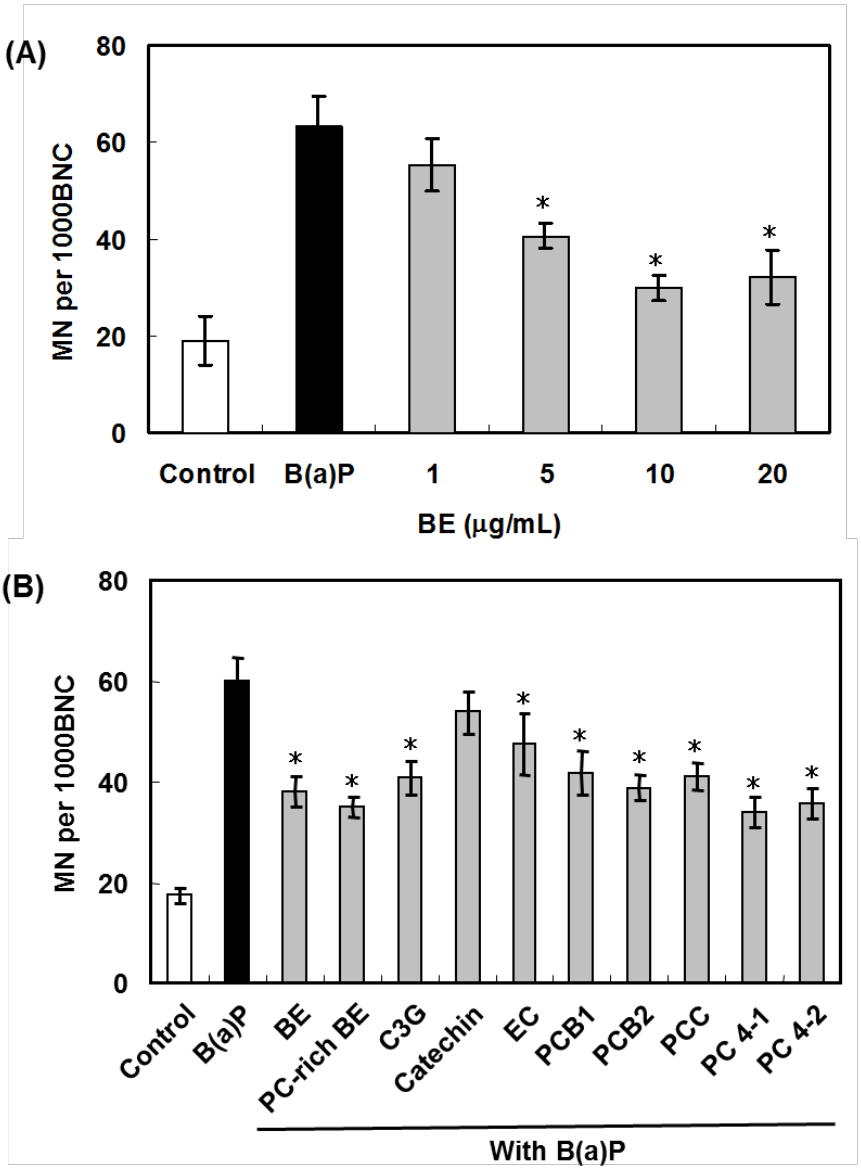


**Fig. appendix B-2. Effects of BE (A) and its polyphenols (B) against B(a)P-induced MN formation in HepG2 cells.** The cells were treated with BE at various concentrations (A), or BE and PC-rich BE at 4.85 μg/mL and their major polyphenols at 10 μM (B) for 24 h. Then, the cells were exposed to B(a)P at 40 μM for further 2 h. Subsequently, the cells were treated with cytochalasin B (3 μg/mL) for another 24 h. Bars represent MN numbers found in three cultures (in each culture, 1000 binucleated cells were evaluated for MN induction). The results are represented as the mean ± SE (n=3). *Indicates significant difference from the corresponding positive control by Dunnett's test ($p$<0.05).

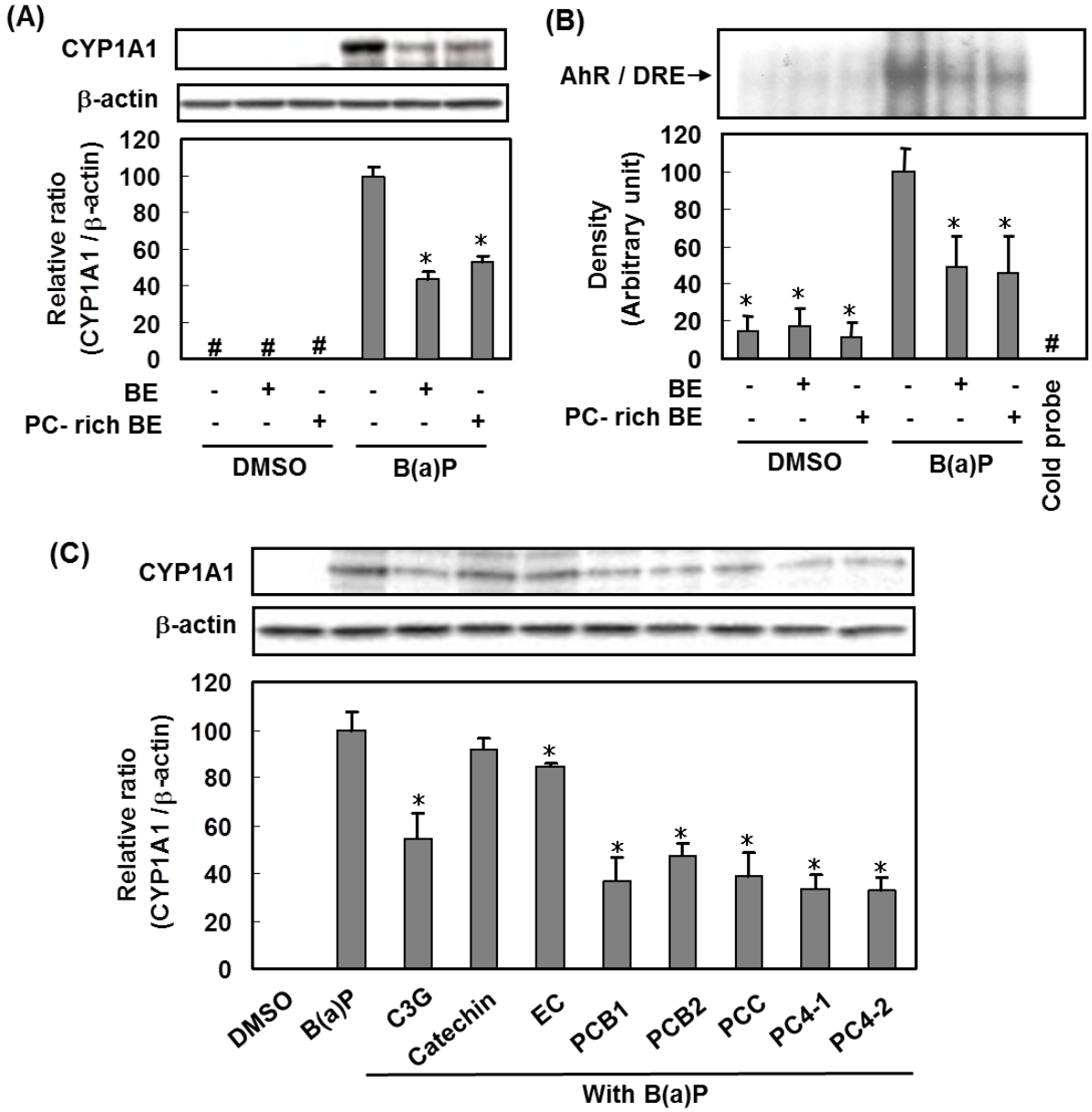


**Fig. appendix B-3. BE (A and B) and its polyphenols (C) suppress CYP1A1 expression (A and C) and AhR transformation (B) induced by B(a)P in HepG2 cells.** The cells were treated with BE (4.85 μg/mL equivalent to 10 μM of C3G) or its components (10 μM) for 1 h. Subsequently, the treated cells were exposed to B(a)P (5 μM) or DMSO for 24 h. CYP1A1 expression was determined by western blotting analysis, whereas AhR transformation was estimated by EMSA. The density of each band was quantified, and the value in the positive control [B(a)P-treated cells] was set to 100%. The results are represented as the mean ± SE (n=3). *Indicates significant difference from the corresponding positive control by Dunnett's test ($p$<0.05).

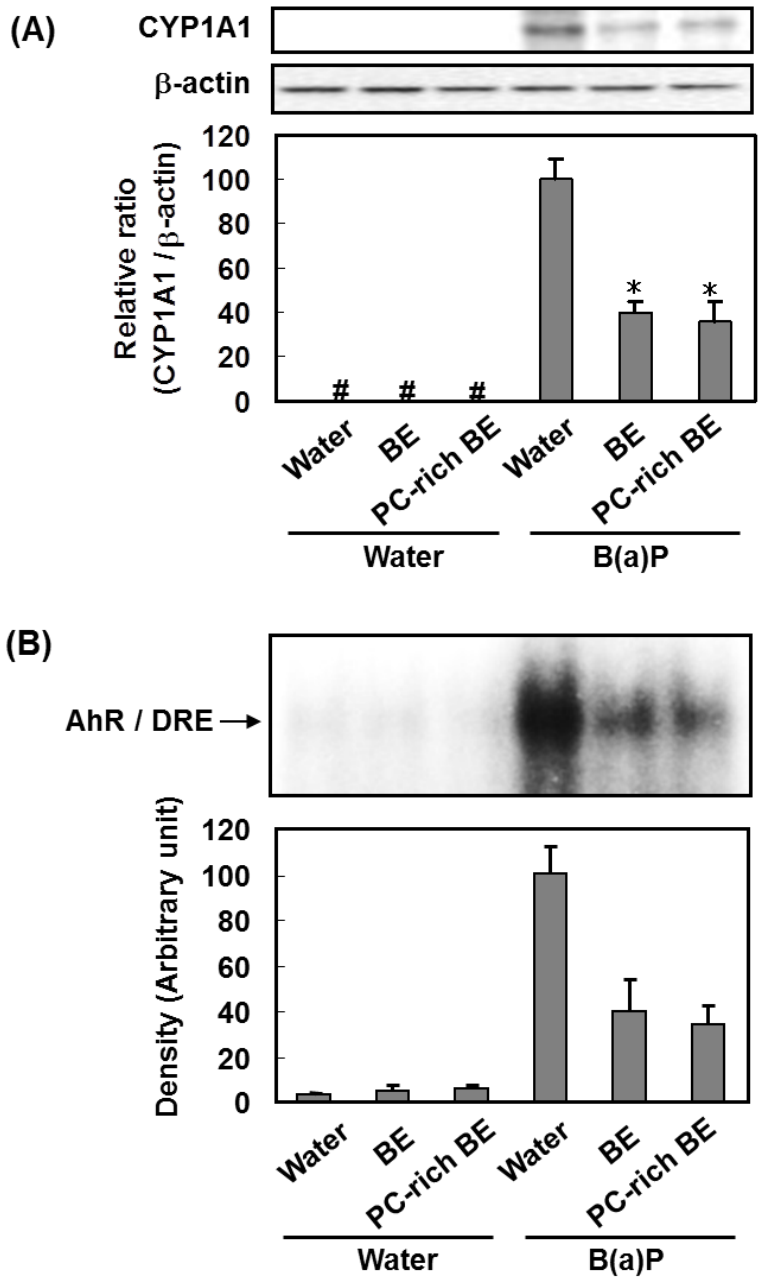


**Fig. appendix B-4. BE and PC-rich BE suppress B(a)P-induced CYP1A1 expression (A) and AhR transformation (B) in ICR mice.** Mice were orally administered BE and PC-rich BE at 1 g/kg of body weight or water (10 mL/kg of body weight) as a vehicle control for 3 consecutive days. Half of the mice were given B(a)P (10 mg/kg of body weight) in corn oil through an intraperitoneal injection 1 h after the administration of BE and PC-rich BE, whereas the remaining were given corn oil (5 mL/kg of body weight) as a vehicle control. After 25 h, hepatic nuclear and post-nuclear fractions were prepared and subjected to EMSA for AhR translocation and western blotting analysis for CYP1A1. The density of each band was quantified, and the value in the positive control was set to 100%. The results are represented as the mean ± SE (n=4). *Indicates significant difference from the corresponding positive control by Dunnett's test ($p$<0.05).

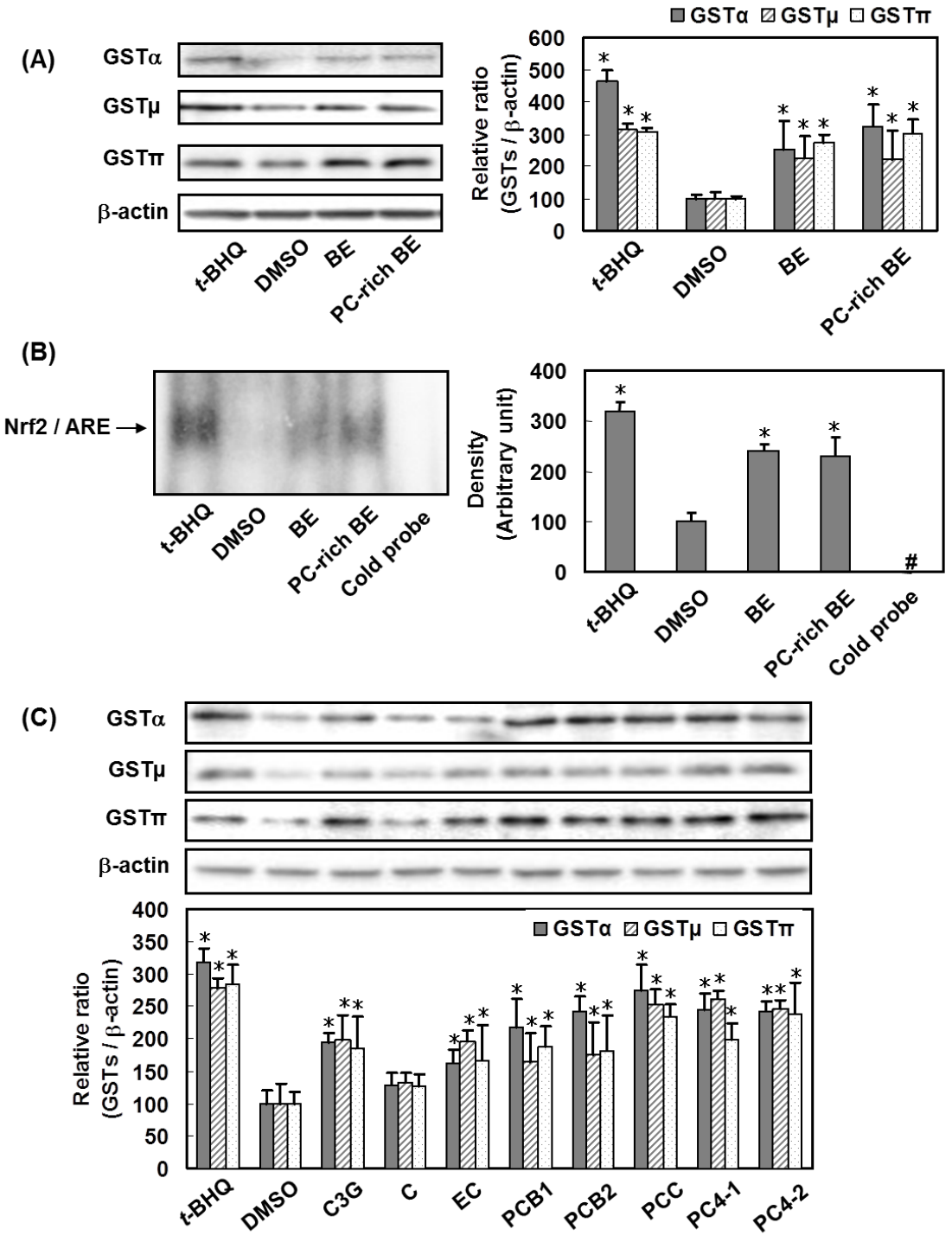


**Fig. appendix B-5. BE (A and B) and its polyphenols (C) enhance GSTα, μ and π expression (A and C) and DNA-binding activity of Nrf2 (B) in HepG2 cells.** The cells were treated with BE (4.85 μg/mL equivalent to 10 μM of C3G) or its components (10 μM) for 24 h. DMSO (final 0.25%) and *t*-BHQ (30 μM) were used as the vehicle and positive controls, respectively. Nuclear and post-nuclear fractions were prepared and subjected to EMSA for Nrf2 and western blotting analysis for GSTs. The density of each band was quantified, and the value in the vehicle control was set to 100%. The results are represented as the mean ± SE (n=3). *Indicates significant difference from the corresponding vehicle control by Dunnett's test ($p$<0.05).

**BE enhances GST expression and DNA-binding activity of Nrf2 in HepG2 cells and livers of ICR mice**

Induction of GST isoforms is considered one of the crucial mechanisms to protect cells against B(a)P-induced DNA damage. Nrf2 has been described as the main transcription factor that binds to the ARE sequence promoter region of the gene encoding for GSTs [Zhu *et al*., 2005]. The author performed both *in vitro* and *in vivo* experiments to evaluate the effects of BE on GST expression and Nrf2 transformation. For the *in vitro* experiments, HepG2 cells were treated with BE and PC-rich BE at 4.85 μg/mL for 24 h. Western blotting results showed that GSTα, μ and π expression were significantly enhanced by 2-3 fold compared with the control group (Fig. appendix B-5A). The EMSA results also showed that these proteins increased DNA-binding activity of Nrf2 (Fig. appendix B-5B). To determine the effects of various components, the author tested the effects of the BE components C3G, catechin, EC and procyanidins (PCB1, PCB2, PCC, PCD1 and PCD2) on GST expression in HepG2 cells. As shown in Fig. appendix B-5C, C3G, EC, PCB1, PCB2, PCC, PCD1 and PCD2 up-regulated GST expression approximately 1.5- to 3-fold compared with the control group.

Similar results were also found from the *in vivo* experiments. After the mice were orally administered BE and PC-rich BE at 1 g/kg of body weight for 1 day or 3 consecutive days, BE and PC-rich BE significantly increased GSTα, μ and π expression in both 1-day and 3-day treatment (Figs. appendix B-6A and 6B, respectively). In addition, induction of GSTs in the 3-day treatment was higher compared with that from the 1-day treatment (Figs. appendix-6A and 6B). As expected, these treatments were observed to increase the DNA-binding activity of Nrf2 (Figs. appendix-6C).

**DISCUSSION**

In the present study, the author first confirmed that BE was non-genotoxic (Fig. appendix B-1). In contrast, BE showed a dose-dependent anti-genotoxic activity against B(a)P-induced MN frequencies (Fig. appendix B-2A). This anti-genotoxic activation was involved in the modulation of drug metabolizing enzyme expression by BE: On the one hand, BE suppressed the expression of CYP1A1 by inhibiting AhR transformation both *in vitro* and *in vivo* (Figs. appendix B-3 and 4). On the other hand, BE enhanced DNA-binding activity of Nrf2 and subsequent induction of GSTs expression both *in vitro* and *in vivo* (Figs. appendix B-5 and 6). The author's finding demonstrated that PCs (dimmer, trimer and tetramer) and C3G, which

are the major polyphenols in BE, contributed to the anti-genotoxic activity of BE (Figs. appendix B-2B, 3C and 5C). From these results, polyphenols in BE have the potential to protect against B(a)P-induced DNA damage.

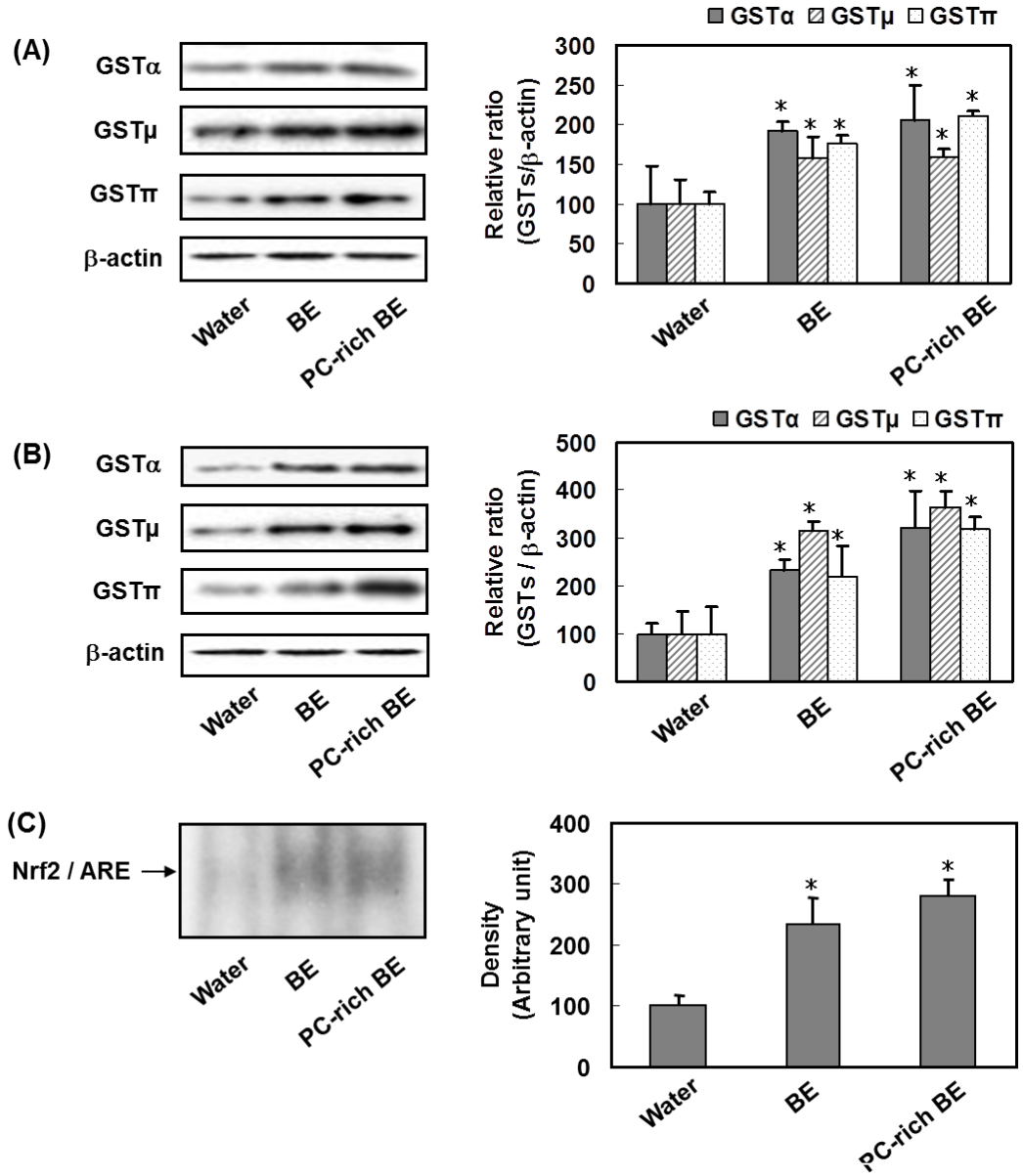


**Fig. appendix B-6. BE and PC-rich BE enhance GSTα, μ and π expression (A and B) and DNA-binding activity of Nrf2 (C) in ICR mice.** Mice were orally administered BE and PC-rich BE at 1 g/kg of body weight or water (10 mL/kg of body weight) as a vehicle control for 1 day (A) or 3 consecutive days (B). Hepatic nuclear and post-nuclear fractions were prepared and subjected to EMSA for Nrf2 and western blotting analysis for GSTs. The density of each band was quantified, and the value of the vehicle control was set to 100%. The results are represented as the mean ± SE (n=4). *Indicates significant difference from the corresponding positive control by Dunnett's test ($p$<0.05).

B(a)P is a five-ring polycyclic aromatic hydrocarbon whose metabolites are mutagenic and highly carcinogenic. Numerous studies have documented B(a)P involved in an increase of cancer risk [Xiong *et al*., 2001; Sinha *et al*., 2005]. It has been known that B(a)P causes cancer through several enzymatic reactions [Jiang *et al*., 2007]. The most important enzyme is CYP1A1. B(a)P induces CYP1A1 through an AhR-mediated pathway [Whitlock, 1999; James and Whitlock, 1999]. Induced CYP1A1 plays a role in metabolic activation of B(a)P to form epoxides, which generate radicals and DNA-adducts [Lodovici *et al*., 2004]. Previous studies have demonstrated that procyanidin-rich cacao polyphenol extract suppresses 3-methylcholanthrene-induced CYP1A1 expression in C57BL/6 mice [Mukai *et al*., 2008]. Anthocyanin-rich pomegranate juice prevents procarcinogen activation through the inhibition of CYP activity [Faria *et al*., 2007]. The author's results showed that BE and its components suppressed CYP1A1 expression both in HepG2 cells and livers of ICR mice, and this suppression was involved in the inhibition of AhR transformation [Figs. Appendix B-3 and 4]. These results suggest that BE and its components down-regulated expression of CYP1A1 by inhibiting the binding between AhR and DRE to reduce metabolic activation of B(a)P. Moreover, it has been reported that certain polyphenols can form complexes with B(a)P and reduce its mutagenicity [Gupta *et al*., 2002; de Mejia *et al*., 1999]. Thus, the anti-genotoxic activity of PCs and C3G of BE are explained by modifying CYP1A1 expression or forming complexes with B(a)P to inhibit metabolic activation of B(a)P and formation of DNA adducts.

The metabolic fate of the prototype carcinogen B(a)P has been extensively studied since the mid-1970s [Conney, 1982]. A series of investigations have clearly demonstrated that B(a)P epoxides, especially (+)-*anti*-7,8-dihydroxy-9,10-tetrahydrobenzo[a]pyrene, are highly reactive towards DNA and are classified as the ultimate carcinogenic metabolites of B(a)P [Gelboin, 1980; Buening *et al*., 1978; Slaga *et al*., 1979]. It is been known that B(a)P epoxides inactivation seems to be by conjugation with GSH, a reaction catalyzed by GSTs [Frova, 2006]. The main physiological activity of the detoxification enzyme GST family is to protect the cells against carcinogens, toxins and free radical oxidants [Lo and Ali-Osman, 2007]. Thus, GSTs have been recognized as important target molecules for a number of chemopreventive and cytoprotective agents, including dietary factors [Lii *et al*., 2006]. Previous studies have shown that naturally occurring flavonoid procyanidin B2 inhibits oxidative stress by inducing Nrf2 nuclear translocation and GSTP1 expression [Rodriguez-Ramiro *et al*., 2012]. Anthocyanin from black rice, berry and potato prevent

oxidative stress and some toxins by inducing GST expression [Hou *et al*., 2010; Milbury *et al*., 2007; Hwang *et al*., 2011]. Notably, BE and its component PCs and C3G also enhanced GST expression in both HepG2 and livers of ICR mice (Figs. Appendix B- 5 and 6). These results indicate that PCs and C3G in BE may help to convert B(a)P epoxides into less harmful compounds by inducing GST expression. It is well known that up-regulation of GSTs is mediated by activation of the nuclear translocation of Nrf2 and its binding to ARE sequence [Zhu *et al*., 2005]. The author's findings have demonstrated that BE and PC-rich BE increased the DNA-binding activity of Nrf2 and its downstream GST expression (Figs. Appendix B-5 and 6). Moreover, the increase in the DNA-binding activity of Nrf2 is correlated with an increase in GST expression. These results indicate that BE may reduce the toxicity of carcinogenic metabolites of B(a)P via enhancing detoxification enzyme GST expression.

In conclusion, BE did not induce genotoxicity to HepG2 cells. On the contrary, BE revealed a strong anti-genotoxicity against B(a)P-induced DNA damage in HepG2 cells. The author found that PCs and C3G were the main active compounds in BE for the suppression of DNA damage. These suppression effects of DNA damage were involved in the modulation of drug metabolizing enzyme expression. The author found that the PCs and C3G were the main active compounds in BE for down-regulation of CYP1A1 and up-regulation of GST expression, indicating that these compounds can effectively protect against B(a)P-induced DNA damage in liver cells. Collectively, BE is not only a safe and effective functional food but also has medicinal properties for the prevention and treatment of chemical-induced DNA damage and cancers.

**Appendix C**

**Black soybean seed coat polyphenols inhibit AAPH-induced production of 8-OHdG in HepG2.**

## INTRODUCTION

Reactive oxygen species (ROS) are well-established physiological molecules controlled by antioxidative defense systems. Cells are naturally provided with an extensive array of protective enzymes to scavenge ROS [Halliwell, 1994]. However, when environmental stress occurs, ROS levels can increase dramatically [Devasagayam *et al.*, 2004]. Oxidants produce extensive oxidative damage to DNA, which, in turn contributes to malignant tumors, diabetes and other degenerative diseases [Sun, 1990; Poulsen *et al.*, 1998]. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is one of the biomarkers of oxidative DNA damage [Helbock *et al.*, 1999]. An accumulation of 8-OHdG has been shown to lead to G: C-to-T: A transversion mutations that are prevalent in mutated in tumor suppressor genes [Shibutani *et al.*,1991; Hussain *et al.*,1998]. In recent years, the 8-OHdG lesions can be detected and analyzed with high sensitivity by liquid chromatography-mass spectrometry-mass spectrometry (LC-MS/MS). Determination and analysis 8-OHdG can be performed in cells, animal organs and human samples as a biomarker of oxidative stress and carcinogenesis [Valavanidis *et al.*, 2009].

The appendix A and B showed that BE and its components inhibited mutagens-induced DNA damage effectively. In appendix C, the author, further, investigated the protective effect of BE and its polyphenols on oxidative DNA damage by estimating the levels of 8-OHdG in HepG2 cells.

## MATERIAL AND METHODS

### Materials

8-OHdG, and a ROS generator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), were purchased from Wako Pure Chemical Industries. 8-mercaptoguanosine (8-SHG) was purchased from Sigma (St. Louis, MD). Other materials were described in appendix A and B.

### DNA Sample preparation

The HepG2 cells were seeded at a density of $5\times10^5$ cells/mL on a 60 mm plastic dish. After a 5 day culture, the cells were incubated with 4.85 μg/mL (equivalent to 10 μM of C3G)

BE and PC-rich BE or 10 μM each polyphenol for 1 or 24 h and then exposed to 25 mM AAPH at 37°C for 3 h. Then, the incubated HepG2 cells were submitted for isolation of the nuclei according to a previous method [Kanazawa *et al.*, 2006] with some modification. Briefly, the cells scraped with 100 μL of PBS were washed three times with ice-cold PBS and centrifuged at 1000×g for 3 min at 4 °C. The pellet was gently homogenized in an ice-cold 5 mM HEPES buffer (pH 7.5) containing 0.25 M sucrose and 0.5 mM EGTA, and again centrifuged at 1000×g for 10 min at 4°C. The pellet was three times washed with the buffer, and repeating the centrifugation. After washing with ice-cold PBS, the nuclei were lysed with 400 μL of TE buffer (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA) containing 0.5% sodium dodecyl sulfate (SDS). The lysate was treated with a final concentration of 0.5 mg/mL of ribonuclease A (Sigma) for 30 min at 50°C, and again with 0.5 mg/mL of proteinase K (Sigma) for 1 h at 50°C. By adding 0.5 M NaCl, DNA was then precipitated in 50% isopropanol and centrifuged at 17,000×g for 15 min. The pellet was dissolved in 35 mM sodium acetate containing 1 mM EDTA, mixed with a final concentration of 500 nM of internal standard 8-SHG and 0.2 mg/mL of nuclease P1 (Sigma) at 37°C for 30 min, and the reaction was stopped by adding 0.1 M Tris-HCl (pH 7.5). The DNA was treated with 3 units of alkaline phosphatase (Sigma) for 1 h at 37°C and centrifuged at 17,000×g for 10 min at 4°C. The supernatant was filtered through a 0.45 μm membrane filter. DNA concentration in the filtered solution was quantified using the NanoDrop® ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and the DNA concentration was adjusted to 40 ng/μL.

**Measurement of 8-OHdG**

The formation of 8-OHdG was detected and quantified by LC-MS/MS (4000 Q TRAP®, AB Sciex, Foster City, CA, USA) using electrospray ionization. HPLC separation was done with a gradient system using the mobile phase consisted of Solvent A (0.1% formic acid in de-ionized water) and Solvent B (acetonitrile). The samples were performed on a 5C18-MS-II 2.0 ID×15 mm column (Chemicals Evaluation and Research Institute, Tokyo, Japan) at a flow rate of 0.2 mL/min. The column oven was maintained at 40°C. The filtered samples were injected (5 μL) on column with initial conditions set to 0% Solvent B for 1 min. and then the concentration of Solvent B was increased linearly to 80% by 13 min. This condition was held for 1 min before returning to the initial mobile phase composition that maintained for the remainder of the assay and returned to initial conditions prior to the next injection. The total

run time, including column re-equilibration, was 20 min. Detection of 8-OHdG and 8-SHG was carried out under the positive electrospray ionization mode. The conditions for detection were obtained by direct infusion of a standard solution in line with the HPLC, set to initial mobile phase conditions. The cone and collision settings were established individually for 8-OHdG and 8-SHG to be used for multiple reaction monitoring (MRM) detection. Peak area of 8-OHdG was corrected by the area of the internal standard, 8-SHG.

**Statistical analysis**

The data are expressed as the mean ± SE. Dunnett's test was used to determine the significance of differences between sample group and control group in LC-MS/MS analysis. The level of statistical significance was set to $p<0.05$.

**RESULTS**

**Establishment of analytical procedure by LC-MS/MS**

To quantify 8-OHdG and 8-SHG (as an internal standard), the author first established the measurement conditions in LC-MS/MS. The MRM channels for 8-OHdG and 8-SHG were found to be 284/168 m/z and 317/185 m/z, respectively, corresponding to the $[M + H]^+$ parent ion and daughter fragment (the data was not shown). The detection limit was determined to be 1 nM (signal-to-noise ratio > 10:1) of 8-OHdG. In order to confirm the 8-SHG can be used as the internal standard to measure 8-OHdG from cells samples, the author, first, measured 8-OHdG (500 nM) and 8-SHG (500 nM) simultaneously, and found the retention time of them was 7.56 min and 7.73 min, respectively (shown in Fig. appendix C-1). Then the 8-OHdG in DNA samples 40 ng/μL from AAPH-treated cells were measured with or without internal standard 8-SHG (500 nM) under the same condition. Fig. appendix C-1B and C showed one of the typical results of DNA sample from AAPH-treated cells. The results exhibit that 8-OHdG and 8-SHG can be detected simultaneously, and there is no 8-SHG existing in DNA samples from cells. Thus, the author identified 8-SHG can be used as the internal standard to detect the formation of 8-OHdG in DNA samples from HepG2 cells.

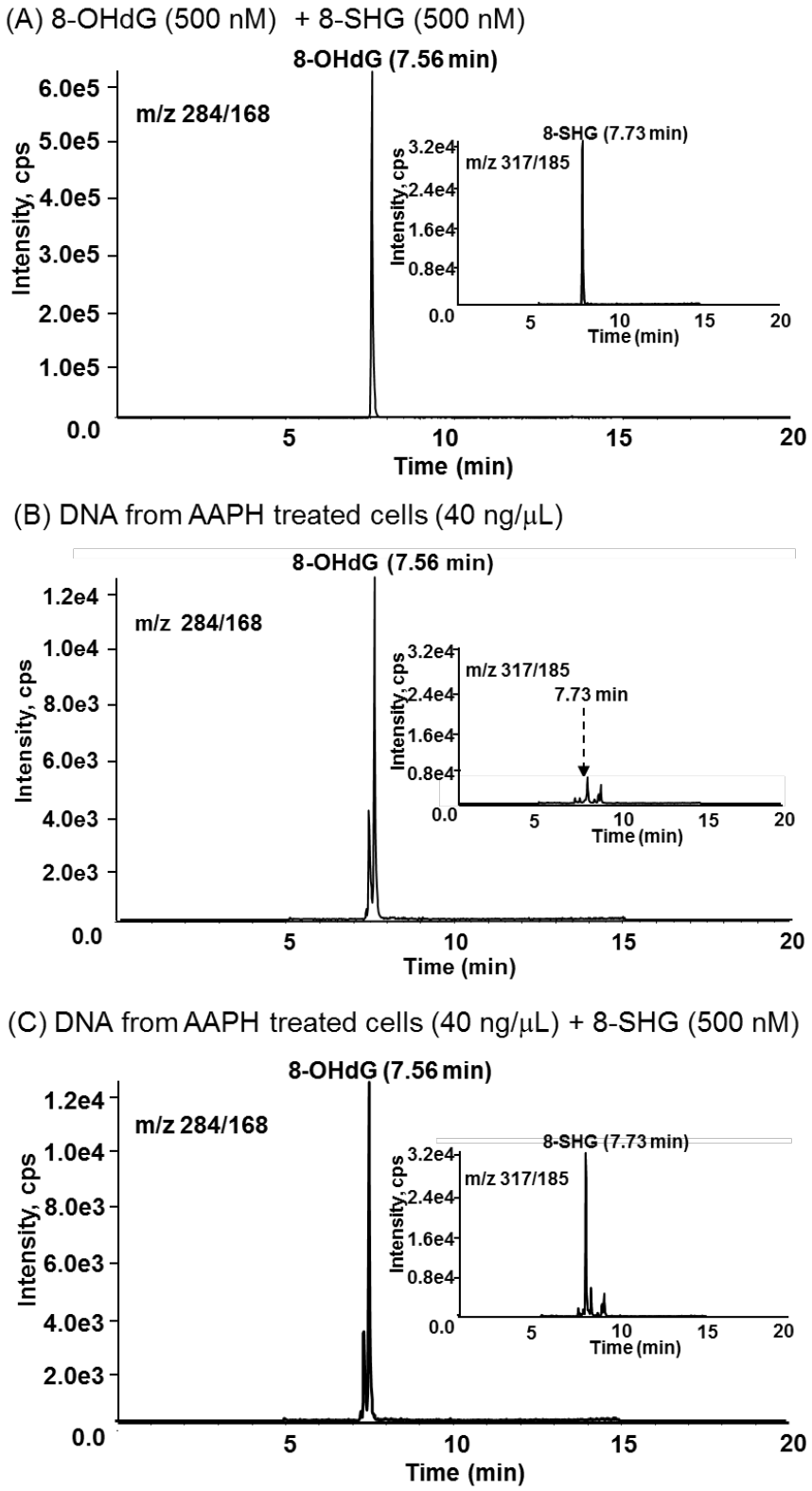


**Fig. appendix C-1. LC-MS/MS chromatogram of reference compounds using multiple reaction monitoring (MRM) mode.** (A) Simultaneous measurement of 8-OHdG (500 nM) and 8-SHG (500 nM). (B) Measurement of DNA sample (40 ng/μL) from the AAPH-treated cells without internal standard 8-SHG. (C) Simultaneous measurement of DNA sample (40 ng/μL) from the AAPH-treated cells with internal standard 8-SHG (500 nM). Typical result was shown from three independent experiments.

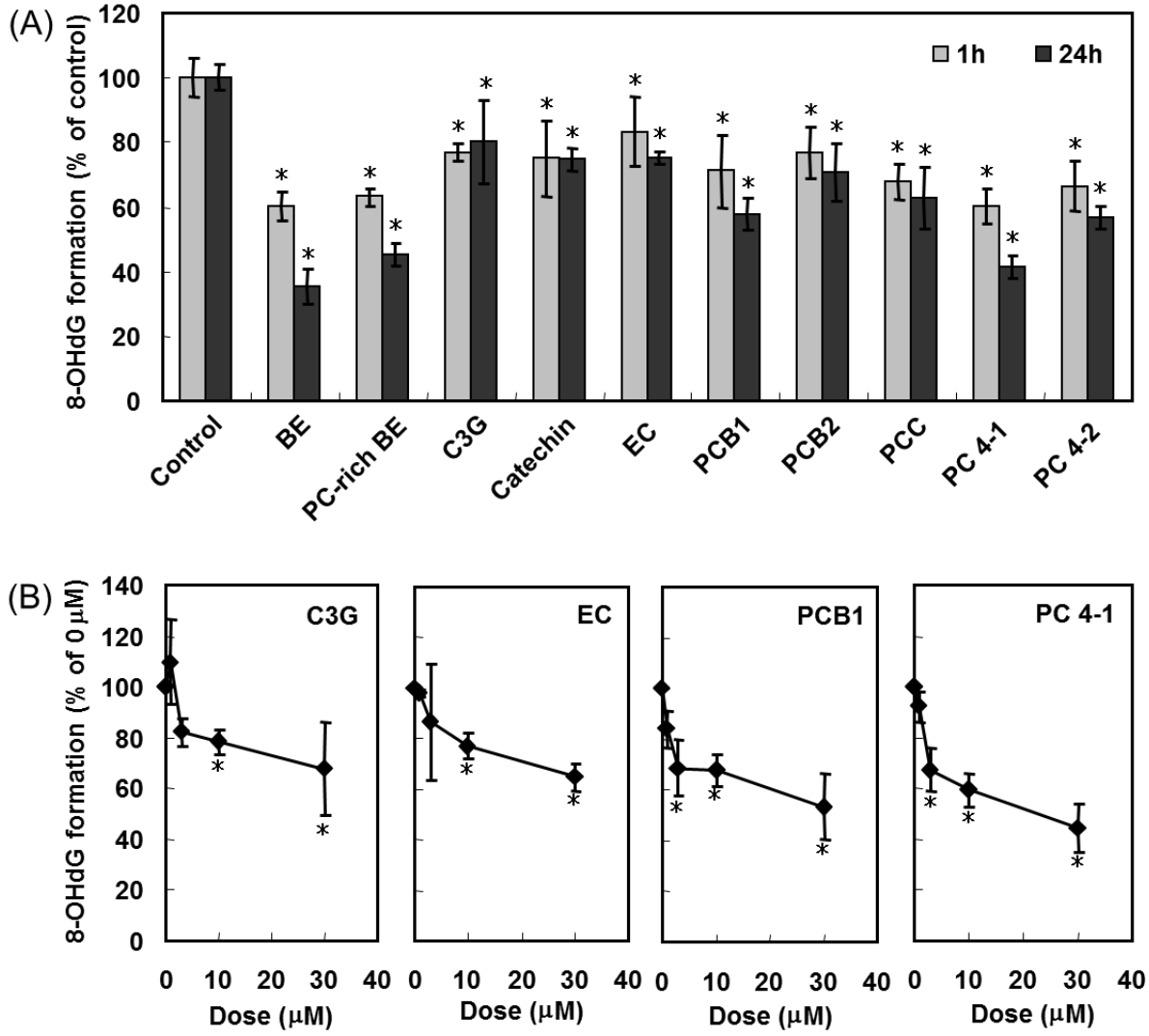


**Fig. appendix C-2. Inhibition effect of BE and its polyphenols on the AAPH-induced 8-OHdG formation in HepG2.** (A) BE and PC-rich BE at 4.85 μg/mL (equivalent to 10 μM C3G) and its polyphenols [C3G, (+)-catechin, EC, PCB1, PCB2, PCC, PC 4-1 and PC 4-2] at 10 μM each were treated to HepG2 cells for 1 or 24 h. (B) HepG2 cells were treated with C3G, EC, PCB1 and PC 4-1 (1, 5, 10 and 30 μM) for 1h. Then these cells were exposed to 25 mM AAPH for another 3 h. Nuclei were isolated from these cells. DNA was extracted, purified and subjected to LC-MS/MS analysis. Three independent experiments were performed. *Significantly different from the 8-OHdG level in DNA sample from the corresponding control cells by Dunnett's test ($p<0.05$).

**Preventive effect of BE and its polyphenols on 8-OHdG formation**

Using the established methods, the author examined the potential protective effect of BE and its polyphenols on the AAPH-induced 8-OHdG formation in HepG2. BE and PC-rich BE (4.85 μg/mL equivalent to 10 μM C3G), C3G, (+)-catechin, EC, PCB1, PCB2, PCC, PC 4-1 and PC 4-2 (10 μM each) significantly suppressed the formation of 8-OHdG by 60 %, 63%,

76%, 75%, 83%, 71%, 76%, 68%, 60% and 66% for 1 h treatment compared with control. Furthermore, they showed higher inhibitory effect for the 8-OHdG formation by 35%, 45%, 80%, 75%, 75%, 58%, 71%, 63%, 41% and 57% for 24 h treatment (Fig. appendix C-2A). The author further, examined the dose-dependent effect of C3G, EC, PCB1 and PC 4-1 against AAPH-induced 8-OHdG formation. As shown in Fig. appendix C-2B, these 4 compounds suppressed the formation of 8-OHdG dose-dependently. C3G and EC significantly decreased the AAPH-induced 8-OHdG formation from 10 μM, while PCB1 and PC 4-1 from 3 μM.

**DISCUSSION**

ROS-induced oxidative DNA damage has been implicated in mutagenesis and carcinogenesis. This oxidative DNA damage is mainly due to the formation of 8-OHdG [Sun, 1990]. Accumulation of 8-OHdG has been exhibited in several carcinoma cells [Miyake *et al*., 2004; Kondo *et al*., 2000; Musarrat *et al*., 1996]. Moreover, 8-OHdG in human muscle, lung and intestine are increasing with aging, smoking habit and chronic diseases, respectively [Hayakawa *et al*., 1991; Bizon *et al*., 2011; D'Inca *et al*., 2004]. Since 8-OHdG is one of the biomarkers to evaluate carcinogenesis, establishment of a high-sensitive analytical method for 8-OHdG is important. The method LC-MS/MS was found to be demonstrably as reliable, sensitive and preciseto detective 8-OHdG. Owing to the complex nature of the sample extract, it is not uncommon for each of these methods to require several labor-intensive clean-up steps prior to analysis. For these reasons, isotopic labeled internal standards are necessary for quantification by mass spectroscopy. However, isotopic labeled internal standards are not stable and would not be cost-effective for a clinical laboratory [Hu *et al*., 2004]. Recently, 8-SHG as a cheap and readily available non-isotopic internal standard was established for LC-MS/MS assay to detect 8-OHdG in urine [Crow *et al*., 2008]. In the present study, the author confirmed that 8-SHG also can be used as an internal standard to detection of 8-OHdG in the cells system (Fig. appendix C-1). Using this established method, the author found that PCs, C3G in addition to (+)-catechin and (-)-epicatechin inhibited the 8-OHdG formation. In particular, PCs presented a strong inhibition effect (Fig. appendix C-2). PCs in beverages, vegetable and fruits have been reported for the prevention activity against DNA damage. For example, PCs in wine protect against hydrogen peroxide-induced oxidative stress in Fao cells [Roig *et al*., 2002], PCs from apple juice reduce oxidative DNA damage *in vivo* [Barth *et al*., 2005] and grape seed PC extract protects DNA from $H_2O_2$-induced DNA lesions [Llopiz *et al*.,

2004]. Therefore, PCs should be the main active compounds in BE that suppressed ROS generator AAPH-induced DNA damage.

In appendix C, the author established a method to detect 8-OHdG formation in HepG2 cells. Using this method, it was found that PCs should be the main active compound in BE for suppression of 8-OHdG formation induced by a ROS generator AAPH. Therefore, BE effective material for functional food and a medicinal material for prevent oxidative DNA damage.

**APPENDIX DISCUSSION**

DNA damage can lead to degenerative diseases [Reardon *et al*., 1992; Lee and Blair, 2001]. Black soybean seed coats are rich in various polyphenols such as catechins, anthocyanins, procyanidins and other flavonoids. Constituents in black soybean seed coat extract (BE) have been reported to possess various physiological functions [Ignasius *et al*., 2009; Kanamoto *et a*l., 2011; Reardon *et al*., 1992; Lee and Blair, 2001]. In this study the author found that BE polyphenols prevent mutagens- and AAPH-induced DNA damage.

In appendix A, Ames test was used to estimate mutagenic and antimutigenic potential of BE polyphenols. The results showed that BE neither exhibited mutagenicity nor any cytotoxicity in *Salmonella tyhimurium*. On the contrary, C3G and procyanidins, the main BE polyphenols contributed to a strong antimutagenicity against B(a)P- and 4NQO-induced DNA damage. To clarify the underlying preventive mechanism of BE on mutagens-induced DNA damage, in appendix B, the author investigated the potential protective effects of polyphenolic extracts from black soybean seed coat on B(a)P-induced DNA damage in HepG2 cells and ICR mice. BE did not bring any genotoxicity to HepG2 cells. On the contrary, BE revealed a strong anti-genotoxicity against B(a)P-induced DNA damage in HepG2. The author found that procyanindins and C3G should be the main active compounds in BE for suppression of DNA damage. These suppression effects of DNA damage was involved in modulation of drug-metabolizing enzymes expression. Procyanidins and C3G were the main active compounds in BE for down-regulation of CYP1A1 and up-regulation of GSTs expressions, indicating that these compounds can protect B(a)P-induced DNA damage in the HepG2 cells effectively. Moreover, the author established a method to detect 8-OHdG in the cells system. Using this established method, the author investigated the BE and BE polyphenols on AAPH-induced the formation of 8-OHdG. The results showed that BE polyphenols suppressed 8-OHdG formation effectively. Procyanidins should be the main active compounds in BE for suppression of 8-OHdG formation induced by a ROS generator AAPH in HepG2 cells.

Together all, BE is not only a safe and effective material for functional food but also a medicinal material for prevention and treatment of chemical-induced DNA damage.

## References


Ahmed, M.H., Byrne, C.D., Current treatment of non-alcoholic fatty liver disease. *Diabetes Obes. Metab.,* 2009, 11, 188-195.

Akihisa, T., Motoi, T., Seki, A., Kikuchi, T., Fukatsu, M., Tokuda, H., Suzuki, N., Kimura, Y., Cytotoxic Activities and Anti-Tumor-Promoting Effects of Microbial Transformation Products of Prenylated Chalcones from Angelica keiskei. *Chem. Biodivers.,* 2012, 9, 318-330.

Akihisa, T., Tokuda, H., Hasegawa, D., Ukiya, M., Kimura, Y., Enjo, F., Suzuki, T., Nishino, H., Chalcones and other compounds from the exudates of Angelica keiskei and their cancer chemopreventive effects. *J. Nat. Prod.,* 2006, 69, 38-42.

Alappat, L., Awad, A.B., Curcumin and obesity: evidence and mechanisms. *Nutr. Rev.,* 2010, 68, 729-738.

Ames, B.N., Endogenous oxidative DNA damage, aging, and cancer. *Free. Radic. Res. Commum.,* 1989, 7, 121-128.

Ames, B.N., Gold, L.S., The causes and prevention of cancer: The role of environment. *Biotherapy*., 1998, 11, 205-220.

Antonescu, C.N., Huang, C., Niu, W., Liu, Z., Eyers, P.A., Heidenreich, K.A., Bilan, P.J., Klip, A., Reduction of insulin-stimulated glucose uptake in L6 myotubes by the protein kinase inhibitor SB203580 is independent of p38MAPK activity. *Endocrinology,* 2005, 146**,** 3773-3781.

Assifi, M.M., Suchankova, G., Constant, S., Prentki, M., Saha, A.K., Ruderman, N.B., AMP-activated protein kinase and coordination of hepatic fatty acid metabolism of starved/carbohydrate-refed rats. *Am. J. Physiol. Endocrinol. Metab.,* 2005, 289, 794-800.

Azevedo, L., Gomes, J.C., Stringheta, P.C., Gontijo, A.M.M.C., Padovani, C.R., Ribeiro, L.R., Salvadori, D.M.F., Black bean (*Phaseolus vulgaris L.*) as a protective agent against DNA damage in mice. *Food Chem. Toxicol*., 2003, 41, 1671-1676.

Bandgar, B.P., Gawande, S.S., Bodade, R.G., Gawande, N.M., Khobragade, C.N., Synthesis and biological evaluation of a novel series of pyrazole chalcones as anti-inflammatory, antioxidant and antimicrobial agents. *Bioorg. Med. Chem*., 2009, 17, 8168-73.

Barth, S.W., Fahndrich, C., Bub, A., Dietrich, H., Watzl, B., Will, F., Briviba, K.,

Rechkemmer, G., Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats. *Carcinogenesis,* 2005, 26, 1414-1421.

Birari, R.B., Gupta, S., Mohan, C.G., Bhutani, K.K., Antiobesity and lipid lowering effects of *Glycyrrhiza* chalcones: Experimental and computational studies. *Phytomedicine,* 2011, 18, 795-801.

Bizon, A., Milnerowicz-Nabzdyk, E., Zalewska, M., Zimmer, M., Milnerowicz, H., Changes in pro/antioxidant balance in smoking and non-smoking pregnant women with intrauterine growth restriction. *Reprod. Toxico.,* 2011, 32, 360-367.

Bleich, S., Cutler, D., Murray, C., Adams, A., "Why is the developed world obese?" *Annu. Rev. Public. Health*, 2008, 29, 273-295.

Bleich, S., Cutler, D., Murray, C., Adams, A., *Why is the developed world obese?* National Bureau of Economic Research. 2007, (No. w12954).

Bogardus, C., Lillioja, S., Mott, D., Reaven, G.R., Kashiwagi, A., Foley, J., Relationship between obesity and maximal insulin-stimulated glucose uptake *in vivo* and *in vitro* in Pima Indians. *Clin. Inves.*, 1984, 73, 800-805.

Bonadonna, R., Groop, L., Kraemer, N., DeFronzo, R.A., Obesity and insulin resistance in man: a dose response study. *Metabolism*., 1990, 39, 452-59.

Bonen A., Chabowski A., Luiken J.A., Glatz, J.F., Is membrane transport of FFA mediated by lipid, protein, or both? Mechanisms and regulation of protein-mediated cellular fatty acid uptake: molecular, biochemical, and physiological evidence. *Physiology (Bethesda),* 2007, 22, 15-29.

Bonnefont, J.P., Djouadi, F., Prip-Buus, C., Gobin, S., Munnich, A., Bastin, J., Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. *Mol. Aspects Med.*, 2004, 25(5), 495-520.

Boss, O., Muzzin, P., Giacobiono, J.P., The uncoupling proteins, a review. *Eur. J. Endocrinol.*, 1998, 139, 1-9.

Bost, F., Aouadi, M., Caron, L., Binétruy, B., The role of MAPKs in adipocyte differentiation and obesity. *Biochimie*., 2005, 87, 51-56.

Bost, F., Aouadi, M., Caron, L., Even, P., Belmonte, N., Prot, M., Binétruy, B., The extracellular signal–regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis. *Diabetes*., 2005, 54, 402-411.

Bouzakri, K., Koistinen, H.A., Zierath, J.R., Molecular mechanisms of skeletal muscle insulin resistance in type 2 diabetes. *Curr. Diabetes. Rev.,* 2005, 1, 167-174.

Bradbury, M.W., Lipid metabolism and liver inflammation. I. Hepatic fatty acid uptake: possible role in steatosis. *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2006, 290, 194-198.

Braissant, O., Wahli, W., Differential expression of peroxisome proliferator-activated receptor-α,-β, and-γ during rat embryonic development. *Endocrinology*, 1998, 139, 2748-2754.

Bray, G.A., Medications for Obesity: Mechanisms and Applications. *Clin. Chest. Med.,* 2009, 3, 525-538.

Brown, M.S., Goldstein, J.L., The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell*, 1997, 89, 331-340.

Browning, J.D., Horton, J.D., Molecular mediators of hepatic steatosis and liver injury. *J. Clin. Invest.*, 2004, 114(2), 147–52.

Buening, M.K., Wislocki, P.G., Levin, W., Yagi, H., Thakker, D.R., Akagi, H., Koreeda, M., Jerina, D.M., Conney, A.H., Tumorigenicity of the optical enantiomers of the diastereomeric benzo[a]pyrene 7,8-diol-9,10-epoxides in newborn mice: exceptional activity of (+)-7beta,8alpha-dihydroxy-9alpha,10alpha-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. *Proc. Natl. Acad. Sci. U. S. A.,* 1978, 11, 5358-5361.

Burgermeister, E., Chuderland, D., Hanoch, T., Meyer, M., Liscovitch, M., Seger, R., Interaction with MEK causes nuclear export and downregulation of peroxisome proliferator-activated receptor γ. *Mol. Cell. Bio.*, 2007, 27, 803-17.

Camp, H.S., Tafuri, S.R., Leff, T., c-Jun N-terminal kinase phosphorylates peroxisome proliferator-activated receptor-gamma1 and negatively regulates its transcriptional activity. *Endocrinology*, 1999, 140, 392-397.

Cao, Z., Umek, R.M., McKnight, S.L., Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes. Dev.*, 1991, 5, 1538-1552.

Carling, D., The AMP-activated protein kinase cascade--a unifying system for energy control. *Trends Biochem. Sci.,* 2004, 29, 18-24.

Celotti, L., Ferraro, P., Furlan, D., Zanesi, N., Pavanello, S., DNA repair in human lymphocytes treated in vitro with (+)-anti- and (+/-)-syn-benzo[a]pyrene diolepoxide. *Mutat. Res.*, 1993, 294, 117-126.

Chen, H., Mo, X., Yu, J., Huang, Z., Alpinetin attenuates inflammatory responses by interfering toll-like receptor 4/nuclear factor kappa B signaling pathway in lipopolysaccharide-induced mastitis in mice. *Int. Immunopharmacol*., 2013, 17, 26-32.

Cheung, O., Sanyal, A.J., Abnormalities of lipid metabolism in nonalcoholic fatty liver disease. *Semin. Liver. Dis.,* 2008, 28, 351–359.

Coditz, G.A., Branch, L.G., Lipnick, R.J., Willett, W.C., Rosner, B., Posner, B.M., Hennekens, C.H., Increased green and yellow vegetable intake and lowered cancer deaths in an elderly population. *Am. J. Clin. Nutr.,* 1985, 4, 132-136.

Conney, A.H., Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. clowes memorial lecture. *Cancer. Res.,* 1982, 42, 4875-4917.

Cook, N.C., Samman, S., Flavonoids chemistry, metabolism, cardioprotective effects and dietary resources. *J. Nutr. Biochem.*, 1996, 7, 66-76.

Crow, B., Bishop, M., Kovalcik, K., Norton, D., George, J., Bralley, J.A., A simple and cost effective method for the quantification of 8-hydroxy-2'-deoxyguanosine from urine using liquid chromatography tandem mass spectrometry. *Biomed. Chromatogr.*, 2008, 22, 394-401.

Darroudi, F., Natarajan, A.T., Metabolic activation of chemicals to mutagenic carcinogens by human hepatoma microsomal extracts in Chinese hamster ovary cells (*in vitro*). *Mutagenesis*, 1993, 8, 11-15.

Deak, M., Clifton, A.D., Lucocq, J.M., Alessi, D.R., Mitogen-and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *The. EMBO. Journal*. 1998, 17(15), 4426-4441.

DeFronzo, R.A., The triumvirate: ß-cell, muscle, liver: a collusion responsible for NIDDM. *Diabetes*., 1988, 37, 667-687.

Denison, M.S., Vella, L.M., Okey, A.B., Hepatic Ah receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Partial stabilization by molybdate. *J. Biol. Chem.,* 1986, 261, 10189–10195.

Despres, J.P., Is visceral obesity the cause of the metabolic syndrome? *Ann. Med*., 2006, 38, 52-63.

Després, J.P., Lemieux, I., Bergeron, J., Pibarot, P., Mathieu, P., Larose, E., Rodés-Cabau, J., Bertrand, O.F., Poirier, P., Abdominal obesity and the metabolic syndrome: contribution

to global cardiometabolic risk. *Arterioscler. Thromb. Vasc. Boil.,* 2008, 28(6), 1039-1049.

Devasagayam, T.P., Tilak, J.C., Boloor, K.K., Sane, K.S., Ghaskadbi, S.S., Lele, R.D., Free Radicals and Antioxidants in Human Health: Current Status and Future Prospects. *J. Assoc. Physicians. Iindia.,* 2004, 52, 794-804.

Dimmock, J.R. Elias, D.W., Beazely, M.A., Kandepu, N.M., Bioactivities of chalcones. *Curr. Med, chem.,* 1999, 6, 1125-1150.

D'Inca, R., Cardin, R., Benazzato, L., Angriman, I., Martines, D., Sturniolo, G.C., Oxidative DNA damage in the mucosa of ulcerative colitis increases with disease duration and dysplasia. *Inflamm. Bowel. Dis.,* 2004, 10, 23-27.

Donnelly, K.L., Smith, C.I., Schwarzenberg, S.J., Jessurun, J., Boldt, M.D., Parks, E.J., Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest*., 2005, 115(5), 1343-1351.

Drahushuk, A.T., McGarrigle, B.P., Larsen, K.E., Stegeman, J.J., Olson, J.R., Detection of CYP1A1 protein in human liver and induction by TCDD in precision-cut liver slices incubated in dynamic organ culture. *Carcinogenesis*, 1998, 19, 1361-1368.

Eaton, S.B., The ancestral human diet: what was it and should it be a paradigm for contemporary nutrition? *Proc. Nutr. Soc.,* 2006, 65, 1-6.

Eckel, R.H., Grundy, S.M., Zimmet, P.Z., The metabolic syndrome. *The Lancet.*, 2005, 365, 1415-1428.

Ehrlich, V., Darroudi, F., Uhl, M., Steinkellner, H., Gann, M., Majer, B.J., Eisenbauer, M., Knasmuller, S., Genotoxic effects of ochratoxin A in human-derived hepatoma (HepG2) cells. *Food Chem. Toxicol.,* 2002, 40, 1085-1090.

Ejaz, A., Wu, D., Kwan, P., Meydani, M. Curcumin inhibits adipogenesis in 3T3-L1 adipocytes and angiogenesis and obesity in C57/BL mice. *J. Nutr.,* 2009, 139, 919-925.

Elferink, C.J., Gasiewicz, T.A., Whitlock, Jr.J.P., Protein-DNA interactions at a dioxin-responsive enhancer. Evidence that the transformed Ah receptor is heteromeric. *J. Biol. Chem.,* 1990, 265, 20708–20712.

Engelman, J.A., Lisanti, M.P., Scherer, P.E., Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis. *J. Biol. Chem.,* 1998, 273, 32111-32120.

Enoki, T., Ohnogi, H., Nagamine, K., Kudo, Y., Sugiyama, K., Tanabe, M., Kobayashi, E., Sagawa, H., Kato, I., Antidiabetic activities of chalcones isolated from a Japanese Herb,

Angelica keiskei. *J. Agric. Food Chem,*. 2007, 55, 6013-6017.

Fabbrini, E., Magkos, F., Mohammed, B.S., Pietka, T., Abumrad, N.A., Patterson, B.W., Okunade, A., Klein, S., Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proc. Natl. Acad. Sci. U. S. A.*, 2009, 106, 15430-15435.

Faria, A., Monteiro, R., Azevedo, I., Calhau, C., pomegranate juice effects on cytochrome P450s expression: in vivo studies. *J. Med. Food*, 2007, 10, 643-649.

Ferguson, B.S., Nam, H., Hopkins, R.G., Morrison, R.F., Impact of reference gene selection for target gene normalization on experimental outcome using real-time qRT-PCR in adipocytes. *Plos One,* 2010. *5*, e15280.

Fischer, M., You, M., Matsumoto, M., Crabb, D.W., Peroxisome proliferator-activated receptor alpha (PPARalpha) agonist treatment reverses PPARalpha dysfunction and abnormalities in hepatic lipid metabolism in ethanol-fed mice. *J. Biol. Chem.,* 2003, 278(30), 27997-8004.

Foretz, M., Ancellin, N., Andreelli, F., Saintillan, Y., Grondin, P., Kahn, A., Thorens, B., Vaulont, S., Viollet, B., Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. *Diabetes.*, 2005, 54, 1331-1139.

Freedman, N.D., Park, Y., Subar, A.F., Hollenbeck, A.R., Leitzmann, M.F., Schatzkin, A., Abnet, C.C., Fruit and vegetable intake and head and neck cancer risk in a large United States prospective cohort study. *Epidemiology*, 2008, 112, 2330-2336.

Freytag, S.O., Paielli, D.L., Gilbert, J.D., Ectopic expression of the CCAAT/enhancer-binding protein alpha promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes. Dev.,* 1994, 8, 1654-1663.

Friedman, J.M. Obesity in the new millennium. *Nature*, 2000, 404, 632-634.

Frova, C., Glutathione transferases in the genomics era: new insights and perspectives. *Biomol. Engin.*, 2006, 23, 149-169.

Fukuda, I., Tsutsui, M., Yoshida, T., Toda, T., Tsuda, T., Ashida, H., Oral toxicological studies of black soybean (*Glycine max*) hull extract: Acute studies in rats and mice, and chronic studies in mice. *Food Chem. Toxicol.*, 2011, 49, 3272-3278.

Furuyashiki, T., Nagayasu, H., Aoki, Y., Bessho, H., Hashimoto, T., Kanazawa, K., Ashida, H., Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARγ2 and C/EBPα in 3T3-L1 cells. *Biosci. Biotechnol. Biochem.,* 2004, 68, 2353-2359.

Galiegue-Zouitina, S., Bailleul, B., Ginot, Y.M., Perly, B., Vigny, P., Loucheux-Lefebvre, M.H., *N*2-Guanyl and *N*6-adenyl arylation of chicken erythrocyte DNA by the ultimate carcinogen of 4-nitroquinoline-1-oxide. *Cancer Res*., 1986, 46, 1858-1863.

Gao, Y., Zhou, Y., Xu, A., Wu, D., Effects of an AMP-activated protein kinase inhibitor, compound C, on adipogenic differentiation of 3T3-L1 cells. *Biol. Pharm. Bull*., 2008, 31, 1716-1722.

Gasbarrini, G., Vero, V., Miele, A., Forgione, A., Hernandez, A. P., Greco, A. V.,Gasbarrini, A., Grieco, A., Nonalcoholic fatty liver disease: defining a common problem. *Eur. Rev. Med. Pharmacol. Sci*., 2005, 5, 253.

Gastaldelli, A., Miyazaki, Y., Pettiti, M., Matsuda, M., Mahankali, S., Santini, E., DeFronzo, R.A., Ferrannini, E. Metabolic effects of visceral fat accumulation in type 2 diabetes. *J. Clin. Endocrinol. Metab*., 2002, 87(11), 5098-5103.

Gehart, H., Kumpf, S., Ittner, A., Ricci, R., MAPK signalling in cellular metabolism: stress or wellness? *EMBO reports*., 2010, 11, 834-840.

Gelboin, H.V., Benzo[a]pyrene metabolism, activation, and carcinogenesis: Role and regulation of mixed-function oxidases and related enzymes. *Physiol. Rev.,* 1980, 60, 1107-1166.

Goldstein, D.J., Beneficial health effects of modest weight loss. *Int. J. Obes. Relat. Metab. Disord.*, 1992, 16, 397-415.

Goldstein, J.L., Brown, M.S., From fatty streak to fatty liver: 33 years of joint publications in theJCI. *J. Clin. Invest.,* 2008, 118, 1220-1222.

Gonzalez-Castejon, M., Rodriguez-Casado, A., Dietary phytochemicals and their potential effects on obesity: a review. *Pharmacol. Res.,* 2011, 64, 438-455.

Gourineni, V., Shay, N.F., Chung, S., Sandhu, A.K., Gu, L., Muscadine grape (*Vitis rotundifolia*) and wine phytochemicals prevented obesity-associated metabolic complications in C57BL/6J mice. *J. Agric. Food Chem.,* 2012, 60,,7674-7681.

Green, H., Kehinde, O., An established preadipose cell line and its differentiation in culture II. *Cell*, 1975, 5, 19-27.

Gupta, S., Saha, B., Giri, A.K., Comparative antimutagenic and anticlastogenic effects of green tea and black tea: a review. *Mutat. Res.,* 2002, 512, 37-65.

Hajer, G.R., Van Haeften, T.W., Visseren, F.L., Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *Eur. Heart. J.*, 2008, 29, 2959-2971.

Hajri T., Abumrad N.A., Fatty acid transport across membranes: relevance to nutrition and metabolic pathology. *Annu. Rev. Nutr.,* 2002, 22, 383-415.

Halliwell, B., Free radicals and antioxidants: a personal view. *Nutr. Rev.*, 1994, 52, 253-265.

Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H., Poutanen, K. Impact of dietary polyphenols on carbohydrate metabolism. *Int. J. Mol. Sci.,* 2010, 11, 1365-402.

Hardie, D.G., AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat. Rev. Mol. Cell. Biol.,* 2007, 8, 774–785.

Harvey, R.G., Historical Overview of Chemical Carcinogenesis. *Chemical. Carcinogenesis,* 2011, 1-26.

Hattemer-Frey, H.A., Travis, C.C., Benzo(a)pyrene: Environmental partitioning and human exposure. *Toxicol. Ind. Health*, 1991, 7, 141-157.

Hawley, S.A., Boudeau, J., Reid, J.L., Mustard, K.J., Udd, L., Makela, T.P., Alessi, D.R., Hardie, D.G., Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol.,* 2003, 2(4), 28.

Hayakawa, M., Torii, K., Sugiyama, S., Tanaka, M., Ozawa, T., Age-associated accumulation of 8-hydroxydeoxyguanosine in mitochondrial DNA of human diaphragm. *Biochem. Biophys. Res. Commun.,* 1991, 179, 1023-1029.

He, W., Li, Y., Xue, C., Hu, Z., Chen, X., Sheng, F., Effect of Chinese medicine alpinetin on the structure of human serum albumin. *Bioorg. Med. Chem.*, 2005, 13, 1837-45.

Helbock, H.J., Beckman, K.B., Ames, B.N., 8-Hydroxydeoxyguanosine and 8-hydroxyguanine as biomarkers of oxidative DNA damage. *Meth. Enaymol.,* 1999, 300, 156-166.

Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C.Z., Uysal, K.T., Maeda, K., Karin, M., Hotamisligil, G.S., A central role for JNK in obesity and insulin resistance. *Nature,* 2002, 420, 333-336.

Holt, R.R., Heiss, C., Kelm, M., Keen, C.L., The potential of flavanol and procyanidin intake to influence age-related vascular disease. *J. Nutr. Gerontol. Geriatr.,* 2012, 31, 290-323

Hotamisligil, G.S., Inflammation and metabolic disorders. *Nature,* 2006, 444(7121), 860-867.

Hou, Z., Qin, P., Ren, G., Effect of anthocyanin-rich extract from black rice (*Oryza sativa L. Japonica*) on chronically alcohol-induced liver damage in rats. *J. Agric. Food Chem.,*

2010, 58, 3191-3196.

Hu, C.W., Wu, M.T., Chao, M.R., Pan, C.H., Wang, C.J., Swenberg, J.A., Wu, K.Y., Comparison of analyses of urinary 8-hydroxy-2′- deoxyguanosine by isotope-dilution liquid chromatography with electrospray tandem mass spectrometry and by enzyme-linked immunosorbent assay. *Rapid. Commun. Mass. Sp.,* 2004, 18, 505-510.

Hu, E., Kim J.B., Sarraf, P., Spiegelman, B.M., Inhibition of adipogenesis through MAPkinase-mediated phosphorylation of PPARgamma. *Science*, 1996, 274, 2100-2103.

Hu, F.B., Manson, J.E., Stampfer, M.J., Colditz, G., Liu, S., Solomon, C.G., Willett, W.C., Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. *N. Engl. J. Med*., 2001, 345, 790-797.

Hu, X., Srivastava, S.K., Xia, H., Awasthi, Y.C., Singh, S.V., An alpha class mouse glutathione *S*-transferase with exceptional catalytic efficiency in the conjugation of glutathione with 7b,8a-dihydroxy-9a,10a-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. *J. Biol. Chem.*, 1996, 271, 32684-32688.

Huang, C., Zhang, Y., Gong, Z., Sheng, X., Li, Z., Zhang, W., Qin, Y., Berberine inhibits 3T3-L1 adipocyte differentiation through the PPARγ pathway. *Biochem. Biophys. Res. Commun.*, 2006, 348, 571-578.

Huang, H.C., Chang, J.H., Tung, S.F., Wu, R.T., Foegh, M.L., Immunosuppressive e.ect of emodin, a free radical generator. *Eur.J. Pharmacol.*, 1998, 211, 359-364.

Hussain, S.P., Harris C.C., Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. *Cancer Res.*, 1998, 58, 4023-4037.

Hwang, J.H., Stein, D.T., Barzilai, N., Cui, M.H., Tonelli, J., Kishore, P., Hawkins, M., Increased intrahepatic triglyceride is associated with peripheral insulin resistance: in vivo MR imaging and spectroscopy studies. *Am. J. Physiol-Endoc. M.,* 2007, 293, 1663–1669.

Hwang, Y.P., Choi, J.H., Yun, H.J., Han, E.H., Kim, H.G., Kim, J.Y., Park, B.H., Khanal, T., Choi, J.M., Chung, Y.C., Jeong, H.G., Anthocyanins from purple sweet potato attenuate dimethylnitrosamine-induced liver injury in rats by inducing Nrf2-mediated antioxidant enzymes and reducing COX-2 and iNOS expression. *Food Chem. Toxicol.,* 2011, 49, 93-99.

Ignasius, R.A., Mary, A., Umar, S., Prihati, S.N., *In vitro* antioxidant activity of anthocyanins of black soybean seed coat in human low density lipoprotein (LDL). *Food Chem.*, 2009, 112, 659-663.

Inagaki, S., Morimura, S., Shigematsu, T., Kida, K., Akutagawa, H., Apoptosis induction by vinegar produced from boiled extract of black soybeans in human monoblastic leukemia U937 cells: Difference in sensitivity to cell toxicity compared to normal lymphocytes. *Food Sci. Techno. Res.*, 2005, 11, 311-317.

James, P., Whitlock, J.R., Induction of cytochrome P4501A1. *Ann. Rev. Pharmacol. Toxicol.*, 1999, 39, 103-125.

Jiang, H., Gelhaus, S.L., Mangal, D., Harvey, R.G., Blair, I.A., Penning, T.M., Metabolism of Benzo(a)pyrene in Human Bronchoalveolar H358 Cells Using Liquid Chromatography-Mass Spectrometry. *Chem. Res. Toxicol.*, 2007, 20, 1331-1341.

Julia Peterson, M.S., Johanna Dwyer, DSc.RD., Flavonoids: Dietary occurrence and biochemical activity. *Nutri. Reserch.*, 1998, 18, 1995-2018.

Kaestner, K.H., Christy, R.J., Lane, M.D., Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. *Proc. Natl. Acad. Sci. U. S. A.*, 1990, 87, 251-255.

Kanamoto, Y., Yamashita, Y., Nanba, F., Yoshida, T., Tsuda, T., Fukuda, I., Nakamura-Tsuruta, S., Ashida, H., A black soybean seed coat extract prevents obesity and glucose intolerance by up-regulating uncoupling proteins and down-regulating inflammatory cytokines in high-fat diet-fed mice. *J. Agric. Food Chem.*, 2011, 59, 8985-8993.

Kanazawa, K., Uehara, M., Yanagitani, H., Hashimoto, T., Bioavailable flavonoids to suppress the formation of 8-OHdG in HepG2 cells. *Arch. Biochem. Biophys.*, 2006, 455, 197-203.

Kang, O.H., Kim, S.B., Seo, Y.S., Joung, D.K., Mun, S.H., Choi, J.G., Kwon, D.Y., Curcumin decreases oleic acid-induced lipid accumulation via AMPK phosphorylation in hepatocarcinoma cells. *Eur. Rev. Med. Pharmacol. Sci.*, 2013, 17(19), 2578-2586.

Kaul, K., Tarr, J.M., Ahmad, S.I., Kohner, E.M., Chibber, R., Introduction to diabetes mellitus. *Adv. Exp. Med. Biol.*, 2012, 771, 1-11.

Kawabata, K., Sawada, K., Ikeda, K., Fukuda, I., Kawasaki, K., Yamamoto, N., Ashida, H., Prenylated chalcones 4hydroxyderricin and xanthoangelol stimulate glucose uptake in skeletal muscle cells by inducing GLUT4 translocation. *Mol. Nutr. Food Res.*, 2011, 55, 467-475.

Kawaguchi, T., Osatomi, K., Yamashita, H., Kabashima, T., Uyeda, K., Mechanism for fatty acid "sparing" effect on glucose-induced transcription: regulation of

carbohydrate-responsive element-binding protein by AMP-activated protein kinase. *J. Biol. Chem.,* 2002, 277, 3829-2835.

Kawai, Y., Nishikawa, T., Shiba, Y., Saito, S., Murota, K., Shibata, N., Kobayashi, M., Terao, J., Macrophage as a target of quercetin glucuronides in human atherosclerotic arteries: Implication in the anti-atherosclerotic mechanism of dietary flavonoids, *J.B.C.,* 2008, 283, 9424-9434.

Kim, E.H., Surh, Y.J., 15-Deoxy-Delta12,14-prostaglandin J2 as a potential endogenous regulator of redox-sensitive transcription factors. *Biochem. pharmacol.,* 2006, 72, 1516-28.

Kim, H.J., Higashimori, T., Park, S.Y., Choi, H., Dong, J., Kim, Y.J., Noh, H.L., Cho, Y.R., Cline, G., Kim, Y.B., Kim, J.K., Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action *in vivo*. *Diabetes.,* 2004, 53, 1060-1067.

Kim, J., Han, D.C., Kim, J.M., Lee, S.Y., Kim, S.J., Woo, J.R., Lee, J.W., Jung, S.K., Yoon, K.S., Cheon, H.G., Kim, S.S., Hong, S.S., Hong, S.H., Kwon, B.M., PPAR γ partial agonist, KR-62776, inhibits adipocyte differentiation via activation of ERK. *Cell Mol. Life. Sci.,* 2009, 66, 1766-81.

Kim, J.M., Kim, J.S., Yoo, H., Choung, M.G., Sung, M.K., Effects of black soybean [*Glycine max* (L.) Merr.] seed coats and its anthocyanidins on colonic inflammation and cell proliferation in *vitro* and in *vivo*. *J. Agric. Food Chem.*, 2008, 56, 8427-8433.

Kim, O.K., Kung, S.S., Park, W.B., Lee, M.W., Ham, S.S., The nutritional components of aerial whole plant and juice of Angelica keiskei., *Korean J. Food Sci. Technol.,* 1992, *25,* 592–596.

Kim, S.J., Jung, J.Y., Kim, H.W., Park, T., Anti-obesity effects of Juniperus chinensis extract are associated with increased AMP-activated protein kinase expression and phosphorylation in the visceral adipose tissue of rats. *Biol. Pharm. Bull.,* 2008, 31, 1415-1421.

Kimura, Y., Taniguchi, M., Baba, K. Antitumor and antimetastatic activities of 4hydroxyderricin isolated from Angelica keiskei roots. *Planta. Med.,* 2004, 70, 211-219.

Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A., Evans, R.M., Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature,* 1992, 358, 771-774.

Knoops, K.T., de Groot, L.C., Kromhout, D., Perrin, A.E., Moreiras-Varela, O., Menotti, A., van Staveren, W.A., Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women. *J. A. M. A.*, 2004, 292, 1433-1439.

Kohjima, M., Higuchi, N., Kato, M., Kotoh, K., Yoshimoto, T., Fujino, T., Yada, M., Yada, R., Harada, N., Enjoji, M., Takayanagi, R., Nakamuta, M., SREBP-1c, regulated by the insulin and AMPK signaling pathways, plays a role in nonalcoholic fatty liver disease. *Int. J. Mol. Med.*, 2008, 21, 507–511.

Kondo, S., Toyokuni, S., Tanaka, T., Hiai, H., Onodera, H., Kasai, H., Imamura, M., Overexpression of the hOGGI gene and high 8-hydroxy-20-deoxyguanosine (8-OhdG ) lyase activity in human colorectal carcinoma: regulation mechanism of the 8-OHdG level in DNA. *Clin. Cancer Res.*, 2000, 6, 1394-1400.

Korenblat, K.M., Fabbrini, E., Mohammed, B.S., Klein, S., Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. *Gastroenterology*, 2008, 134, 1369–1375.

Koteish, A., Diehl, A.M., Animal models of steatosis Semin. *Liver Dis*., 2001, 21, 89–104.

Kotzka, J., Müller-Wieland, D., Roth, G., Kremer, L., Munck, M., Schürmann, S., Knebel, B., Krone, W., Sterol regulatory element binding proteins (SREBP)-1a and SREBP-2 are linked to the MAP-kinase cascade. *J. Lip. Res*., 2000, 41(1), 99.

Kozawa, M., Morita, N., Baba, K., Hata, K., The structure of xanthoangelol, a new chalcone from the roots of *Angelica keiskei* KOIDZUMI (Umbelliferae). *Chem. Pharm. Bull.*, 1977, 25, 515–516.

Kuroyanagi, M., Noro, T., Fukushima, S., Aiyama, R., Ikuta, A., Itokawa, I., Morita, M., Studies of constituents of seeds of *Alpinia katsumadai* Hayata. *Chem. Pharm. Bull.*, 1983, 31, 1544-50.

Lage, R., Diéguez, C., Vidal-Puig, A., López, M., AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends. Mol. Med*., 2008, 14, 539-549.

Lakka, H.M., Laaksonen, D.E., Lakka, T.A., Niskanen, L.K., Kumpusalo, E., Tuomilehto, J., Salonen, J.T., The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. *J. A. M. A.*, 2002, 288, 2709–2716.

Lee, C.H., Olson, P., Evans, R.M., Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. *Endocrinology*, 2003, 144, 2201-2207.

Lee, S.E., Shin, H.T., Hwang, H.J., Kim, J.H., Antioxidant activity of extracts from Alpinia

katsumadai seed. *Phytother. Res.*, 2003, 17, 1041-1047.

Lee, S.H., Blair, I.A., Oxidative DNA Damage and Cardiovascular Disease, Trends Cardiovasc. *Med.*, 2001, 11, 148-155.

Lee, W.J., AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARα and PGC-1. *Biophys. Res. Commun.*, 2006, 340, 281-295.

Lefterova, M. I., Lazar, M. A., New developments in adipogenesis. *Trends. Endocrinol. Metab.*, 2009, 20,107-114.

Li, Y., Xu, S., Mihaylova, M.M., Zheng, B., Hou, X., Jiang, B., Park, O., Luo, Z., Lefai, E., Shyy, J.Y., Gao, B., Wierzbicki, M., Verbeuren, T.J., Shaw, R.J., Cohen, R.A., Zang, M., AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell. Metabolism.*, 2011, 13(4), 376–388.

Ligibel, J., Obesity and breast cancer. *Oncology (Williston Park)*, 2011, 25, 994-1000.

Lii, C.K., Huang, C.Y., Chen, H.W., Chow, M.Y., Lin, Y.R., Huang, C.S., Tsai, C.W., Diallyl trisulfide suppresses the adipogenesis of 3T3-L1 preadipocytes through ERK activation. *Food. Chem. Toxicol.*, 2012, 50, 478-484.

Lii, K.C., Tsai, C.W., Wu, C.C., Garlic allyl sulfides display differential modulation of rat cytochrome P450 2B1 and the placental form glutathione S-transferase in various organs. *J. Agric. Food. Chem.*, 2006, 54, 5191-5196.

Lim, S.S., Jung, S.H., Ji, J., Shin, K.H., Keum, S.R., Synthesis of flavonoids and their effects on aldose reductase and sorbitol accumulation in streptozotocin‐induced diabetic rat tissues. *J. Pharm. Pharmacol*., 2001, 53, 653-668.

Lin, C.T., Senthil Kumar, K.J., Tseng, Y.H., Wang, Z.J., Pan, M.Y., Xiao, J.H., Chien, S.C., Wang, S.Y., Anti-inflammatory activity of Flavokawain B from Alpinia pricei Hayata. *J. Agric. Food. Chem.* 2009, 57, 6060-6065.

Livak, K.J., Schmittgen, T.D., Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 2001, 25, 402-408.

Llopiz, N., Puiggros, F., Cespedes, E., Arola, L., Ardevol, A., Blade, C., Salvado, M.J., Antigenotoxic effect of grape seed procyanidin extract in Fao cells submitted to oxidative stress. *J. Agric. Food Chem.*, 2004, 52, 1083-1087.

Lo, H.W., Ali-Osman, F., Genetic polymorphism and function of glutathione S-transferases in tumor drug resistance. *Curr. Opin. Pharmacol.*, 2007, 7, 367-374.

Lodovici, M., Luceri, C., Guglielmi, F., Bacci, C., Akpan, V., Fonnesu, M.L., Boddi, V., Dolara, P., Benzo(a)pyrene diolepoxide (BPDE)-DNA adduct levels in leukocytes of smokers in relation to polymorphism of CYPA11, GSTM1, GSTP1, GSTT1, and mEH. *Cancer. Epidemiol. Biomarkers. Prev.,* 2004, 13, 1342-1348.

Lois, K., Young, J., Kumar, S., Obesity; epiphenomenon or cause of metabolic syndrome? *Int. J. Clin. Pract.,* 2008, 62, 932–938.

Lu, C., Zhu, W., Shen, C.L., Gao, W., Green tea polyphenols reduce body weight in rats by modulating obesity-related genes. *Plos One*, 2012, 7, e38332.

Luo, Z., Sha, A.K., Xiang, X., Ruderman, N.B., AMPK the metabolic syndrome and cancer. *Trends. Parmacol. Sci.,* 2005, 26, 69-76.

Madsen, L., Petersen, R.K., Sorensen, M.B., Jorgensen, C., Hallenborg, P., Pridal, L., Fleckner, J., Amri, E.Z., Krieg, P., Furstenberger, G., Berge, R.K., Kristiansen, K., Adipocyte differentiation of 3T3-L1 preadipocytes is dependent on lipoxygenase activity during the initial stages of the differentiation process. *J. Biochem.* 2003, 375, 539–549.

Malterud, K.E., Farbrot, T.L., Huse, A.E., Sund, R.B., Antioxidant and radical scavenging effects of anthraquinone and anthrones. *Pharmacology*, 1993, 47, 77-85.

Marchesini, G., Bugianesi, E., Forlani, G., Cerrelli, F., Lenzi, M., Manini, R., Rizzetto, M., Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology*, 2003, 37, 917-923.

Maron, D.H., Ames, B.N., Revised methods for the Salmonella mutagenicity test. *Mutat. Res.,* 1983, 113, 173-215.

Matteoni, C.A., Younossi, Z.M., Gramlich, T., Boparai, N., Liu, Y.C., McCullough, A.J., Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology*, 1999, 116(6), 1413–9.

McGarry, J.D., Brown, N.F., The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur. J. Biochem.,* 1997, 244, 1–14.

Mejia, E.G., Castano-Tostado, E., Loarca-Pina, G., Antimutagenic effects of natural phenolic compounds in beans. *Mutat. Res.*, 1999, 441, 1-9.

Merrill, G.F., Kurth, E.J., Hardie, D.G., Winder, W.W., AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am. J. Physiol.*, 1997, 273, 1107-1112.

Milbury, P.E., Graf, B., Curran-Celentano, J.M., Blumberg, J.B., Bilberry (*Vaccinium*

*myrtillus*) anthocyanins modulate heme oxygenase-1 and glutathione S-transferase-pi expression in ARPE-19 cells. *Invest. Ophthalmol. Vis. Sci.*, 2007, 48, 2343-2349.

Miyake, H., Hara, I., Gleave, I.M.E., Eto, H., Protection of androgen-dependent human prostate cancer cells from oxidative stress-induced DNA damage by overexpression of clusterin and its modulation by androgen. *Prostate*., 2004, 61, 318-323.

Miyazawa, S., Hayashi, H., Hijikata, M., Ishii, N., Furuta, S., Kagamiyama, H., Osumi, T., Hashimoto, T., Complete nucleotide sequence of cDNA and predicted amino acid sequence of rat acyl-CoA oxidase. *J. Biol. Chem.*, 1987, 262(17), 8131-8137.

Montagut, G., Onnockx, S., Vaqué, M., Bladé, C., Blay, M., Fernández-Larrea, J., Pujadas, G., Salvadó, M.J., Arola, L., Pirson, I., Ardévol, A., Pinent, M., Oligomers of grape-seed procyanidin extract activate the insulin receptor and key targets of the insulin signaling pathway differently from insulin. *J. Nutr. Biochem.*, 2010, 21, 476-481.

Mortelmans, K., Zeiger, E., The Ames Salmonella/microsome mutagenicity assay. *Mutat. Res*., 2000, 455, 29-60.

Mukai, R., Fukuda, I., Nishiumi, S., Natsume, M., Osakabe, N., Yoshida, K., Ashida, H., Cacao polyphenol extract suppresses transformation of an aryl hydrocarbon receptor in C57BL/6 mice. *J. Agric. Food Chem.*, 2008, 56, 10399-10405.

Mullen, W., Edwards, C.A., Crozier, A., Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions, *Brit. J. Nutr.*, 2006, 96, 107-116.

Musarrat, J., Arezina-wilson, J., Wani, A.A., Prognostic and aetiological relevance of 8-hydroxyguanosine in human breast carcinogenesis. *Eur. J. Cancer*, 1996, 32A, 1209-1214.

Nakamura, T., Tokushima, T., Kawabata, K., Yamamoto, N., Miyamoto, M., Ashida, H., Absorption and metabolism of 4-hydroxyderricin and xanthoangelol after oral administration of Angelica keiskei (Ashitaba) extract in mice. *Arch. Biochem. Biophys.*, 2012, 521(1-2):71-76.

Nishikawa, S., Yasoshima, A., Doi, K., Nakayama, H., Uetsuka, K., Involvement of sex, strain and age factors in high fat diet-induced obesity in C57BL/6J and BALB/cA mice. *Exp. Anim.*, 2007, 56, 263–272.

Nishiumi, S., Yamamoto, N., Kodoi, R., Fukuda, I., Yoshida, K., Ashida, H., Antagonistic and agonistic effects of indigoids on the transformation of an aryl hydrocarbon receptor.

*Arch. Biochem. Biophys.*, 2008, 47*0*, 187-199.

Nunoshiba, T., Demple, B., Potent intracellular oxidative stress exerted by the carcinogen 4-nitroquinoline-N-oxide. *Cancer Res.*, 1993, 53, 3250-3252.

Ohkura, N., Nakakuki, Y., Taniguchi, M., Kanai, S., Nakayama, A., Ohnishi, K., Sakata, T., Nohira, T., Matsuda, J., Baba, K., Atsumi, G., Xanthoangelols isolated from Angelica keiskei inhibit inflammatory-induced plasminogen activator inhibitor 1 (PAI-1) production. *Biofactors*., 2011, 37, 455-461.

Ohkura, N., Nakakuki, Y., Taniguchi, M., Kanai, S., Nakayama, A., Ohnishi, K., Sakata, T., Nohira, T., Matsuda, J., Baba, K., Atsumi, G., Xanthoangelols isolated from Angelica keiskei inhibit inflammatory-induced plasminogen activator inhibitor 1 (PAI-1) production. *Biofactors*., 2011, 37, 455-461.

O'Neill, H.M., Holloway, G.P., Steinberg, G.R., AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: Implications for obesity. *Mol. Cell Endocrinol.*, 2012, in press, doi: 10.1016/j.mce.2012.06.019.

Pang, J., Choi, Y., Park, T., Ilex paraguariensis extract ameliorates obesity induced by high-fat diet: potential role of AMPK in the visceral adipose tissue. *Arch. Biochem. Biophys.*, 2008, 476, 178-185.

Park, S.H., Gammon, S.R., Knippers, J.D., Paulsen, S.R., Rubink, D.S., Winder, W.W., Phosphorylation–activity relationships of AMPK and acetyl-CoA carboxylase in muscle. *J. Appl. Physiol.*, 2002, 92(6), 2475–2482.

Peng, C.H., Liu, L.K., Chuang, C.M., Chyau, C.C., Huang, C.N., Wang, C.J., Mulberry water extracts possess an anti-obesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. *J. agric. food chem.*, 2011, 59(6), 2663-2671.

Poulsen, H.E., Prieme, H., Loft, S., Role of oxidative DNA damage in cancer initiation and promotion. *Eur. J. Canc. Prev.*, 1998, 7, 9-16.

Rayalam, S., Della-Fera, M.A., Baile, C.A., Phytochemicals and regulation of the adipocyte life cycle. *J. Nutr. Biochem.*, 2008, 19, 717-726.

Reardon, W., Ross, R.J., Sweeney, M.G., Luxon, L.M., Pembrey, M.E., Harding, A.E., Trembath, R.C., Diabetes mellitus associated with a pathogenic point mutation in mitochondrial DNA. *Lancet*., 1992, 340, 1376-1379.

Ribe, D., Yang, J., Patel, S., Koumanov, F., Cushman, S.W., Holman, G.D., Endofacial competitive inhibition of glucose transporter-4 intrinsic activity by the mitogen-activated protein kinase inhibitor SB203580. *Endocrinology,* 2005, 146**,** 1713-1717.

Ricquier, D.F., Bouillaud, F., The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and At UCP. *Bouillaud Biochem J.*, 2000, 345, 161-179.

Robertson, I.G.C., Guthenberg, C., Mannervik, B., Jernstrom, B., Differences in stereo selectivity and catalytic efficiency of three human glutathione transferases in the conjugation of glutathione with 7b,8a-dihydroxy-9a,10a-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. *Cancer Res*., 1986, 46, 2220–2224.

Robinson, Megan J., Melanie H. Cobb., "Mitogen-activated protein kinase pathways." *Curr. Opin. Cell biol*., 1997, 9, 180-186.

Rodriguez, J.W., Kirlin, W.G., Wirsiy, Y.G., Matheravidathu, S., Hodge, T.W., Urso, P., Maternal exposure to benzo(a)pyrene alters development of T lymphocytes in offspring. *Immunopharmacol. Immunotoxicol*., 1999, 21, 379-396.

Rodriguez-Ramiro, I., Ramos, S., Bravo, L., Goya, L., Martin, M.A., Procyanidin B2 induces Nrf2 translocation and glutathione S-transferase P1 expression via ERKs and p38-MAPK pathways and protect human colonic cells against oxidative stress. *Eur. J. Nutr.*, 2012, 51, 881-892.

Roig, R., Cascon, E., Arola, L., Blade, C., Salvado, M.J., Procyanidins protect Fao cells against hydrogen peroxide-induced oxidative stress. *Biochim. Biophys. Acta.*, 2002, 1572, 25-30.

Rosen, E.D., Hsu, C.H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., Spiegelman, B.M. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev*., 2002, 16, 22–26.

Sabio, G., Cavanagh-Kyros, J., Ko, H.J., Jung, D.Y., Gray, S., Jun, J.Y., Barrett, T., Mora, A., Kim, J.K., Davis, R.J., Prevention of steatosis by hepatic JNK1. *Cell. Metab.*, 2009, 10, 491-498.

Sabio, G., Davis, R.J., cJun NH(2)-terminal kinase 1 (JNK1): roles in metabolic regulation of insulin resistance. *Trends. Biochem. Sci.*, 2010, 35**,** 490-496.

Sakaue, H., Ogawa, W., Nakamura, T., Mori, T., Nakamura, K., Kasuga, M., Role of MAPK phosphatase-1 (MKP-1) in adipocyte differentiation. *J. Biol. Chem.*, 2004, 279, 39951-39957.

Sale, E.M., Atkinson, P.G., Sale, G.J., Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. *EMBO. J.*, 1995, 14, 674-684.

Sanyal, A.J., Mechanisms of disease: pathogenesis of nonalcoholic fatty liver disease. *Nat. Clin. Pract. Gastroenterol. Hepatol.*, 2005, 2, 46-53.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., Jiménez, L., Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food. Sci. Nutr.*, 2005, 45, 287-306.

Schimmack, G., Defronzo, R.A., Musi, N., AMP-activated protein kinase: Role in metabolism and therapeutic implications. *Diabetes. Obes. Metab.*, 2006, 8, 591–602.

Schoket, B., Papp, G., Lévay, K., Mracková, G., Kadlubar, F.F., Vincze, I., Impact of metabolic genotypes on levels of biomarkers of genotoxic exposure. *Mutat. Res.*, 2001, 482, 57-69.

Shaw, R.J., Kosmatka, M., Bardeesy, N., Hurley, R.L., Witters, L.A., DePinho, R.A., Cantley, L.C., The tumor suppressor LKB1 kinase directly activates AMPactivated kinase and regulates apoptosis in response to energy stress. *Pro. Natl. Acad. Sci. U. S. A.*, 2004, 101(10), 3329–3335.

Shibutani, S., Takeshita, M., Grollman, A.P., Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature*, 1991, 349, 431- 434.

Singh, S.V., Hu, X., Srivastava, S.K., Singh, M., Xia, H., Orchard, J.L., Zaren, H.A., Mechanism of inhibition of benzo[a]pyrene-induced forestomach cancer in mice by dietary curcumin. *Carcinogenesis*, 1998, 19, 1357-1360.

Sinha, R., Kulldorff, M., Gunter, M.J., Strickland, P., Rothman, N., Dietary benzo(a)pyrene intake and risk of colorectal adenoma. *Cancer Epidemiol. Biomarkers. Prev.*, 2005, 14, 2030-2034.

Slaga, T.J., Bracken, W.J., Gleason, G., Levin, W., Yagi, H., Jerina, D.M., Conney, A.H., Marked differences in the skin tumor-initiating activities of the optical enantiomers of the diastereomeric benzo(a)pyrene 7,8-diol-9,10-epoxides. *Cancer. Res.* 1979, 39, 67-71.

Su X., Abumrad N.A., Cellular fatty acid uptake: a pathway under construction. *Trends Endocrinol. Metab.*, 2009, 20, 72-77.

Sun, Y., Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radic. Biol. Med.*, 1990, 8, 583-599.

Surwit, R.S., Feinglos, M.N., Rodin, J., Sutherland. A, Petro, A.E., Opara, E.C., Kuhn, C.M.,

Rebuffe-Scrive, M. Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. *Metabolism.*, 1995, 44, 645-651.

Takahashi, M., Ikemoto, S., Ezaki, O. Effect of the fat/carbohydrate ratio in the diet on obesity and oral glucose tolerance in C57BL/6J mice. *J. Nutr. Sci. Vitaminol.*, 1999, 45, 583–593.

Tang, B., Du, J., Wang, J., Tan, G., Gao, Z., Wang, Z., Wang, L., Alpinetin suppresses proliferation of human hepatoma cells by the activation of MKK7 and elevates sensitization to cis-diammined dichloridoplatium. *Oncol. Rep.*, 2012, 27, 1090-6

Targher, G., Bertolini, L., Rodella, S., Tessari, R., Zenari, L., Lippi, G., Arcaro, G., Nonalcoholic fatty liver disease is independently associated with an increased incidence of cardiovascular events in type 2 diabetic patients. *Diabetes Care*, 2007, 30, 2119–2121.

Tilg, H., Moschen, A.R., Inflammatory mechanisms in the regulation of insulin resistance. *Mol. Med.*, 2008, 14(3-4), 222.

Tontonoz, P., Graves, R.A., Budavari, A.I., Erdjument-Bromage, H., Lui, M., Hu, E., Tempst, P., Spiegelman, B.M., Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormonereceptors, PPAR gamma and RXR alpha. *Nucl. Acids Res.*, 1994, 22, 5628-5634.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., Spiegelman, B.M., mPPARγ2: tissue-specific regulator of an adipocyte enhancer. *Genes. Dev.*, 1994, 8, 1224-1234.

Tontonoz, P., Hu, E., Spiegelman, B.M., Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell*, 1994, 79, 1147-1156.

Tontonoz, P., Spiegelman, B.M., Fat and beyond: the diverse biology of PPARγ. *Annu. Rev. Biochem.*, 2008, 77, 289-312.

Ueki, K., Kondo, T., Kahn, C.R., Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. *Mol. Cell. Biol.*, 2004, 24, 5434-5446.

Valavanidis, A., Vlachogianni, T., Fiotakis, C., 8-hydroxy-2’-deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *J. Environ. Sci. Health*, 2009, 27, 120-139.

Vernooij, J.W., van der Graaf, Y., Visseren, F.L., Spiering, W., Smart study group, The prevalence of obesity-related hypertension and risk for new vascular events in patients

with vascular diseases. *Obesity (Silver Spring)*, 2012, 20, 2118-23.

Vucenik, I., Stains, J. P., Obesity and cancer risk: evidence, mechanisms, and recommendations. *Ann. N. Y. Acad. Sci.*, 2012, 1271, 37-43.

Wanders, R.J.A., Ferdinandusse, S., Jansen, G.A., Vreken, P., Waterham, H.R., Van Roermund, C.W.T., Van Grunsven, E.G., Peroxisomal fatty acid alpha-and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochem. Soc. Trans.*, 2001, 29(2), 250-266.

Wang, N.D., Finegold, M.J., Bradley, A., Ou, C.N., Abdelsayed, S.V., Wilde, M.D., Taylor, L.R., Wilson, D.R., Darlington, G.J., Impaired energy homeostasis in C/EBP alpha knockout mice. *Science*, 1995, 269, 1108-1112.

Wang, T., Wang, Y., Kontani, Y., Kobayashi, Y., Sato, Y., Mori, N., Yamashita, H., Evodiamine improves diet-induced obesity in a uncoupling protein-1-independent manner: involvement of antiadipogenic mechanism and extracellularly regulated kinase/mitogen-activated protein kinase signaling. *Endocrinology*, 2008, 149, 358-66.

Wang, T., Wang, Y., Yamashita, H., Evodiamine inhibits adipogenesis via the EGFR-PKCα-ERK signaling pathway. *FEBS. Letters*, 2009, 583, 3655-9.

Weisburger, J.H., Lifestyle, health and disease prevention: the underlying mechanisms. *European journal of cancer prevention: the official journal of the European Cancer Prevention Organisation (ECP).*, 2002, 11, S1.

Whitlock, J.P.Jr., Induction of cytochome P4501A1, Annu. *Rev. Pharmacol. Toxicol.*, 1999, 39, 103-125.

Williamson, G., Manach, C., Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.*, 2005, 81, 243-255.

Winder, W.W., Hardie, D.G., AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am. J. Physiol.*, 1999, 277, 1-10.

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

Wu, X., Zhang, L., Gurley, E., Studer, E., Shang, J., Wang, T., Wang, C., Yan, M., Jiang, Z., Hylemon, P.B., Sanyal, A.J., Pandak, W.M., Zhou, H., Prevention of free fatty acid–induced hepatic lipotoxicity by 18β‐glycyrrhetinic acid through lysosomal and mitochondrial pathways. *Hepatology*., 2008, 47(6), 1905-1915.

Wu, Z., Rosen, E.D., Brun, R., Hauser, S., Adelmant, G., Troy, A.E., McKeon, C., Darlington, G.J., Spiegelman, B.M., Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol. Cell*, 1999, 3, 151-158.

Xiong, P., Bondy, M.L., Li, D.H., Shen, H.B., Wang, L.E., Singletary, S.E., Spitz, M.R., Wei, Q.Y., Sensitivity to benzo(a)pyrene diol-epoxide associated with risk of breast cancer in young women and modulation by glutathione S-transferase polymorphisms. *Cancer. Rev.* 2001, 61, 8465-8469.

Xiong, Y., Collins, Q.F., An, J., Lupo, E., Liu, H.Y., Liu, D., Robidoux, J., Liu, Z., Cao, W., p38 mitogen-activated protein kinase plays an inhibitory role in hepatic lipogenesis. *J. B. C*., 2007, 282, 4975-4982.

Yamamoto, N., Kawabata, K., Sawada, K., Ueda, M., Fukuda, I., Kawasaki, K., Murakami, A., Ashida, H., Cardamonin Stimulates Glucose Uptake through Translocation of Glucose Transporter-4 in L6 Myotubes. *Phytother. Res.*, 2011, 25, 1218-1224.

Ye, J.M., Iglesias, M.A., Watson, D.G., Ellis, B., Wood, L., Jensen, P.B., Sorensen, R.V., Larsen, P.J., Cooney, G.J., Wassermann, K., Kraegen, E.W., PPARalpha /gamma ragaglitazar eliminates fatty liver and enhances insulin action in fat-fed rats in the absence of hepatomegaly. *Am. J. Physiol. Endocrinol. Metab.*, 2003, 284(3), 531-540.

Yeh, W.C., Cao, Z., Classon, M., McKnight, S.L., Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes. Dev.*, 1995, 9, 168-181.

Yoshimura, M., Sano, A., Kamei, J.I., Obata, A., Identification and quantification of metabolites of orally administered naringenin chalcone in rats, *J. Agr. Food Chem.*, 2009, 57, 6432-6437.

You, T., Yang, R., Lyles, M. F., Gong, D., Nicklas, B.J. Abdominal adipose tissue cytokine gene expression: relationship to obesity and metabolic risk factors. *Am. J. Physiol. Endocrinol. Metab.*, 2005, 288(4), 741-747.

Yu, Y.S, Hsu, C.L., Yen, G.C., Anti-inflammatory effects of the roots of Alpinia pricei Hayata and its phenolic compounds. *J. Agric. Food. Chem.*, 2009, 57, 7673-80.

Zhang, B.B., Zhou, G., Li, C., AMPK: an emerging drug target for diabetes and the metabolic syndrome. *Cell Metab.*, 2009, 9, 407-416.

Zhang, T., Jiang, S., He, C., Kimura, Y., Yamashita, Y., Ashida, H., Black soybean seed coat

polyphenols prevent B(a)P-induced DNA damage through modulating drug-metabolizing enzymes in HepG2 cells and ICR mice. *Mutat. Res.*, 2013, 752, 34-41.

Zhang, Y., Wang, G.-J., Song, T.T., Murphy, P.A., Hendrich, S., Urinary disposition of the soybean isoflavones daidzein, genistein and glycitein differs among humans with moderate fecal isoflavone degradation activity, *J. Nutr.*, 1999, 129, 957-962.

Zhou G., Myers R., Li Y., Chen Y., Shen X., Fenyk-Melody J., Wu, M., Centre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F. Goodyear, L.J. Moller, D. E., Role of AMP activated protein kinase in mechanism of metformin action. *J. Clin. Invest*., 2001, 108, 1167-1674.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J., Moller, D.E., Role of AMP-activated protein kinase in mechanism of metformin action. *J. Clin. Invest.*, 2001, 108, 1167-117

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Centre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J. Moller, D.E., Role of AMP activated protein kinase in mechanism of metformin action. *J. Clin. Invest.*, 2001, 108, 1167-1674.

Zhou, P., Gross, S., Liu, J.H., Yu, B.Y., Feng, L.L., Nolta, J., Piwnica-Worms, D., Qiu, S.X., Flavokawain B The hepatotoxic constituent from kava root, induces GSH-sensitive oxidative stress through modulation of IKK/NF-κB and MAPK signaling pathways. *J. FASEB.*, 2010, 24, 4722-4732.

Zhu, H., Itoh, K., Yamamoto, M., Zweier, J. L., Li, Y., Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS. Lett.*, 2005, 579, 3029-3036.

Reaven, G. M., Insulin resistance: the link between obesity and cardiovascular disease. *Med. Clin. North Am.*, 2011, *95*, 875-892.

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## List of Publication

1. **Tianshun Zhang**, Keisuke Sawada, Norio Yamamoto, Hitoshi Ashida, 4-Hydroxyderricin and xanthoangelol from Ashitaba (*Angelica keiskei*) suppress differentiation of preadiopocytes to adipocytes via AMPK and MAPK pathways. *Molecular Nutrition & Food Research*, 2013, *57*, 1729-1740.

2. **Tianshun Zhang,** Songyan Jiang , Chao He, Yuki Kimura, Yoko Yamashita, Hitoshi Ashida, Black soybean seed coat polyphenols prevent B(a)P-induced DNA damage through modulating drug-metabolizing enzymes in HepG2 cells and ICR mice, *Mutation Research*, 2013, *752*, 34-41.

3. **Tianshun Zhang**, Kyuichi Kawabata, Rei Kitano, Hitoshi Ashida, Preventive effects of black soybean seed coat polyphenols against DNA damage in *Salmonella typhimurium*, *Food Science and Technology Research*, 2013, *19*, 685-690.

4. Yamashita, Yoko, Wang, Lihua, **Tianshun, Zhang,** Nakamura, Toshiyuki, Ashida, Hitoshi, Fermented tea improves glucose intolerance in mice by enhancing translocation of glucose transporter 4 in skeletal muscle, *Journal of Agricultural Food chemistry*. 2012, *60*, 11366-11371.