



Studies on the functions of quercetin and its derivatives on glucose and lipid metabolism

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DOCTORAL DISSERTATION

**Studies on the functions of quercetin and its
derivatives on glucose and lipid metabolism**

（ケルセチンとその誘導体による糖代謝と脂質代謝に関する研究）

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Abbreviations

ACC, acetyl-CoA-carboxylase

AICAR, 5-amino-1- β -d-ribofuranosyl-imidazole-4-carboxamide

AMP, Adenosine 5'-monophosphate

AMPK, adenosine monophosphate-activated protein kinase

AUC, area under the curve

aPKC, atypical protein kinase C

BAT, brown adipose tissue

BSA, bovine serum albumin

CAR, constitutive androstane receptor

C/EBP, CCAAT/enhancer-binding protein

CPT1, carnitine palmitoyltransferase 1

CVD, cardiovascular diseases

2DG, 2-deoxy-d-glucose

DM, diabetes mellitus

DMSO, dimethyl sulfoxide

DTT, dithiothreitol

FAS, fatty acid synthase

FBS, fetal bovine serum

FXR, farnesoid X receptor

GLUT, glucose transporter

G6Pase, glucose 6-phosphatase

HF, high fat

HOMA-IR, homeostasis model assessment of insulin resistance

HPLC, high-performance liquid chromatography

IL-15, interleukin-15

IR, insulin receptor

IRS1, insulin receptor substrate 1

JAK2, Janus kinase 2

KRH, Krebs-Ringer HEPES buffer

MEM, minimum essential medium

OGTT, oral glucose tolerance test

PBS, phosphate buffered saline

PEPCK, phosphoenolpyruvate carboxykinase

PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator-1alpha

PI3K, phosphoinositide 3-kinase

PKB/Akt, protein kinase B

PKC, protein kinase C

PPAR, peroxisome proliferator-activated receptor

PRDM16, PR domain containing 16

SDS, sodium dodecyl sulfate

SE, standard error

SIRT1, sirtuin 1

SREBP, sterol regulatory element-binding protein

STAT, signal transducer and activator of transcription

T2DM, type 2 diabetes mellitus

TEA, triethanolamine hydrochlorid

UCP, uncoupling protein.

WAT, white adipose tissue

Chapter 1: General introduction

Preface

Diabesity is the term for diabetes occurring in obese persons, which demonstrated a direct relationship between body mass index (BMI) and diabetes. The reason of that is type 2 diabetes mellitus (T2DM) and obesity have become highly prevalent metabolic disorders diseases all over the world. T2DM, obesity and their chronic complications including cardiovascular diseases (CVD), stroke, and retinopathy, have been regarded as major threats to the human health system [Cao, L., *et al.*, 2016; El-Bassossy, HM., *et al.*, 2017]. Rapid increases in the incidence of the diseases are strongly associated with the shifted of global dietary patterns and lifestyle factors as excess intake of high-fat and high-calorie foods and lack of energy expenditure in a sedentary lifestyle [Hubert, K. and Stephan, M., 2017]. Currently, identification of potential dietary substance from fruits, herbs, and vegetables is regarded as a new strategy for diabetes and obesity treatment. Polyphenols have been regarded as ideal substitutes to medications, because most of the drugs have certain side effects [Wang, S., *et al.*, 2014]. In this study, quercetin and its derivatives are chosen by the author, due to they possess various beneficial health effects against diabetes, obesity, and cancer *in vitro* and *in vivo* [Zhao, L., *et al.*, 2017; Li, X., *et al.*, 2018]. Even though the functions and bioavailabilities of quercetin and its derivatives have been reported, their molecular mechanisms still need further exploration [Arts, IC., *et al.*, 2004; Murota, K., *et al.*, 2010]. Hence, in this chapter, the author mainly presented and summarized the background of this dissertation, including diabetes mellitus, obesity, and related molecular targets of polyphenols for regulation of energy metabolism.

Diabetes mellitus and insulin resistance

Diabetes mellitus (DM) is characterized by hyperglycemia, hyperinsulinemia, and insulin resistance. Furthermore, there are over 280 million diabetic patients in 2010, not only distribute in advanced countries, but also in developing countries, sometimes coexisting with undernutrition [Ogurtsova, K., *et al.*, 2017]. The population of diabetic patients will parallel with aging and obesity in 2035 [Riobó Serván, P., *et al.*, 2013]. The total economic cost of diabetes is estimated to reach \$245 billion per year, which poses a significant burden to our economy [Handorf, AM., *et al.*, 2015]. The vast majority of cases of diabetes fall into two broad etiopathogenetic categories (type 1 and type 2 DM). In one category, type 1 diabetes (T1DM) is an autoimmune pathologic diabetes, resulting from the destruction of the β -cells of pancreas by genetic markers. T1DM is caused by the deficiency of insulin secretion and mainly onset in children and adolescents, presenting with ketoacidosis as the initial manifestation [Guthrie, RA., and Guthrie, DW., 2004]. On the other hand, T2DM, which accounts for 90-95% of patients, is characterized by insulin resistance. The pathogenesis has been reported that the insulin-sensitive peripheral tissues have resisted the insulin action resulting in a state, which greater amounts of insulin is required to maintain a normal biological response, such as facilitating cellular glucose uptake, and modulating carbohydrate, lipid and protein metabolism [Wilcox, G., *et al.*, 2005]. Insulin resistance is induced by genetic factors and environmental factors, as hyper-caloric diet, obesity, and sedentarism [Riobó Serván, P., *et al.*, 2013]. The pathogenesis is slow and not easy to detect without clinical symptoms. During this long time of asymptomatic period, changes in pathologic and functional have been induced by abnormal carbohydrate metabolism and hyperglycemia in related target tissues [American Diabetes Association, 2012].

Current treatments for T1DM are exogenous insulin therapy, whole-pancreas transplant, and insulin gene therapy [Handorf, AM., *et al.*, 2015]. However, these treatments still present significant drawbacks. Moreover, T2DM tends to be alleviated by pharmacological treatment and non-pharmacological treatment. Regarding therapeutic drugs, agents for T2DM treatment raised the insulin concentration in a glucose independent manner, while this persistently excessive insulin concentration induce an increase in body weight. After the long time administration, weight gain also could exacerbate the course of T2DM [Pfeiffer, AF., *et al.*, 2014]. Hence, novel and fully-sustainable therapy is still required. Non-pharmacological treatment mainly refers to healthy diets (caloric restriction) and appropriate physical exercise to ameliorate mitochondrial functions, inhibit proliferation and improve the insulin efficacy [Pfeiffer, AF., and Klein, HH., 2014].

Obesity

Obesity, especially abnormal or excessive fat accumulation distributed predominantly in the abdominal region, is an increasing global public health issue. Obesity is a heterogeneous disorder with a spectrum of traits and central to metabolic syndrome. The clinical manifestation of obesity is usually associated with physical inactivity, high level of triglyceride, impaired fasting glucose, hypertension, and subclinical inflammation [Kathleen, M., *et al.*, 2015; Tuomilehto. J, *et al.*, 2001]. Furthermore, obesity itself causes certain degree of insulin resistance [Dattilo, AM., and Kris-Etherton, PM., 1992].

Adipose tissue is an energy storage organ, while increasing recognition indicated that adipose tissue is also an endocrine and immune organ. There are two major types of adipose tissues: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT

secretes over 50 adipokines, and involves in inflammation, angiogenesis and glucose metabolism [Kiess, W., *et al.*, 2008; Laclaustra, M., *et al.*, 2007]. In the obese state, content and infiltration of macrophage in WAT significantly increased, due to they are positively associated with expression of pro-inflammatory chemokines and cytokines [Bruun, JM., *et al.*, 2005]. Among these chemokines and cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6 and IL-8, are involved in potentially contributing to obesity-related insulin resistance [Havel, PJ., *et al.*, 2004; Lucas, S., *et al.*, 2009; Kern, PA., *et al.*, 1995]. BAT is different from WAT in morphology. BAT contains a large number of mitochondria, and has relative with glucose homeostasis, fatty acid oxidation, and energy expenditure to maintain an adequate core body temperature [Calderon-Dominguez, M., *et al.*, 2015; Ikeda, K., *et al.*, 2018; Saely, CH., *et al.*, 2012]. Thus, BAT is the foremost site of non-shivering thermogenesis. This heat production process primarily depends on uncoupling protein (UCP) 1 activity [Crichton, PG., *et al.*, 2017]. UCP 1 acts as a transmembrane protein to drive ATP synthesis [Ricquier, D., *et al.*, 2011]. Though WAT and BAT have significant differences in morphology and functional properties, it has been reported that WAT can transfer into BAT by a process called “browning or beiging” in response to prolonged cold exposure or β -adrenergic stimulation [Lee, YH., *et al.*, 2014]. In the context of obesity, beige adipocytes generation is reduced, which contributes to decrease energy expenditure, weaken the thermogenic capacity, and impair adipose tissue insulin sensitivity [Villarroya, F., *et al.*, 2018]. Hence, promising strategy to decrease the onset of obesity is promoting energy expenditure, including the modulation of thermogenesis and formation of beige adipocytes.

Glucose homeostasis

The main energy source of organisms is glucose [Mergenthaler, P., *et al.*, 2013]. Hence, tissues constantly need glucose. Lower concentration of blood glucose causes seizures and irreversible cell damage, while high blood glucose also causes glucotoxicity, resulting in blindness and cardiac vascular diseases [Scheepers, A., *et al.*, 2004]. Blood glucose concentration has to be maintained at a steady level (80-100 mg/dL) [Ayala, JE., *et al.*, 2010]. Thus, maintaining these narrow range is referred to as glucose homeostasis. Maintaining of glucose homeostasis requires a number of physiological processes, including intestinal absorption from diet, glycogenolysis in liver, glyconeogenesis in the liver and kidney and glucose excretion [Murugan, AC., *et al.*, 2015]. T2DM and obesity are established risk factors for abnormal glucose homeostasis, which induced hyperglycemia and insulin resistance. The abnormal glucose homeostasis tend to be recovered by promoting the translocation of glucose transporters in the muscle to increase their glucose uptake and to store the glucose as glycogen in the muscle and suppressing glucose and free fatty acid release from the liver and kidney into the circulation [Bano, G., *et al.*, 2013].

Glucose transporters

Glucose uptake is closely related with glucose transporters, which play essential roles in maintaining glucose homeostasis. Due to the hydrophilicity, glucose can passively diffusion through the plasma membrane (PM). There are two different types of glucose transporter proteins, sodium dependent glucose transporters (SGLTs) and the facilitative glucose transporters (GLUTs) in the plasma membrane [Bell, GI., *et al.*, 1990; Scheepers, A., *et al.*, 2004]. The SGLT family comprises Na⁺-dependent glucose co-transporters (SGLT1 and SGLT2), the glucose sensor (SGLT3), multivitamin transporters (SGLT4 and SGLT6), and the thyroid iodide transporters (SGLT5).

Regarding SGLT1 and SGLT2, the transport is a cotransport of glucose molecules and sodium ions process.

On the other hand, members of the GLUTs exhibit a striking and specific effect on regulating glucose uptake [Joost, HG., and Thorens, B., 2001]. Now 14 different members of GLUT family have been detected in human genome, which can be divided into 3 subfamilies according to sequence similarities and characteristic elements [Joost, HG., and Thorens, B., 2001]. The major function of Class 1 GLUTs is regulating the glucose redistribution to maintain glucose homeostasis. GLUT1 is ubiquitously, and exhibits high expression in erythrocytes membrane [Koranyi, L., *et al.*, 1991]. Due to highly-expressed GLUT1, blood contains effectively glucose carrying capacity to brain, which is absolutely relies on glucose as a fuel source [Yeh, WL., *et al.*, 2008]. In addition, GLUT1 expresses in brain astrocytes as well. Supplying glycolytically-derived lactate to neurons is also regarded as the mainly responsibility of GLUT1 [Pellerin, L., *et al.*, 2002]. GLUT2 predominantly expresses in pancreatic β -cells, liver, and kidney [Fukumoto, H., *et al.*, 1988]. GLUT2 also transports fructose and glucosamine [Uldry, M., *et al.*, 2002]. Cooperation with GLUT1, GLUT3 is a mainly regulator of glucose uptake utilized into neurons and parenchymal cells of the brain [Leino, RL., *et al.*, 1997]. GLUT4 is predominantly expressed in skeletal muscle, heart, and adipose tissues [Fukumoto, H., *et al.*, 1989; Simpson, IA., *et al.*, 1986]. GLUT4 translocation to the plasma membrane, displays a 10-20-fold increase after insulin stimulation [Bryant, NJ., *et al.*, 2002]. In patients with T2DM, glucose uptake of muscle is decreased drastically [Bogardus, C., *et al.*, 1984; Bonadonna, RC., *et al.*, 1990]. Hence, the author regards GLUT4 translocation to the plasma membrane and its mechanism in muscle as a major strategy for T2DM treatment.

Insulin-dependent signaling pathway for GLUT4 translocation

In skeletal muscle, upon the insulin stimulation, GLUT4 translocates to the cell membrane through an intracellular cascade reaction [Fukumoto, H., *et al.*, 1989; Simpson, IA., *et al.*, 1986]. The specific heterotetramer of insulin receptor (IR) is activated by insulin and provides a structural change leading to autophosphorylation of a tyrosine kinase domain of β subunits [Boucher, J., *et al.*, 2014]. The activation of the IR subsequently activated several protein intermediates such as insulin receptor substrate 1 (IRS1), and phosphatidylinositol-3-kinase (PI3K). PI3K is a dimer composed of a regulatory subunit (p85) with two SH2 domains and a catalytic subunit with one SH3 domain [Yu, J., *et al.*, 1998]. The activated PI3K not only catalyzes the addition of phosphate groups to the inositol ring of phosphoinositides at position 3 resulting in producing phosphatidylinositol 3, 4, 5-triphosphate [PI(3, 4, 5)P₃], but also provides phosphorylation of AKT and atypical protein kinase C (aPKC) [Manna, P., *et al.*, 2015]. In mammals, there are three isoforms in AKT family, AKT1-AKT3. Specifically, AKT2 reveals a pivotal role in glucose metabolism, because systemic AKT2KO mice show hyperglycemia and impaired glucose tolerance [Cho, H., *et al.*, 2001]. In addition to inducing GLUT4 translocation to the plasma membrane, virtually AKT is involved in all of insulin's metabolic effects. For example, phosphorylation of glycogen synthase kinase 3 (GSK-3 β), downstream factor of AKT, leads to the activation of glycogen synthase [Patel, S., *et al.*, 2008]. Upon insulin stimuli, AKT translocates to the plasma membrane preferentially binding to PI(3, 4, 5)P₃ than other phosphoinositides. The binding of PIP₃ induces a conformational change in AKT and exposures regulatory phosphorylation sites Thr308 in the kinase domain and Ser473 in the C-terminal domain [Milburn, CC., *et al.*, 2003; Rowland, AF., *et al.*, 2011]. Akt is

not only activated its phosphorylation at Thr308 by phosphoinositide-dependent kinase-1 (PDK1), but also phosphorylation at Ser473 by mammalian target of rapamycin (mTOR) in complex with rictor (rictor/mTOR) [Frödin, M., *et al.*, 2002; Sarbassov, DD., *et al.*, 2005]. Hence, a complex cascade consisting of various kinases has been demonstrated to induce GLUT4 translocation to the plasma membrane.

Insulin-independent signaling pathway for GLUT4 translocation

1. Adenosine monophosphate-activated protein kinase (AMPK)-signaling pathway

AMPK promotes lipid accumulation and gene transcription, as well as the regulation of glucose metabolism to maintain the metabolic energy balance of the whole body [Long, YC., *et al.*, 2006; Gruzman, A., *et al.*, 2009]. The complex of AMPK is a family member of heterotrimeric serine/threonine protein kinases consisting of subunits α ($\alpha 1$ and $\alpha 2$), β ($\beta 1$ and $\beta 2$), and γ ($\gamma 1$, $\gamma 2$ and $\gamma 3$) [Gruzman, A., *et al.*, 2009]. Due to the presence of isoforms, AMPK expresses in different tissues and demonstrates different functions under different conditions [Dasgupta, B., *et al.*, 2016]. In skeletal muscles, AMPK facilitates glucose uptake and induces GLUT4 translocation by phosphorylating Rab-GTPase-activating protein TBC1D1, independent of insulin-pathway [Chavez, JA., *et al.*, 2008]. AMPK is activated by phosphorylating threonine residue (Thr172) of AMPK's subunits α , which is activated by the contraction of skeletal muscle and the reduction in ratios of ATP/AMP and creatine/phosphocreatine [Chen, ZP., *et al.*, 2000; Fujii, N., *et al.*, 2000; Winder, WW. and Hardie, DG. 1996]. Furthermore, liver kinase B1 (LKB1), calcium/calmodulin-dependent protein kinase kinase II (CAMKK2), and TGF β -activated kinase 1 (TAK1) are confirmed upstream factors of AMPK phosphorylation [Woods, A., *et al.*, 2005; Neumann, D., *et al.*, 2018]. Hence, activated AMPK would be expected to have beneficial effects in reducing the

risk of T2DM, and atherosclerotic CVD. However, multiple molecular mechanisms of AMPK activated by polyphenols are not fully clarified

2. Janus kinase (JAK)/signal transducer and activator of transcription (STAT)-signaling pathway

JAK/STAT pathway is known to regulate mediating the action of cytokines, interferons, and their gene expression [O'Shea, JJ., *et al.*, 2015; Schindler, C., *et al.*, 2007]. Recently, this description of the function of JAK/STAT pathway has been extended, as the pathway transduces extracellular signals into transcriptional programs to regulate cell growth and differentiation [Darnell, JE Jr., *et al.*, 1997; Nakajima, K., *et al.*, 1996]. Activated JAK2 and JAK3 alter intracellular signaling to result in the activation of STATs, such as STAT1, STAT3, and STAT5, that participate in multiple biological responses, including regulating chromatin conformation in the nucleus and affecting oxidative phosphorylation in the mitochondria [Thomas, SJ., *et al.*, 2015; Villarino, AV., *et al.*, 2015]. The deregulation of JAK/STAT signaling contributes to induce various diseases in the different tissues, as lymphocytes, adipocytes, neuronal cells, cardiomyocytes, hepatocytes, stem cells [von Manstein, V., *et al.*, 2013]. It has been reported that muscle-specific deficiency of STAT3 and/or STAT5 increases lipid accumulation in skeletal muscle and induces dyslipidemia and hyperglycemia [Gurzoy, EN., *et al.*, 2016]. Recently, JAK/STAT pathway has attracted with considerable interest in energy metabolism and DM. For example, IL-15 induces GLUT4 translocation by activating JAK3/STAT3 signaling pathway in muscle cells [Krolopp, JE., *et al.*, 2016].

Major regulators of lipid metabolism

Lipid metabolism could be divided into catabolism and anabolism [Baraona, E., *et al.*, 1979]. Adipose tissue and liver play important role on maintaining the stability of

the lipid metabolism. The secreted hormones from adipose tissue, such as leptin and adiponectin, have various lipid metabolic effects on body weight regulation [Stern, JH., *et al.*, 2016]. In the liver, excess carbohydrates in food are converted to triglycerides [Glimcher, LH., *et al.*, 2009]. In starvation or long-term endurance exercise, synthetic triglyceride converts to glycerol and fatty acid and decomposes to molecules of ATP [Hardie, DG., *et al.*, 2012]. In order to maintain this lipid metabolism balance of the whole body, many transcription factors and several protein intermediates are involved, constituting a complex network in these processes. AMPK not only regulates glucose metabolism and insulin sensitivity to maintain the metabolic glucose balance in the muscle, but also is an essential target for promoting energy metabolism and repressing adipogenesis by regulating fatty acid synthesis and oxidation [Wang, S., *et al.*, 2015; Cho, BY., *et al.*, 2017], and reducing lipogenesis in the WAT and liver [Cho, BY., *et al.*, 2017; Moseti, D., *et al.*, 2016]. In WAT, AMPK regulates the expression levels of carnitine palmitoyltransferase 1 (CPT1), acetyl-CoA carboxylase (ACC), sterol regulatory element binding protein 1 (SREBP1), peroxisome proliferator activated receptor γ (PPAR γ), and CCAAT/enhancer binding proteins (C/EBPs) [Wang, S., *et al.*, 2015; Cho, BY., *et al.*, 2017]. UCP2 and CPT1, downstream factors of AMPK, are reported to be regulators of mitochondrial fatty acid oxidation [Cho, BY., *et al.*, 2017; Zhang, T., *et al.*, 2015]. SREBP1, PPAR γ , and C/EBPs are associated with lipogenesis and adipocyte differentiation [Cho, BY., *et al.*, 2017; Moseti, D., *et al.*, 2016; Brun, RP., *et al.*, 1996]. UCP1 [Zhang, T., *et al.*, 2015], PPAR γ coactivator α (PGC-1 α) [Puigserver, P., *et al.*, 2003], PR-domain containing protein 16 (PRDM16) [Chi, J., *et al.*, 2016], and sirtuin 1 (SIRT1) [Son, MJ., *et al.*, 2016] have relationship with the regulation of mitochondrial biogenesis, thermogenesis and brown fat differentiation.

In the liver, AMPK inhibits fatty acid synthesis, enhances fatty acid oxidation and

improves lipid homeostasis through inhibition of ACC and activation of CPT1 and PPAR α [Viollet, B., *et al.*, 2006]. In addition, hepatic glucose metabolism is also regulated by AMPK phosphorylation [Miao, J., *et al.*, 2006; Wagner, M., *et al.*, 2005; Kulkarni, SR., *et al.*, 2013]. Gluconeogenic enzyme expression and the enzymatic activity of gluconeogenesis, such as PEPCK and G6Pase have been reported to be suppressed by FXR and CAR activation in metabolic disorder mice, which are downstream factors of AMPK [Miao, J., *et al.*, 2006; Wagner, M., *et al.*, 2005; Kulkarni, SR., *et al.*, 2013; Mo, L., *et al.*, 2016].

The effects of polyphenols on glucose and lipid metabolism

Numerous studies have demonstrated that natural products rich in polyphenols, such as fruit, herbs and spices, have various beneficial effects for the prevention and treatment human diseases without side effects [Tsao, R., 2010]. Various botanical extracts and polyphenols have been reported to regulate glucose homeostasis and energy metabolism through different mechanisms, as decreasing energy absorption in the small intestine, increasing insulin secretion in pancreatic β -cells, regulating hepatic gluconeogenesis and fatty acid oxidation, promoting glucose uptake in muscle, and modulating lipogenesis and lipolysis in adipose tissue [Hajiaghaalipour, F., *et al.*, 2015]. Catechins, present in teas, wine, or cocoa, have been verified to inhibit carbohydrate digestion and glucose absorption in the intestine through decreasing the activities of SGLT 1, α -amylase, and α -glucosidase [Shimizu, M., *et al.*, 2000]. Cyanidin-3-glucoside, one of the anthocyanins, significantly stimulates insulin secretion from rodent pancreatic β -cells (INS-1832/13 cells) *in vitro* [Jayaprakasam, B., *et al.*, 2005]. EGCg suppresses hepatic gluconeogenesis and lipid accumulation and increases the activity of hepatic glucokinase and insulin sensitivity [Collins, QF., *et al.*, 2007].

Furthermore, in insulin-sensitive tissues namely muscles and adipose tissues, EGCg, procyanidin, piperine, 4-hydroxyderricin and xanthoangelol affect glucose metabolism by inducing GLUT4 translocation through different pathways *in vitro* and *in vivo* [Ueda-Wakagi, M., *et al.*, 2018; Yamashita, Y., *et al.*, 2016; Maeda, A., *et al.*, 2018; Kawabata, K., *et al.*, 2011]. Cacao extract increased fatty acid oxidation by regulating AMPK and ACC phosphorylation [Yamashita, Y., *et al.*, 2012]. Cinnamon, ashitaba, and coffee suppress diet-induced body fat accumulation by downregulating the adipogenesis and related proteins expression in mice [Cheng, DM., *et al.*, 2012; Cao, H., *et al.*, 2007; Zhang, T., *et al.*, 2015; Murase, T., *et al.*, 2011]. Taken together, these results indicate that polyphenols have potential to prevent and ameliorate lifestyle diseases that relating to energy metabolism disorder.

Quercetin and its derivatives

Polyphenols are widely distributed and exist naturally as plant metabolites. It has been noted that polyphenols are the largest source of human dietary antioxidants with a typical daily intake of roughly 1 g/day [Kühnau, J., *et al.*, 1976; Mullie, P., *et al.*, 2007]. Quercetin, an attractive natural flavonol, presents as various glycoside forms rather than their aglycone forms, such as rutin (quercetin-3-O- β -rutinoside) and isoquercitrin (quercetin-3-O- β -glucoside) in plants and plant-derived foods [Arts, IC., *et al.*, 2004]. After intake of quercetin glycosides, following chewing, digestion, and absorption, sugar moieties could be released quercetin from its glycosides. For example, quercetin glycosides are hydrolyzed to liberate quercetin aglycone form at the intestinal mucosa [Németh, K., *et al.*, 2003]. Furthermore, the large intestine, the liver and the kidneys, also contribute to quercetin metabolism, as rising glucuronidated, methylated and sulfated forms of quercetin [Guo, Y., *et al.*, 2015; Lesser, S., *et al.*, 2004; Nemeth,

K., *et al.*, 2007]. Quercetin glucosides possess various health beneficial effects against diabetes, obesity, and cancer [Zhao, L., *et al.*, 2017; Li, X., *et al.*, 2018]. It has been reported that administration of quercetin decreases the marker levels of oxidative stress in diabetic male rats [Roslan, J., *et al.*, 2017]. Supplementation with quercetin-rich onion peel extract upregulated the mRNA expression of CPT1 in 3T3-L1 cells and adipose tissue [Moon, J., *et al.*, 2013]. Nonetheless, it has been reported that the mechanisms of quercetin and its derivatives regulating energy metabolism are different. Isoquercitrin and quercein-4-*O*- β -glucoside regulate SGLT1 in a competitive manner (dependent on glucose concentration), but quercein-3-*O*- β -galactoside, rutin and the aglycone quercetin are devoid of effect [Ader, P., *et al.*, 2001; Cermak, R., *et al.*, 2004; Wolfram, S., *et al.*, 2002]. In addition, in agreement with this observation, quercetin and isoquercitrin decrease GLUT2-mediated Na⁺-independent diffusive uptake of glucose through different mechanisms to affect glucose absorption in the intestinal: quercetin is a potent inhibitor of GLUT2-mediated glucose transport (independent on glucose concentration), while isoquercitrin seems to be a competitive inhibitor of GLUT2-mediated glucose transport [Chen, CH., *et al.*, 2007; Song, J., *et al.*, 2002]. Therefore, it is necessary to clarify the mechanisms of quercetin and its glucosides on glucose and lipid metabolism.

Regarding the bioavailabilities of quercetin and its derivatives, it is necessary to emphasize that the bioavailability of quercetin is lower in the organism [Hollman, PC., *et al.*, 1995]. The bioavailability of quercetin is associated with the type of sugar moiety present. Pharmacokinetic studies on quercetin and its glucosides are well documented. The absorption rate of quercetin is reportedly 9%–20% in humans [Graefe, EU., *et al.*, 2001; Manach, C., *et al.*, 2004]. It has been detected that the concentration of quercetin metabolites is approximately 1.5 μ M in the plasma within 2 h after intake of quercetin

monoglucosides, and basal concentrations of quercetin aglycone in the blood were reported to be from 300 to 750 nM after consumption of 80–100 mg of quercetin equivalent in humans [de Vries, JH., *et al.*, 1998; Mullen, W., *et al.*, 2006; Manach, C., *et al.*, 2004; Zhao, G., *et al.*, 2013]. The bioavailability of isoquercitrin has been reported using a high-performance liquid chromatography (HPLC), and the concentration of conjugated metabolites instantly increases in the plasma and reaches a maximal level 1.5 h after intake and decreases thereafter [Murota, K., *et al.*, 2010]. Isorhamnetin shows a constant level for 6 h after oral administration quercetin glucoside [Murota, K., *et al.*, 2010]. On the other hand, quercetin conjugates and isorhamnetin do not reveal significant increases in the plasma during 6 h after intake of rutin [Murota, K., *et al.*, 2010]. These data indicate that quercetin and its metabolites are absorbed and metabolized in the site of upper intestine. Moreover, the range of muscle concentrations of quercetin was only detected from 0.1 nM to 163 nM in rats [Xie, Y., *et al.*, 2014]. Furthermore, only nM level of quercetin is absorbed by Caco-2 cells [Boyer, J., *et al.*, 2005].

Recent researches have shown that the bioavailabilities of dietary flavonoids are enhanced by enzymatic modification, because of their oligoglycoside moieties exert appropriate hydrophilic properties in the intestinal fluid and are readily digested to produce aglycone form at the intestinal mucosa membranes [Kohara, A., *et al.*, 2017; Park, HY., *et al.*, 2013]. Enzymatically modified isoquercitrin (EMIQ), a mixture of quercetin monoglucosides, derives from rutin via enzymatic hydrolysis (Fig. GI-1) [Murota, K., *et al.*, 2010]. Concomitant with a series of reactions including removal of the rhamnosyl group and subsequent treatment with glycosyltransferase, EMIQ absorption in humans is similar to quercetin glycosides from natural sources [Makino, T., *et al.*, 2009; Olthof, MR., *et al.*, 2000; Erlund, I., *et al.*, 2000]. The bioavailability

of EMIQ is 17-folds greater than quercetin and 3-times greater than isoquercitrin [Kohara, A., *et al.*, 2017; Murota, K., *et al.*, 2010]. Therefore, EMIQ is an efficient glycosidic form of quercetin and a safe food additive, which is acknowledged by the US Food & Drug Administration (FDA) [Morita, R., *et al.*, 2011; Murota, K., *et al.*, 2010].

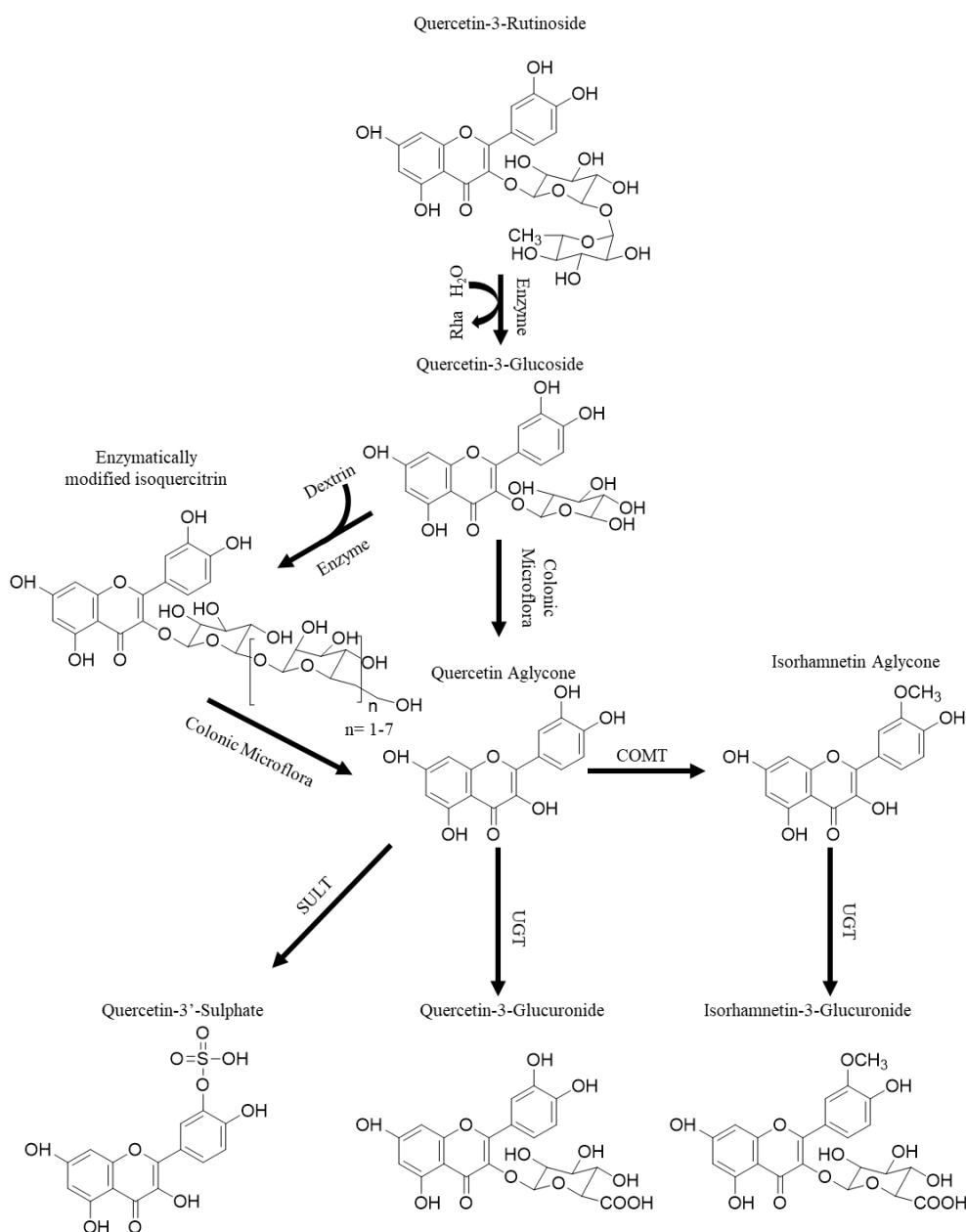


Figure. 1-1. Schematic representation of quercetin biotransformation. Adapted from several references. Abbreviations: COMT, catechol-O-methyl transferase; LPH, lactase phlorizin hydrolase; SULT, sulfotransferase; UGT, uridine 5'-diphospho-glucuronosyltransferase.

Aim of this dissertation

This dissertation aimed to investigate the potential prevention effects of quercetin and its derivatives on anti-hyperglycemia and anti-obesity and explore their underlying molecular mechanisms *in vitro* and *in vivo*. In the next chapter (Chapter 2), the author declared that quercetin and isorhamnetin significantly increased glucose uptake via translocation of GLUT4 in L6 myotubes through different pathways *in vitro*. *In vivo*, the author measured incorporated amounts of quercetin and isorhamnetin after oral administration of EMIQ (10-1000 mg/kg BW) to ICR mice using the HPLC coupled with a diode array detector. GLUT4 translocation was induced by EMIQ in the skeletal muscles of mice. Absorbed quercetin existed mainly as conjugates, but certain amounts of quercetin aglycone were detected: plasma concentrations of quercetin aglycone form were 4.95 and 6.80 nM after administration of EMIQ at 10 and 100 mg/kg BW, while muscle concentrations of aglycone form of quercetin and isorhamnetin were 0.26 and 0.44 nmol/g, respectively, after administration of EMIQ (100 mg/kg BW). In Chapter 3, the author to clarify the prevention of quercetin and its derivatives, as isoquercitrin, rutin, and EMIQ, at a lower concentration range on hyperglycemia through a single oral administration *in vivo*. Pre-administration of isoquercitrin [1 µg or 1 mg/kg body weight (BW)], rutin (1 µg/kg BW), and EMIQ (1 mg/kg BW) suppressed postprandial hyperglycemia at 15 min after the glucose loading. Of these quercetin derivatives, isoquercitrin was the most effective compound for anti-hyperglycemia. The underlying mechanism is that pre-administration of isoquercitrin (1 µg or 1 mg/kg BW), rutin (1 µg or 1 mg/kg BW), and EMIQ (1 mg/kg BW) promoted GLUT4 translocation to the plasma membrane in skeletal muscle of mice through the AMPK-dependent signaling pathway. In Chapter 4, the author investigated whether quercetin and its glucoside

EMIQ would contribute to regulate energy metabolism in C57BL/6 mice for two weeks. EMIQ repressed the lipid accumulation and regulated energy metabolism through activating a fundamental metabolic factor, AMPK. EMIQ lowered mesenteric and epididymal fat deposition by inhibiting adipogenesis, promoting energy expenditure and increasing thermogenesis-related proteins expression, through AMPK phosphorylation in WAT. In the liver, EMIQ induced AMPK phosphorylation to upregulate fatty acid oxidation. In the muscle, EMIQ suppressed chronic hyperglycemia accompanied by GLUT4 translocation to the plasma membrane via the AMPK signaling pathway. In Chapter 5, the author investigated the effects of quercetin and its glucosides (isoquercitrin, rutin, and EMIQ) on anti-hyperglycemia and anti-obesity. Mice were fed a control or high-fat diet supplementation with quercetin, isoquercitrin, rutin and EMIQ for thirteen weeks. The author confirmed that quercetin and its glucosides prevented HF diet-induced insulin resistance by promoting GLUT4 translocation in skeletal muscle, and also prevented obesity by activating AMPK-dependent signaling pathway in adipose tissue and liver. Finally, general discussion of this dissertation was summarized (Chapter 6). Taken together, this dissertation demonstrated the vital mechanisms of quercetin and its derivatives on energy metabolism. The author concludes that quercetin and its derivatives have the potential to regulate energy metabolism.

Chapter 2 : Quercetin and its metabolite isorhamnetin promote glucose uptake through different signaling pathways in myotubes

Introduction

The prevalence of diabetes is alarmingly high, and the population of patients already reach 285 million [Ali, R., *et al.*, 2016]. Younger individuals also could be affected by this disease as a result of diet, behavior, and obesity [Benhalima, K., *et al.*, 2011]. Recent reports regard GLUT4 translocation to the plasma membrane as a novel way to improve insulin resistance. GLUT4 is an insulin-sensitive glucose transporter, which plays an important role in regulation of hyperglycemia in the postprandial state. GLUT4 rapidly translocates to the plasma membrane upon insulin stimulation. This translocation is activated by various upstream targets, including insulin receptor substrate 1 (IRS1), phosphoinositide 3-kinase (PI3K), and Akt [Bhattacharya, S., *et al.*, 2007; Qu, W., *et al.*, 2011]. Furthermore, exercise and energy depletion also increase glucose uptake and induce GLUT4 translocation through AMPK phosphorylation [Ojuka, EO., *et al.*, 2012; Röckl, KS., *et al.*, 2007]. In the last two decades, JAK/STAT pathway has been reported to involve in intricate biological responses, not only oncogenesis and apoptosis, but also glucose metabolism. IL-15, a cytokine secreted by mononuclear phagocytes, is implicated in modulating the activation of T cells and exerts effects on regulation of glucose metabolism in skeletal muscle cells [Amoyel, M., *et al.*, 2014; Krolopp, JE., *et al.*, 2016; Rückert, R., *et al.*, 2009].

It has been reported that flavonoids have a potency in term of promoting GLUT4

translocation. In the author's laboratory, Ueda reported that propolis extract promotes glucose uptake and GLUT4 translocation to the plasma membrane in skeletal muscle of mice [Ueda, M., *et al.*, 2013]; Epigallocatechin gallate increases GLUT4 translocation through PI3K phosphorylation in skeletal muscle [Ueda, M., *et al.*, 2008]; and procyanidin promotes GLUT4 translocation through activation of PI3K and AMPK phosphorylation in skeletal muscle of mice [Yamashita, Y., *et al.*, 2016]. Quercetin and isorhamnetin are considered to have a potency to prevent various diseases [Arias, N., *et al.*, 2017; Hu, S., *et al.*, 2015; Liu, Y., *et al.*, 2017; Ruan, Y., *et al.*, 2015]. Due to the poor bioavailability of quercetin, clarifying the effective concentration of quercetin and isorhamnetin on GLUT4 translocation and underlying mechanisms at a physiological concentration range is necessary.

EMIQ, a mixture of quercetin glycosides, manufactured from rutin by enzymatic glycosylation and possesses high water solubility [Makino, T., *et al.*, 2013; Murota, K., *et al.*, 2010]. Pharmacokinetic studies suggested that EMIQ is metabolized to liberate aglycone and conjugated forms of quercetin at the intestinal mucosa membranes and their plasma concentrations were increased after intake of EMIQ [Murota, K., *et al.*, 2010]. It has been reported that EMIQ is an effective antioxidant and has chemopreventive potential against the oxidative DNA damage and the development of hepatocellular lesions and tumors [Fujii, Y., *et al.*, 2013; Morita, R., *et al.*, 2011; Nishimura, J., *et al.*, 2010; Shimada, Y., *et al.*, 2010]. Furthermore, EMIQ also has ergogenic effect on preventing disused muscle atrophy in mice [Kohara, A., *et al.*, 2017]. Hence, EMIQ is an attractive quercetin glucoside with high bioavailability.

In this chapter, the author investigated whether quercetin and isorhamnetin increase glucose uptake and promote GLUT4 translocation to the plasma membrane and underlying mechanisms in cultured L6 myotubes. However, although EMIQ is an

attractive quercetin glucoside, its physiologic mechanism is not fully understood. Hence, the author examined whether EMIQ promotes GLUT4 translocation to the plasma membrane after oral administration in ICR mice, and verified the effective plasma and muscle concentrations of quercetin and isorhamnetin.

Material and methods

Materials

L6, the rat myoblast cell line, was obtained from Health Science Research Resources Bank (Osaka, Japan). DMSO, nobiletin and quercetin were from Wako Pure Chemical Industries (Osaka, Japan) and isorhamnetin was from Extrasynthese (Genay, France). AICAR, leptin, BAPTA-AM, resazurin, 2-deoxyglucose, glucose-6-phosphate dehydrogenase Resazurin, sulfatase from *abalone entrails* (Type VIII), and β -glucuronidase from *Escherichia coli* (Type IX-A) were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA), Blocking-One and Blocking One-P solutions were obtained from Nacalai Tesque (Kyoto, Japan). Reduced serum medium (Opti-MEM) and STO-609 were from Santa Cruz Biotechnology (Santa Cruz, CA). Minimum essential medium (MEM) was from Nissui Pharmaceutical (Tokyo, Japan). Protease and phosphatase inhibitor cocktails were purchased from Roche Diagnostics (Tokyo, Japan). Lipofectamine™ RNAiMAX was obtained from Invitrogen Life Technologies (Burlington, Ontario, Canada). All other reagents used were of the highest grade available commercially. For western blot analysis, anti-GLUT4 mouse IgG, anti-Akt rabbit IgG, anti-phospho-AMPK α (Thr172) rabbit IgG, anti-AMPK α rabbit IgG, anti-JAK2 rabbit IgG, anti-phospho-JAK2 rabbit IgG, anti-STAT3 mouse IgG, anti-phospho-STAT3 (Tyr705) rabbit mAb, anti-mouse IgG, and anti-rabbit IgG antibodies were from Cell Signaling Technology (Danvers, MA). Anti-STAT1, anti-STAT1

(phosphoY701), anti-STAT5a, and anti-STAT5a (phosphoY694) antibodies were from Abcam (Cambridge, MA). Anti-IRS1 rabbit IgG antibody was from Upstate Cell Signaling Solution (Lake Placid, NY). Anti-phospho-IRS1 (Ser307) and anti-phosphotyrosine mouse mAb antibodies were purchased from BD Transduction Laboratories (San Diego, CA). Anti-IR rabbit IgG, anti- β -actin rabbit IgG, anti-phospho-Akt (Thr308) rabbit IgG and anti-phospho-Akt (Ser473) rabbit IgG antibodies were from sigma Chemical (St. Louis, MO). For siRNA assay, the following siRNAs were used: 5'-GCA UAU GCU GCA GGU AGA-3' and 5'-UCU ACC UGC AGC AUA UGC-3' for AMPK α 1 and AMPK α 2, respectively; 5'-CCA CCC AAU CAU GUC UUC CAC AUA G-3' and 5'-CUA UGU GGA AGA CAU GAU UGG GUG G-3' for JAK2; 5'-GCU GGC AUU CUG GAC UGC AAG UAG A-3' and 5'-UCU ACU UGC AGU CCA GAA UGC CAG C-3' for JAK3 and 5'-AUU CUA UCA CUA GCG UGA CUU-3' for the control.

Equipment

HPLC equipped with a DGU-20A 3R degas unit, LC-20AD XR binary pump, SIL-20AC XR auto sampler, diode array detector (SPD-M20A), column oven and communications bus module connected to a LC work station was performed (Shimadzu Corporation, Kyoto, Japan). A Cadenza CL-C18 column and a guard column were ϕ 4.6 mm $\times$ 250 mm and ϕ 5 mm $\times$ 2 mm from Imtakt, respectively (Kyoto, Japan).

Chromatographic conditions

Aqueous phosphoric acid (2: 10000, v/v, phosphoric: MilliQ water) was used as mobile phase A and acetonitrile was mobile phase B. Sample separation was processed with a gradient system. The initial injection volume was set at 10 μ l and the flow rate is 1.0 ml/min. The gradient program was 20% B from 0 to 13 min, 50-80% B from 13 to 33 min, 100% B from 33 to 43 min, 20% B from 43 to 60 min. Absorbance of the

quercetin and isorhamnetin were monitored at 370 nm using a UV detector.

Limit of detection and limit of quantification

The ratio of the absolute amount of analyte to noise equal to 3:1 was defined as the limit of detection (LOD) and the ratio of the absolute amount of analyte to noise equal to 10:1 was defined as the limit of quantification (LOQ).

Cell culture

L6 myoblasts were maintained in MEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C. Cells (2.2×10^4 cells/ml) were seeded into 96-well plates or 60-mm dishes. After 2 days, myoblasts were supplemented with MEM containing 2% FBS for 7 days.

Animal treatment

All animal experiments were approved by the Institutional Animal Care and Use Committed of Kobe University (Permission 29-05-02) and carried out according to the guidelines for animal experiments at Kobe University Animal Experimentation Regulation. Male ICR mice (5 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and housed in an air-conditioned room ($23 \pm 2^\circ\text{C}$) under a 12 h light-dark cycle with free access to water and commercial chow (Research Diets, Tokyo, Japan). Mice were acclimatized for 1 week. To measure quercetin and isorhamnetin concentrations, the mice were sacrificed 90 min after the administration of EMIQ at 10, 100, or 1000 mg/kg body weight, or water as a vehicle control after 18 h fasting. Plasma and soleus muscle were collected and kept at -80°C until use.

2DG uptake assay

Glucose uptake assay was performed as previously described [Yamamoto, N., *et al.*, 2010]. The differentiated L6 myotubes on 96-well plates were maintained in serum-free MEM containing 0.2% w/v bovine serum albumin (BSA) for 18 h and then treated

with indicated concentrations of quercetin, isorhamnetin (0.01–10³ nM), DMSO (vehicle control) and insulin (100 nM, positive control) for 4 h, respectively.

Preparation of the plasma membrane and whole protein fractions

The differentiated L6 myotubes were maintained in serum-free MEM containing 0.2% w/v BSA for 18 h. Quercetin or isorhamnetin (0.1–10 nM), DMSO (final 0.1%), insulin (100 nM), AICAR (1 mM), leptin (10 nM) or IL-15 (100 ng/ml) were treated to myotubes, respectively. The plasma membrane fraction and whole protein fractions were prepared from quercetin- and isorhamnetin-treated myotubes as previously described [Nishiumi, S., *et al.*, 2007]. *In vivo*, the plasma membrane fraction and tissue lysate were prepared [Nishiumi, S., *et al.*, 2007].

Western blotting

Equal amounts of proteins were separated and transferred by SDS-polyacrylamide gels. After blocking, the membrane was incubated with an appropriate primary antibody overnight and followed by secondary antibody for 1 h. Individual band density was determined using ImageJ image analysis software.

Immunoprecipitation

Equal amounts of cell lysate was incubated with Anti-IR antibody (1:100) for 2 h at 4°C. Then, the mixture was incubated with protein A/G plus-agarose beads (10 µl) in a rotator overnight at 4°C. These reaction mixture was separated by SDS-polyacrylamide gels.

siRNA experiments

Myotubes were transfected with siRNA for 48 h at the end of differentiation period. Subsequently, quercetin or isorhamnetin in the indicated concentrations were treated to cells in fresh Opti-MEM.

Determination of plasma concentration of quercetin and isorhamnetin in the mice

orally administrated EMIQ

To determine quercetin and isorhamnetin concentrations in the plasma, the plasma was treated with deconjugation enzymes as previously described [Wang, L., *et al.*, 2018; Yamashita, Y., *et al.*, 2016]. Briefly, plasma was mixed with ascorbic acid (50 μ l, 20% (w/v)) to prevent oxidation during the extraction procedure. Next, 2 μ l of nobiletin (1 mM) was added to tubes and used as an internal standard. The plasma was incubated with potassium phosphate buffer (125 μ l, 75 mM, and pH 6.8) with or without β -glucuronidase (500 U/sample) and sodium acetate buffer (125 μ l, 200 mM, and pH 5.0) with or without sulfatase (10 U/sample) in the tubes for 2 h at 37°C. After hydrolyzed, protein in the sample was precipitated by centrifuging at 3000 $\times$ g for 15 min. Solid phase extraction (SPE) was involved in separate quercetin and isorhamnetin. Sep-Pak C18 Vac Cartridge (1 cc, 50 mg, Waters, MA, USA) was preconditioned with 5 ml of methanol and 5 ml of MilliQ water. Mixture was implemented in cartridge. Cartridge was then washed with 5 ml of 10% (v/v) methanol and 2 ml 95% (v/v) methanol. The solution was evaporated *in vacuo*, dissolved in 50% methanol (50 μ l) and subjected to HPLC.

Determination of muscle concentration of quercetin and isorhamnetin in the mice orally administrated EMIQ

Tissue (50 mg) was homogenized using a motor homogenizer (PRO Scientific Inc, Connecticut, USA) with 300 μ l phosphate buffer saline as previously described [Wang, L., *et al.*, 2018]. The homogenate sample was mixed with ascorbic acid (25 μ l, 2% (w/v)) and nobiletin (2 μ l, 1 mM). Then the mixture was removed to perfluoroalkoxy alkanes (PFA) centrifuge tubes (15 ml, Savillex, Eden Prairie, MN, USA) and was reacted with potassium phosphate buffer (375 μ l, 75 mM, and pH 6.8) with or without β -glucuronidase (500 U/sample) for 1 h at 37°C. After that the mixture was hydrolyzed

with sodium acetate buffer (750 μ l, 200 mM, and pH 5.0) with or without sulfatase (10 U/sample) for another 1 h. To separate quercetin and isorhamnetin, acetonitrile (1 ml) was used for precipitating the protein. After centrifuging, supernatant was transferred to new PFA tube and was reacted with ethyl acetate (1 ml) to extract the quercetin and isorhamnetin. The ethyl acetate layer was removed to different PFA tube after vortexing and centrifuging. This step was replicated for another two times. Collected ethyl acetate layer was dried by evaporation and dried material was dissolved in methanol (50 μ l, 50% (v/v)) and analyzed.

Calibration curves

Calibration curves were prepared with plasma or muscle obtained from ICR mice administrated distilled water with known concentration of quercetin or isorhamnetin (0-3.2 μ M). The peak area ratios of quercetin or isorhamnetin to nobiletin were used to produce the linear regression equation in each calibration curve.

Statistical analysis

Data represent mean $\pm$ SD (Fig. (2-1)-(2-7)) or mean $\pm$ SE (Fig. 2-8). Statistical significance was analyzed using Dunnett, Student's *t* or Tukey–Kramer multiple-comparison tests, and the level of significance was $p < 0.05$.

Results

Quercetin and isorhamnetin promoted glucose uptake in L6 myotubes

The author investigated the effect of quercetin and isorhamnetin on glucose uptake in L6 myotubes. As shown in Figure 2-1, Quercetin (0.1 and 1 nM) and isorhamnetin (1 nM) significantly increased glucose uptake compared to the DMSO treatment. Glucose uptake was decreased by both compounds at 10 nM. However, 1 μ M and 10 μ M quercetin and isorhamnetin significantly increased glucose uptake again. These

results indicated that a biphasic increase in glucose uptake was promoted by quercetin and isorhamnetin in L6 myotubes.

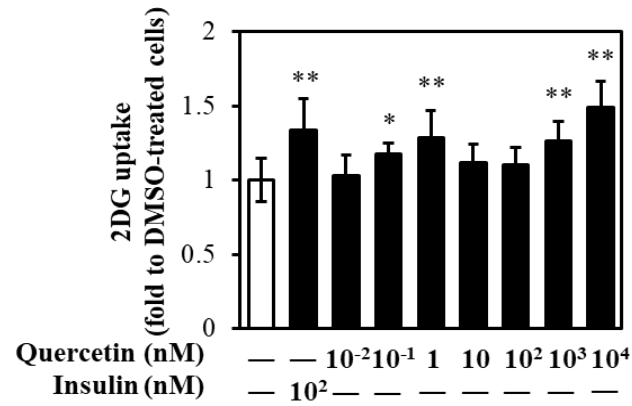
Effect of quercetin and isorhamnetin on GLUT4 translocation

The author next assessed the effect of quercetin and isorhamnetin on GLUT4 translocation in L6 myotubes. As would be expected, quercetin and isorhamnetin (except 0.1 nM isorhamnetin) significantly induced translocation of GLUT4 to the plasma membrane (Fig. 2-2A). Moreover, quercetin and isorhamnetin did not affect the expression of GLUT4 in the whole fraction (Fig. 2-2A). Furthermore, quercetin-induced GLUT4 translocation exhibited a bell-shaped curve in a time-dependent manner (Fig. 2-2B). Quercetin-induced GLUT4 translocation was significantly increased at 15 min and reached plateau from 30 to 60 min (Fig. 2-2B). From these results, quercetin and isorhamnetin promoted translocation of GLUT4 to the plasma membrane without affecting GLUT4 expression in L6 myotubes.

Effect of quercetin and isorhamnetin on the insulin signaling pathway in L6 myotubes

Next, the author investigated the molecular mechanisms of both compounds-induced GLUT4 translocation to the plasma membrane. Insulin receptor (IR), IRS1, PI3K, and Akt are important targets of insulin pathway. IR phosphorylation was not increased by quercetin and isorhamnetin (Fig. 2-3A). Interestingly, quercetin at 10 nM significantly increased phosphorylation of IRS1, PI3K and Akt at Thr308 and Ser473, which are downstream factors of IR (Fig. 2-3B). Akt phosphorylation at Ser473 was also significantly increased by quercetin and isorhamnetin at higher concentrations (Fig. 2-7). Surprisingly, PI3K phosphorylation also increased by 0.1 nM isorhamnetin, while expression level of these proteins were not affected by quercetin and isorhamnetin.

(A)



(B)

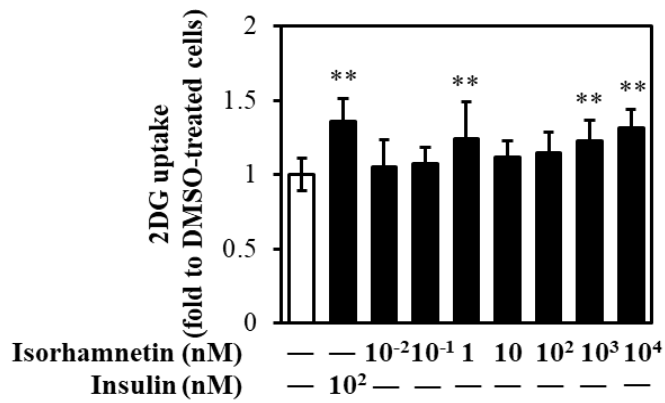


Figure 2-1. Quercetin and isorhamnetin promoted glucose uptake in L6 myotubes. Glucose uptake was measured in L6 myotubes treated with quercetin (A) and isorhamnetin (B) for 4 h. Data are shown as the mean $\pm$ SD (n = 3). * p < 0.05 and ** p < 0.01 vs control cells by Dunnett's multiple comparison test.

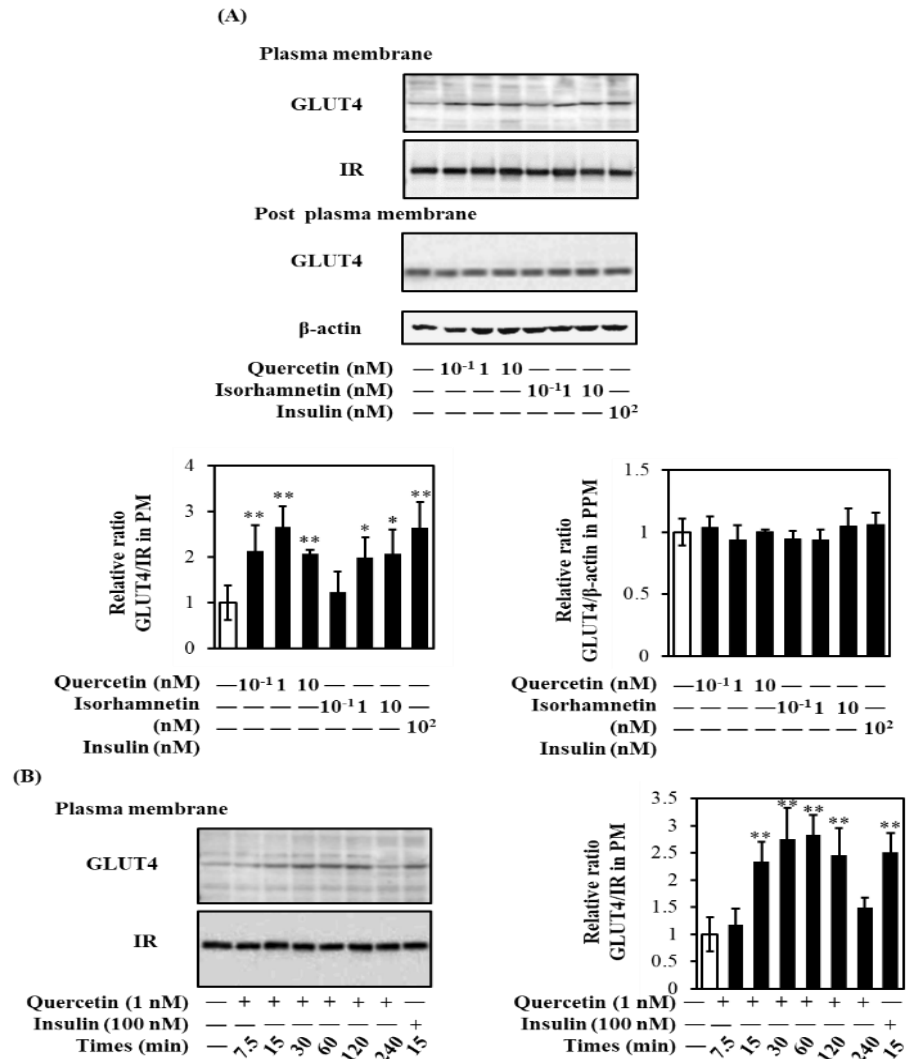


Figure 2-2. Quercetin and isorhamnetin induced GLUT4 translocation in L6 myotubes. (A) Quercetin and isorhamnetin at 10^{-1} , 1, 10 nM were treated to L6 myotubes for 15 min, respectively. The expression of GLUT4 and IR β in the plasma membrane fraction and GLUT4 and β -actin in the cell lysate were determined by western blot analysis. (B) L6 myotubes were treated with quercetin (1 nM) for the indicated times (0–240 min), respectively. The expression of GLUT4 and IR β in the plasma membrane fraction in the cell lysate were determined by western blot analysis. Data are shown as the mean $\pm$ SD (n = 3). * p < 0.05 and ** p < 0.01 vs control cells by Dunnett's multiple comparison test.

Effect of quercetin and isorhamnetin on the AMPK signaling pathway in L6 myotubes

AMPK phosphorylation has been reported to trigger GLUT4 translocation as an insulin-independent mechanism [Röckl, KS., *et al.*, 2007]. Quercetin at 0.1 nM and 1 nM significantly increased phosphorylation of AMPK and ACC (Fig. 2-4A). Therefore, AMPK upstream factors, such as LKB1, CaMKK β , and intracellular-free calcium (Ca^{2+}), were examined by the author. Quercetin-induced AMPK phosphorylation was abolished by CaMKK β inhibitor STO-609 (Fig. 2-4B). In contrast, phosphorylation of LKB1 was not activated by quercetin and isorhamnetin (Fig. 2-4C). As shown in Fig. 2-4D, quercetin-induced AMPK phosphorylation was not affected by the intracellular Ca^{2+} chelator 1,2-Bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM). From these results, 0.1 and 1 nM quercetin principally activated AMPK phosphorylation, and 10 nM quercetin activated phosphorylation of IRS1, PI3K and Akt in L6 cells.

Effect of quercetin and isorhamnetin on the JAK/STAT signaling pathway in L6 myotubes

Recently, JAK/STAT pathway has been reported to maintain glucose homeostasis and induce GLUT4 translocation to the plasma membrane [Krolopp, JE., *et al.*, 2016]. As shown in Fig. 2-5, JAK2 and JAK3 phosphorylation were significantly increased by isorhamnetin, but not quercetin (Fig. 2-5A and B). Concurrently, isorhamnetin at 10 nM and 1 nM significantly activated phosphorylation of STAT3 and STAT5, but not STAT1, respectively (Fig. 2-5C). Furthermore, JAK2 phosphorylation was significantly increased by both quercetin and isorhamnetin at 1 μM and 10 μM (Fig. 2-7).

The author further confirmed whether these compounds induce GLUT4 translocation through activation of AMPK α , JAK2, and JAK3 by siRNA. These data

indicated that quercetin- and AICAR-induced GLUT4 translocation, but not isorhamnetin-induced GLUT4 translocation, were significantly decreased after treatment with siRNA for AMPK (Fig. 2-6A). On the contrary, isorhamnetin- and leptin-induced GLUT4 translocation were decreased by siJAK2, while quercetin-induced GLUT4 translocation was not attenuated by siJAK2 (Fig. 2-6B). As shown in Fig. 2-6C, siRNA for JAK3 only slightly decreased isorhamnetin-induced GLUT4 translocation. These results illustrated that quercetin increased AMPK phosphorylation to induce GLUT4 translocation to the plasma membrane, while isorhamnetin primarily promoted translocation of GLUT4 through phosphorylation of JAK2.

Effect of enzymatically modified isoquercitrin (EMIQ) on GLUT4 translocation *in vivo*

EMIQ is an artificial quercetin glycoside with high bioavailability [Murota, K., *et al.*, 2010]. Thus, the author first investigated the effects of EMIQ in high concentration on inducing GLUT4 translocation to the plasma membrane in mice. As shown in Fig. 2-8, GLUT4 translocation was significantly increased by oral administration of EMIQ at 10 and 100 mg/kg body weight in skeletal muscles of mice. On the contrary, the expression levels of GLUT4 was not promoted by EMIQ in tissue lysate (Fig. 2-8). These results indicated that low concentrations of EMIQ were more effective than high concentration of EMIQ at 1000 mg/kg body weight.

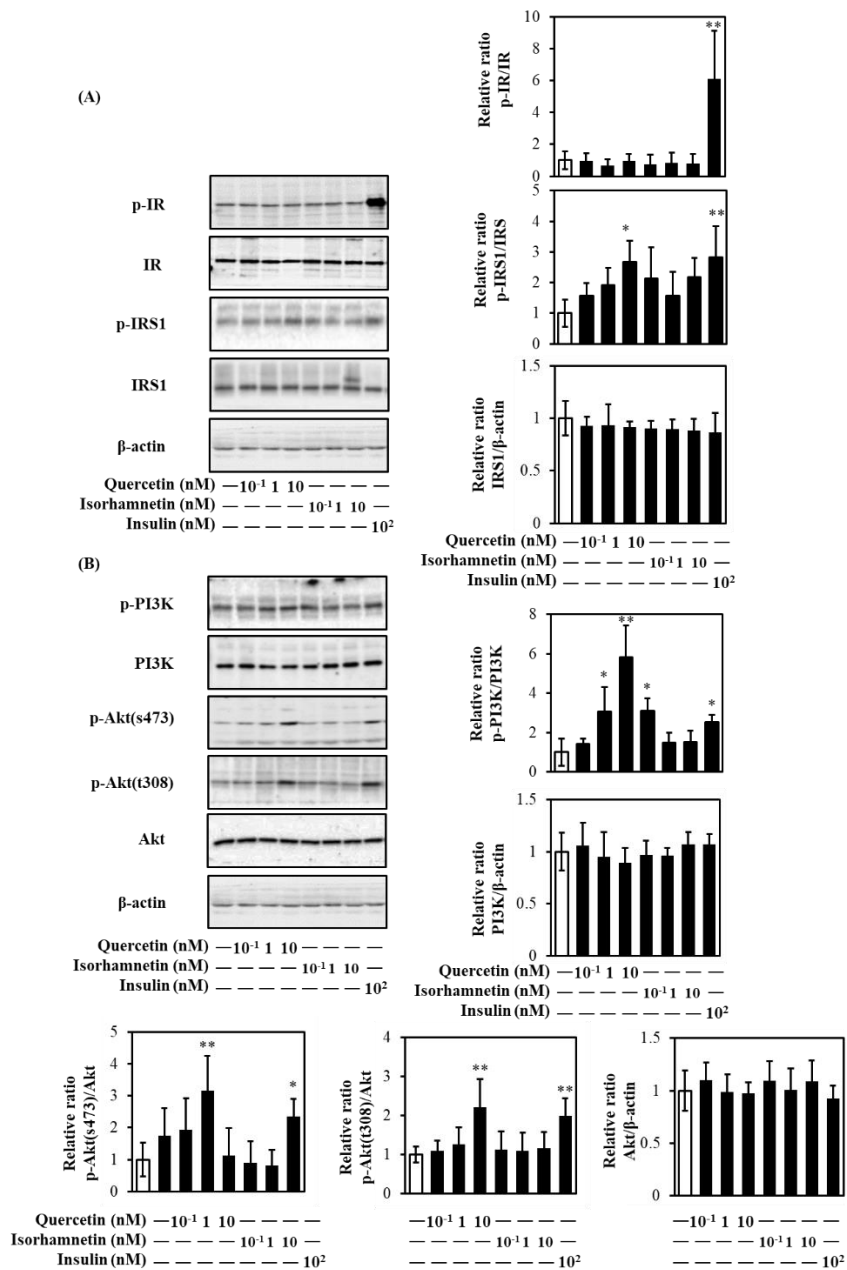


Figure 2-3. Effect of quercetin and isorhamnetin on the insulin signaling pathway in L6 myotubes.

Quercetin and isorhamnetin at 10⁻¹, 1, 10 nM were treated to L6 myotubes for 15 min, respectively. (A) The phosphorylation and expression levels of (A) IR and IRS1, and (B) PI3K and Akt were detected by western blot analysis. Data are shown as the mean ± SD (n = 3). **p* < 0.05 and ***p* < 0.01 vs control cells by Dunnett's multiple comparison test.

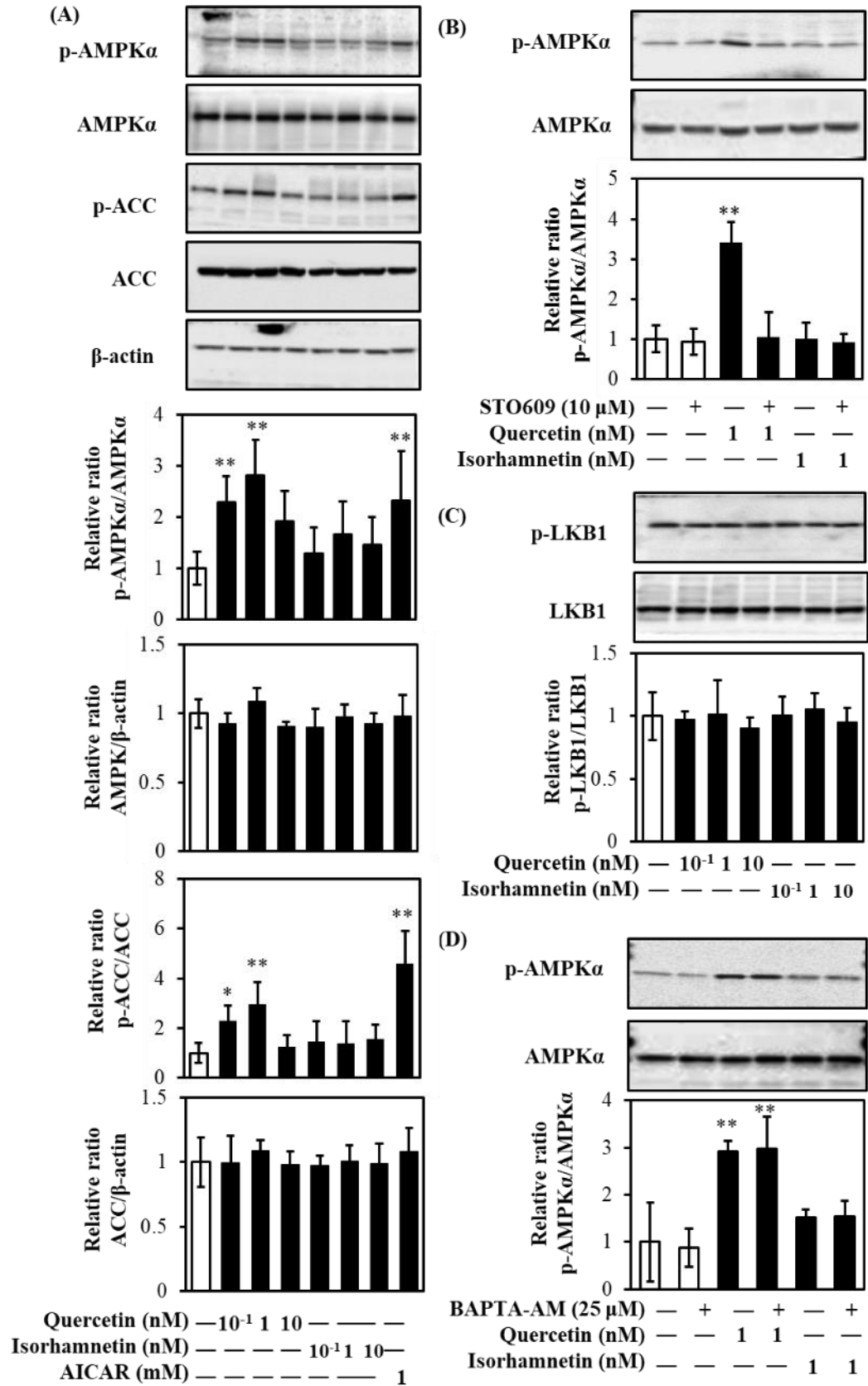


Figure 2-4. Effect of quercetin and isorhamnetin on the AMPK signaling pathway in L6 myotubes.

Quercetin and isorhamnetin at 10^{-1} , 1, 10 nM were treated to L6 myotubes for 15 min in the absence or presence of STO-609 or BADPA-AM, respectively. (A) Quercetin and isorhamnetin at 10^{-1} , 1, 10 nM were treated to L6 myotubes for 15 min. The phosphorylation and expression levels of AMPK and ACC were detected by western blot analysis. (B) Quercetin and isorhamnetin at 1 nM were treated to L6 myotubes for 15 min in the presence of STO-609. The phosphorylation and expression levels of AMPK were detected by western blot analysis. (C) Quercetin and isorhamnetin at 10^{-1} , 1, 10 nM were treated to L6 myotubes for 15 min. The phosphorylation and expression levels of LKB1 were detected by western blot analysis. (D) Quercetin and isorhamnetin at 1 nM were treated to L6 myotubes for 15 min in the presence of BADPA-AM. The phosphorylation and expression levels of AMPK were detected by western blot analysis. Data are shown as the mean $\pm$ SD ($n = 3$). $*p < 0.05$ and $**p < 0.01$ vs control cells by Dunnett's multiple comparison test.

The plasma concentrations of quercetin and isorhamnetin in mice

The plasma concentrations of aglycone and conjugated forms of quercetin and isorhamnetin were measured by the author. The concentrations were 4.95 ± 0.82 nM and 6.80 ± 2.00 nM for quercetin aglycone and 11.69 ± 3.99 nM and 54.89 ± 27.44 nM for total quercetin concentrations in the plasma, 90 min after oral administration of EMIQ at 10 and 100 mg/kg body weight, (Table. 2-3). Furthermore, after administration of EMIQ at 10, and 100 mg/kg body weight, isorhamnetin aglycone was not detected in the plasma (Table. 2-3). Compared to the concentrations of aglycone forms of quercetin and isorhamnetin, total concentrations of compounds were higher, which suggested that only certain amounts of quercetin and isorhamnetin existed in their aglycone form in the plasma and most of them presented in their conjugated forms. These results suggested that EMIQ was digested to liberate certain amounts of quercetin and isorhamnetin.

The muscle concentrations of quercetin and isorhamnetin in mice

Furthermore, the author quantified the muscle concentrations of aglycone and conjugated forms of quercetin and isorhamnetin in the same mice. In the muscle, the concentrations of aglycone were 0.26 ± 0.18 and 2.57 ± 0.94 nmol/g tissue for quercetin and 0.44 ± 0.38 and 6.11 ± 2.54 nmol/g tissue for isorhamnetin, after oral administration of EMIQ at 100, and 1000 mg/kg body weight, respectively (Table 2-4). Quercetin and isorhamnetin were observed minor increases in the muscle after enzymatic hydrolysis with β -glucuronidase and sulfatase. The total concentrations of quercetin were 0.19 ± 0.07 , 0.49 ± 0.42 , and 4.77 ± 1.26 nmol/g tissue, and the total concentrations of isorhamnetin were 0.22 ± 0.17 , 0.77 ± 0.41 , and 7.99 ± 2.69 nmol/g tissue, respectively. The minor increases between free and total compounds in the muscle suggested that the quercetin and isorhamnetin mainly existed in their aglycone forms in the muscle.

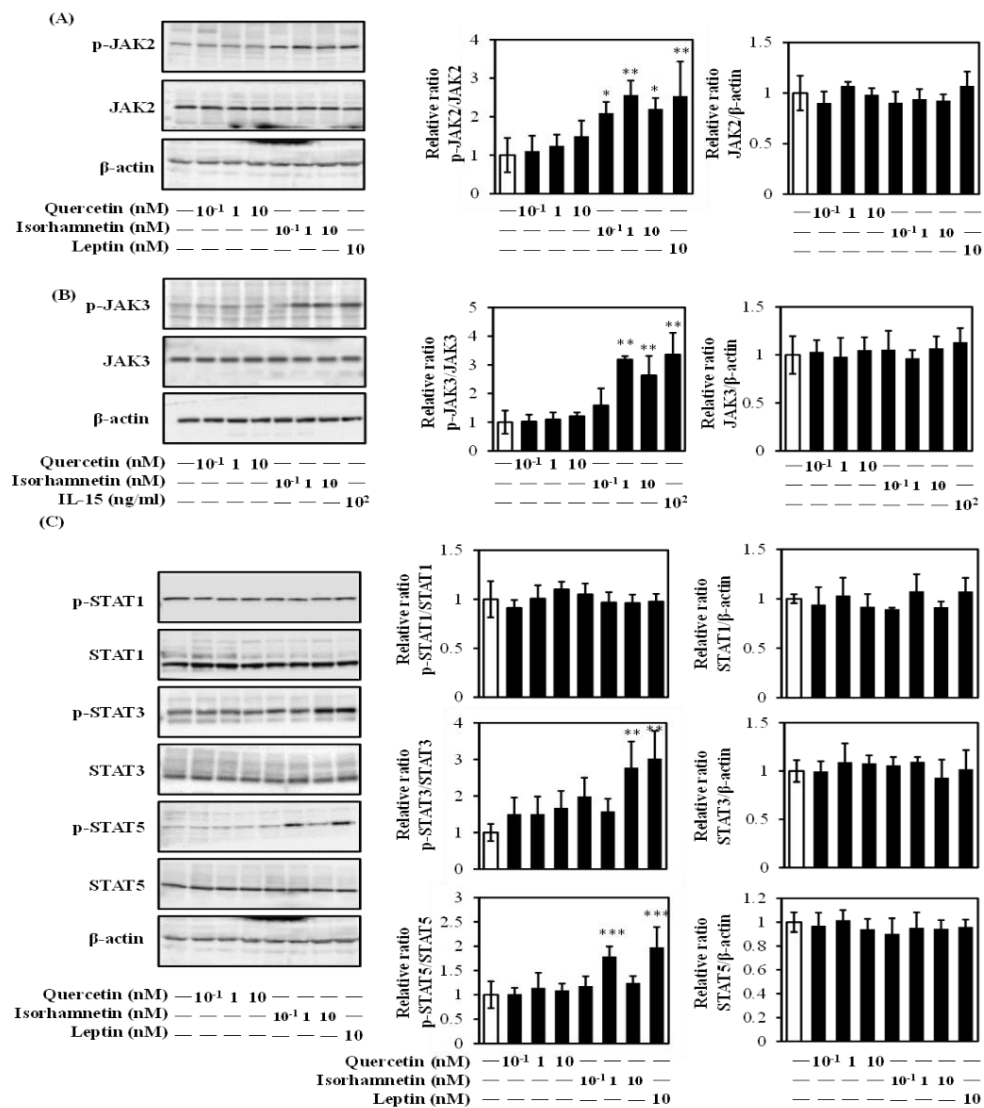


Figure 2-5. Effect of quercetin and isorhamnetin on the JAK/STAT signaling pathway in L6 myotubes. Quercetin and isorhamnetin at 10⁻¹, 1, 10 nM were treated to L6 myotubes for 15 min, respectively. (A) The phosphorylation and expression levels of (A) JAK2, (B) JAK3 and (C) STAT1, STAT3 and STAT5 were detected by western blot analysis. Data are shown as the mean $\pm$ SD (n = 3). **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 vs control cells by Dunnett's multiple comparison test.

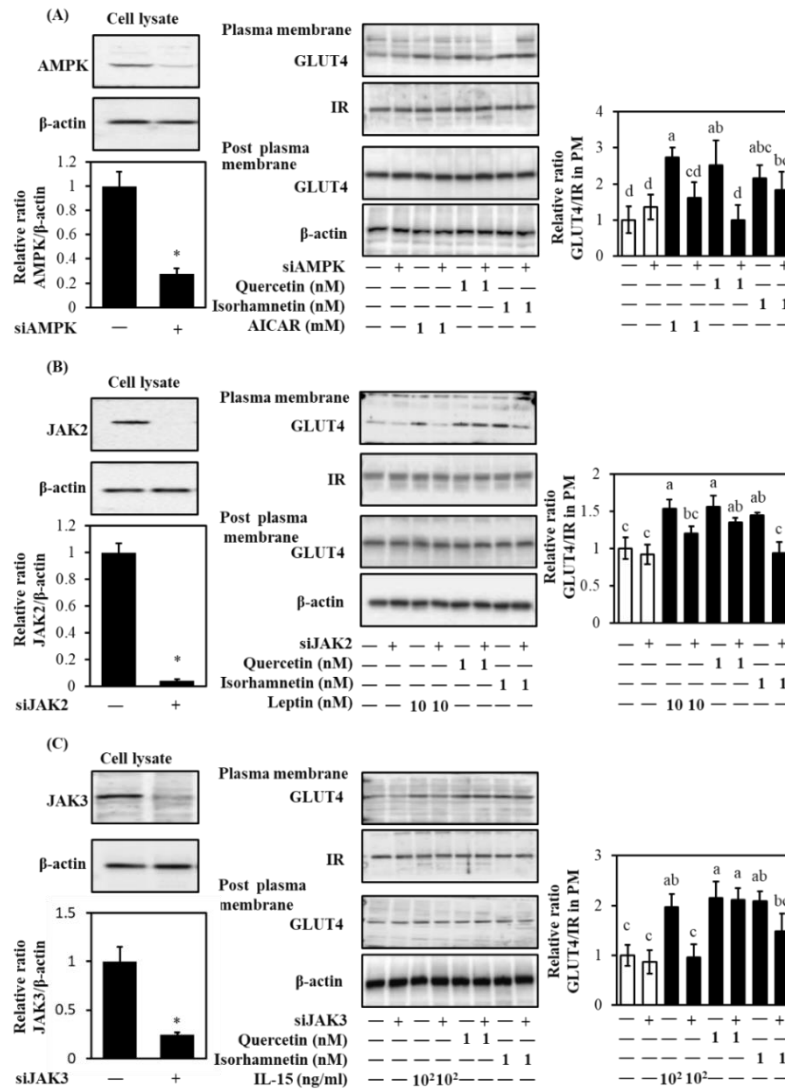


Figure 2-6. Effect of quercetin and isorhamnetin on GLUT4 translocation via AMPK- and JAK/STAT-dependent pathways. L6 myotubes were transfected with siRNAs targeting (A) AMPK α , (B) JAK2, (C) JAK3, or control siRNA and treated with 1 nM quercetin and isorhamnetin for 15 min, respectively. GLUT4 translocation and its expression were detected by western blot analysis. Data were shown as the mean $\pm$ SD ($n = 3$). * $p < 0.05$ vs control cells by student's t -test. Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ($p < 0.05$).

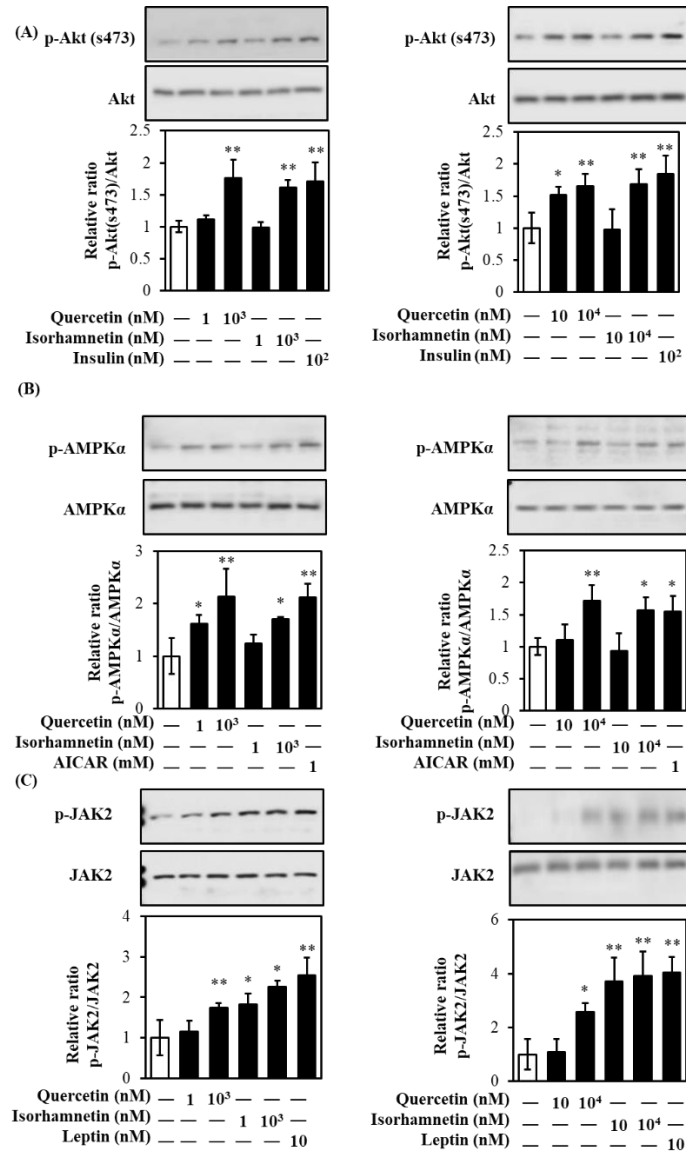


Figure 2-7. Effect of quercetin and isorhamnetin on the insulin-, AMPK-, and JAK/STAT- signaling pathway in L6 myotubes. Quercetin and isorhamnetin at 1, 10, 10³, and 10⁴ nM, were treated to L6 myotubes for 15 min, respectively. The phosphorylation and expression levels of (A) Akt, (B) AMPK and (C) JAK2 were detected by western blot analysis. Data were shown as the mean $\pm$ SD (n = 3). * p < 0.05 and ** p < 0.01 vs control cells by Dunnett's multiple comparison test.

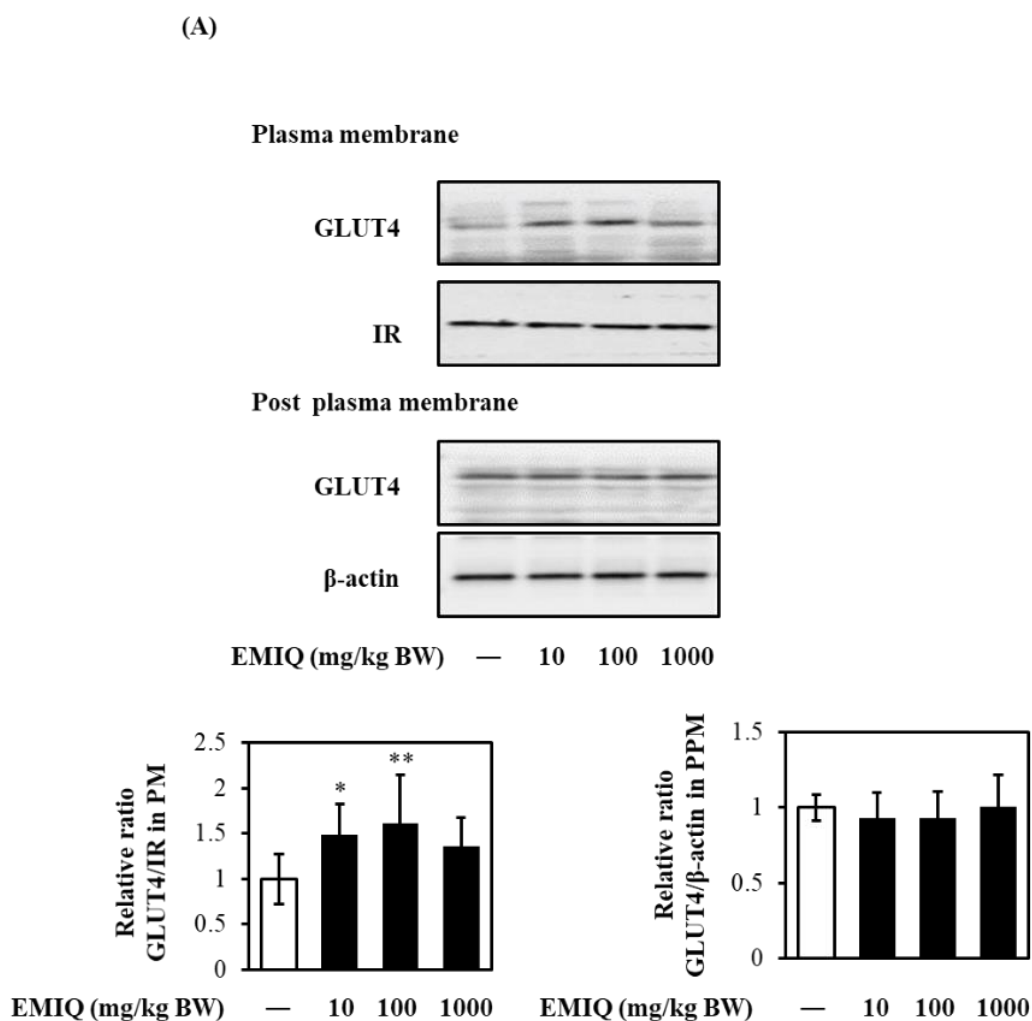


Figure 2-8. EMIQ induced GLUT4 translocation in mice. (A) EMIQ or water alone were orally administrated to ICR mice. The expression of GLUT4 and IR β in the plasma membrane fraction and GLUT4 and β -actin in the tissue lysate were determined by western blot analysis. Data are shown as the mean $\pm$ SE (n = 5). * p < 0.05 and ** p < 0.01 vs control group by Dunnett's multiple comparison test.

Table 2-1. Limit(s) of detection (LOD) and limit (s) of quantification (LOQ) of quercetin and isorhamnetin detected by UV detectors in plasma

	LOD ^a (nM)	LOQ ^b (nM)
Compound	HPLC-UV ^c	HPLC-UV ^c
Quercetin	1.38	4.61
Isorhamnetin	1.68	5.61

Table 2-2. Limit(s) of detection (LOD) and limit (s) of quantification (LOD) of quercetin and isorhamnetin detected by UV detectors in muscle

	LOD ^a (nmol/g)	LOQ ^b (nmol/g)
Compound	HPLC-UV ^c	HPLC-UV ^c
Quercetin	0.038	0.128
Isorhamnetin	0.039	0.130

- LOD is defined as the absolute amount of analyte when its signal-to-noise ratio (S/N) is 3.
- LOQ is defined as the absolute amount of analyte when its signal-to-noise ratio (S/N) is 10.
- Absorbance monitored at 370 nm.

Table 2-3. Concentrations of quercetin and isorhamnetin in plasma of mice after orally administration of EMIQ.

Compounds		EMIQ 10 mg/kg body weight	EMIQ 100 mg/kg body weight	EMIQ 1000 mg/kg body weight
Quercetin	Aglycone form	4.95 ± 0.82	6.80 ± 2.00	138.43 ± 45.14
	Conjugated form	11.69 ± 3.99	54.89 ± 27.44	571.60 ± 225.41
Isorhamnetin	Aglycone form	N.D.	N.D.	130.74 ± 51.52
	Conjugated form	2.75 ± 2.77	14.62 ± 4.62	351.83 ± 139.88

ICR mice were orally administrated EMIQ at 10, 100, or 1000 mg/kg body weight, or water as a vehicle control after 18 h fasting. Blood was collected from a cardiac puncture 90 min after the administration. Plasma was prepared and used for measurement of quercetin and isorhamnetin by HPLC with or without deconjugation.

Discussion

Nowadays, polyphenols and polyphenol-rich food materials have attracted more attention from scientists. It is reported that procyanidin-rich cacao liquor procyanidin extract [Yamashita, Y., *et al.*, 2012], glabridin [Sawada, K., *et al.*, 2014], and epigallocatechin gallate [Ueda-Wakagi, M., *et al.*, 2018] prevent hyperglycemia by different mechanisms. Quercetin and isorhamnetin consumption have been associated with reducing risks of cardiovascular disease, cancer, and obesity [Hu, S., *et al.*, 2015; Liu, Y., *et al.*, 2017; Roslan, J., *et al.*, 2017; Ruan, Y., *et al.*, 2015]. The bioavailability

of quercetin and isorhamnetin are poor [Burak, C., *et al.*, 2017; Formica, JV., *et al.*, 1995; Khor, CM., *et al.*, 2017; Moon, YJ., *et al.*, 2008; Xie, Y., *et al.*, 2014]. Hence, it is necessary to detect their functions within a physiological concentration range. In this chapter, the author confirmed that glucose uptake and GLUT4 translocation were promoted by quercetin and isorhamnetin in L6 myotubes.

A biphasic increase of glucose uptake was induced by quercetin and isorhamnetin. Glucose uptake was increased in a dose-dependent manner from 0.01 nM to 1 nM and decreased at 10 nM and 100 nM by both compounds (Fig. 2-1). However, 1 μ M and 10 μ M quercetin and isorhamnetin significantly increased glucose uptake again (Fig. 2-1). The speculation of this complicated condition is that quercetin and isorhamnetin form macromolecules by migration and various non-covalent interactions, including hydrogen bonds, van der Waals forces, electrostatic interactions, and hydrophobic interactions in the solvent [Park, KH., *et al.*, 2017]. The solubility and absorption rate of quercetin and isorhamnetin are affected in relative tissues [Loftssona, T., and Duchêne, D., 2007]. Serotonylation of Rab and/or Rho protein is another possibility. A similar complicated glucose uptake is increased by serotonin through serotonylation of the small GTPase Rab4 protein in L6 myotubes [Ai-zoairy, R., *et al.*, 2017]. Further studies are needed to clarify this underlying mechanism.

In this chapter, CaMKK β /AMPK/ACC signaling were phosphorylated by quercetin at 0.1 nM and 1 nM (Fig. 2-4). While, quercetin activated phosphorylation of CaMKK β was not suppressed by BAPTA-AM, which indicated that quercetin did not affect the concentration of intracellular calcium. Similarly, it has been reported that glucose uptake is increased by calcium in muscle cells through both CaMKK β /AMPK-dependent and -independent mechanisms [Öberg, AI., *et al.*, 2011]. Furthermore, CaMKK β phosphorylation has been reported to be activated by androgen, ghrelin, and

AMP/ATP [Dhanya, R., *et al.*, 2017; Karacosta, L.G., *et al.*, 2012].

Table 2-4. Concentrations of quercetin and isorhamnetin in muscle of mice after orally administration of EMIQ. (Unit: nmol/g)

Compounds		EMIQ 10 mg/kg body weight	EMIQ 100 mg/kg body weight	EMIQ 1000 mg/kg body weight
Quercetin	Aglycone form	N.D.	0.26 ± 0.18	2.57 ± 0.94
	Conjugated form	0.19 ± 0.07	0.49 ± 0.42	4.77 ± 1.26
Isorhamnetin	Aglycone form	N.D.	0.44 ± 0.38	6.11 ± 2.54
	Conjugated form	0.22 ± 0.17	0.77 ± 0.41	7.99 ± 2.69

ICR mice were orally administrated EMIQ at 10, 100, or 1000 mg/kg body weight, or water as a vehicle control after 18 h fasting. Muscle was collected 90 min after the administration. Muscle was prepared and used for measurement of quercetin and isorhamnetin by HPLC with or without deconjugation.

The author discovered that JAK2/STAT pathway has a direct relations with GLUT4 translocation induced by isorhamnetin in skeletal muscle cells (Fig. 2-5 and 2-6). JAK2 phosphorylation was induced by isorhamnetin, while isorhamnetin at 10 nM and 1 nM activated STAT3 and STAT5 phosphorylation, respectively (Fig. 2-5). The possible explanation is that STAT1, STAT3 and STAT5 are regulated by JAK2 by different mechanisms. It has been reported that phosphorylation of JAK2 and STAT3 were increased by leptin in a rat model, on the contrary, phosphorylation of JAK2 and STAT5 were regulated by growth hormone in flounder [Carvalho, JB., *et al.*, 2003; Fuentes, EN., *et al.*, 2012]. Notably, isorhamnetin at 0.1 nM increased phosphorylation of PI3K, but did not affect Akt phosphorylation and GLUT4 translocation to the plasma membrane (Fig. 2-3B). One conceivable reason is that SH2B domain concurrently regulates the phosphorylation of JAK2 and PI3K [Carvalho, JB., *et al.*, 2003; Duan, C., *et al.*, 2004; Wu, L., *et al.*, 2017]. Indeed, leptin is reported to regulate SH2B domain and induce JAK2, IRS1, IRS2, and PI3K phosphorylation [Fuentes, EN., *et al.*, 2012].

Furthermore, PTP1B also connects the JAK/STAT- and insulin-pathways [Yang, Z., *et al.*, 2018]. It has been reported that insulin resistance is ameliorated by Fudan-Yueyang *Ganoderma lucidum* extract through decreasing PTP1B expression and increasing PI3K phosphorylation [Yang, Z., *et al.*, 2018].

In this chapter, the author found that PI3K phosphorylation was activated by quercetin at 1 nM (Fig. 2-3B). This condition could be explained by differential sensitivity of factors [D'Alessandris, C., *et al.*, 2007]. Similar condition also happened in propolis extract, which was reported to activate PI3K phosphorylation rather than aPKC phosphorylation [Ueda, M., *et al.*, 2013]. In addition, quercetin at 10 nM did not increase glucose uptake, but induced GLUT4 translocation (Figs. 2-1 and 2-2A). This results illustrated that increased glucose uptake dose not completely correlate with translocation of GLUT4. One possible explanation involves phosphorylation of p38 mitogen-activated protein kinase (MAPK), which is a necessary factor connecting GLUT4 translocation and increased glucose uptake [Niu, W., *et al.*, 2003]. Indeed, it has been reported that inactivation of p38 MAPK did not affect GLUT4 translocation to the plasma membrane, but decreased glucose uptake [Sweeney, G., *et al.*, 1999]. Moreover, another possible factor is intracellular delivery of phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P₃], which promotes GLUT4 translocation without affecting glucose uptake [Shisheva, A., *et al.*, 2003]. In addition, it has been reported that SOCS3, SHP1, SHP2, and IRS2 all could induce the similar condition [Bartoe, JL., and Nathanson, NM., 2002; Ijuin, T., and Takenawa, T., 2003; Sasaki-Suzuki, N., *et al.*, 2009]. Therefore, discrepancies between quercetin-induced GLUT4 translocation and quercetin-increased glucose uptake need to be clarified in the future.

In vivo, oral administration of EMIQ (10 and 100 mg/kg body weight) increased translocation of GLUT4, but did not affect the expression level of GLUT4 in the muscle

(Fig. 2-8). Furthermore, the plasma concentration of quercetin aglycone were 4.95 and 6.80 nM, respectively (Table, 2-3). In the same mice, the author could not detect aglycone form of isorhamnetin. It has been reported that EMIQ is a safe additive, which was orally given up to 1600 mg/kg body weight/day in a repeated oral toxicity study in rats for 13 weeks [Tamano, S., *et al.*, 2001]. Hence, the 1000 mg/kg body weight of EMIQ used in this chapter is non-toxic. In addition, it has been confirmed that the bioavailability of EMIQ is higher than that of quercetin aglycone [Makino, T., *et al.*, 2009]. Thus, the author used EMIQ in this study to clarify the effective concentrations in the plasma and muscle. The results of plasma concentrations of quercetin aglycone in mice were matched with the *in vitro* data (Fig.2-3). Furthermore, Mullen's report is also considerably consistent with the author's results [Mullen, W., *et al.*, 2002]. Aglycone form of isorhamnetin could not be detected after 90 min administration of EMIQ at 10 and 100 mg/kg body weight. On the other hand, conjugated forms of quercetin and isorhamnetin are greater than its aglycone form. Results in this chapter were consistent with the prior studies suggesting that quercetin glucuronide and sulfate forms were the primary compounds in plasma of mice [Hirano, T., *et al.*, 2009; Murota, K., 2010]. EMIQ was immediately hydrolyzed and converted to aglycone form quercetin by α -glucosidases (e.g., maltase-glucoamylase) in the intestinal mucosa of rats [Arts, IC., *et al.*, 2004]. Liberated quercetin suffered further metabolism, including conjugation to glucuronides, sulfates and methylate in the small intestine [Murota, K., *et al.*, 2010]. Isorhamnetin is one of methylated forms of quercetin. Hence, quercetin-3-glucuronide, isorhamnetin-3-glucuronide and quercetin-3'-sulfate were the major metabolites [Mullen, W., *et al.*, 2002; Nakamura, T., *et al.*, 2018]. Meanwhile, a very minor component of quercetin aglycone, upon reaching the intestine, could be absorbed from the intestinal lymphatics without conjugation and appeared in the plasma and

tissues without being metabolized [Bokkenheuser, VD., *et al.*, 1987; Nakamura, T., *et al.*, 2018]. Mullen *et al* identified 17 conjugates in rats after oral ingestion of [2-¹⁴C] quercetin-4'-glucoside [Mullen, W., *et al.*, 2002]. Furthermore, the plasma concentration of isorhamnetin appeared at a lower level. These similar results have been reported that the concentration of isorhamnetin was kept constant for another 6 h in the plasma after intake of EMIQ [Murota, K., *et al.*, 2010]. Furthermore, the plasma concentrations of isorhamnetin aglycone and its conjugates were also lower than those of both quercetin aglycone and its conjugate forms. Hence, the author speculated that the aglycone forms of quercetin and isorhamnetin were partially absorbed and undergo further biotransformation in the intestine.

Muscle occupies 45% of body weight in human. However, the author only detected very slight conjugations of quercetin and isorhamnetin in the muscle, which indicated that conjugations of quercetin and isorhamnetin were transferred to muscle rather than conjugated in the muscle (Table. 2-4). These results agreed with the theory that after a single oral dose of radioactively labeled quercetin or quercetin-4-glucoside, high concentration of quercetin is detected in the gastrointestinal tract, liver, and kidney rather than in the lung, muscle and heart [Mullen, W., *et al.*, 2002; Ueno, I., *et al.*, 1983]. Furthermore, high concentrations of quercetin conjugates were also found in heart of rat after diets with 0.5% quercetin for 2 weeks, indicating that quercetin and its conjugates need certain time to reach muscle [Morrice, PC., *et al.*, 2000].

In conclusion, the author found that quercetin and isorhamnetin increased glucose uptake and induced GLUT4 translocation to the plasma membrane through different mechanisms in muscle cells. Furthermore, EMIQ consumption increased the plasma and muscle concentrations of aglycone and conjugate forms of quercetin and isorhamnetin with EMIQ revealing similar effects as quercetin on inducing GLUT4

translocation. Hence, the author's findings provide the mechanism by which quercetin and isorhamnetin help prevent hyperglycemia.

Chapter 3: Prevention effects of quercetin derivatives on hyperglycemia in mice after a single administration

Introduction

Chronic hyperglycemia and insulin resistance are the characteristics of diabetes mellitus [Benhalima, K., *et al.*, 2011; Kong, MY., *et al.*, 2007]. The level of hyperglycemia is changing all the time depending on the metabolic cause [American Diabetes Association., 2009]. Temporary hyperglycemia is asymptomatic and harmless. A series of pathological and functional changes without producing any permanent effects are induced by transient hyperglycemia [American Diabetes Association., 2014]. Acute hyperglycemia, an extremely high glucose level is a medical emergency accompanied with serious complications in diabetes [Pasquel, FJ., *et al.*, 2014]. Now the widely accepted method of antihyperglycemia is effective glycemic control. However, administration of steroids, which are common clinical drugs, have been reported to have potential threats on increasing insulin resistance and further impairing glucose uptake capacity in peripheral tissues, such as increasing hepatic glucose production and depressing glucose intolerance [Hwang, JL., *et al.*, 2014].

The onset of hyperglycemia is closely related to insufficient of glucose metabolism. Epidemiological evidences and many clinical studies have demonstrated that aggressive correction of hyperglycemia using polyphenols reduces morbidity and mortality in multiple clinical situations [Ataie, A., *et al.*, 2016; Tressera-Rimbau, A., *et al.*, 2017]. Quercetin, the most distributed polyphenol, is presents as various glycoside forms in natural food, such as isoquercitrin and rutin. Quercetin glycosides primarily absorbed as aglycone form in small and large intestines. For instance, isoquercitrin was

hydrolyzed to aglycone by lactase phlorizin hydrolase (LPH), β -glucosidase on the outside of the brush-border membrane, and then absorbed in small intestine [Day, AJ., *et al.*, 2000]. On the other hand, rutin, which has a rutinoside moiety, was scarcely hydrolyzed and absorbed in large intestine. It has been reported that rutin was hydrolyzed to aglycone and its phenolic degradation products by colonic microflora [Aura, AM., *et al.*, 2002]. EMIQ has been recognized to be safe food additives with high bioavailability [Murota, K., *et al.*, 2010]. EMIQ is absorbed into the body similar to quercetin glycosides from natural sources [Murota, K., *et al.*, 2010]. These quercetin glycosides possess various favorable effects against hyperglycemia, obesity and cancers. In recent years, epidemiological evidences and several clinical studies have demonstrated quercetin shows protective effect on DNA damage in alloxan induced type 2 diabetic mice [Alam, MM., *et al.*, 2014]. Isoquercitrin inhibits adipocyte differentiation in 3T3-L1 cells [Lee, SH., *et al.*, 2011]. Rutin, a typical dietary quercetin glucoside, inhibits proliferation and decreases migration of human cancerous cells [Ben Sghaier, M., *et al.*, 2016]. EMIQ reveals anti-allergic effects in mice [Makino, T., *et al.*, 2013]. However, it is unknown whether quercetin glucosides suppress hyperglycemia and their underlying mechanisms are not fully understood yet.

Skeletal muscle plays a critical role in maintaining glucose homeostasis, which accounts for approximately 80% of insulin-stimulated glucose uptake [DeFronzo, RA., *et al.*, 1988]. In Chapter 2, the author investigated whether quercetin at physiological concentrations increased glucose uptake and induced GLUT4 translocation *in vitro*. In this chapter (Chapter 3), the author expanded investigation to clarify the anti-hyperglycemic effect of the quercetin and its glucosides at low concentration range in mice and its possible mechanism.

Materials and Methods

Materials

Most of the materials used were the same as showed in Chapter 2. Rutin was obtained from Wako Pure Chemical Industries (Osaka, Japan). EMIQ and isoquercitrin were products of San-Ei Gen F. F. I. Co., Ltd., (Osaka, Japan). Plasma glucose was measured using a commercial kit (Labassay® Glucose Wako kit, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Antibodies were the same as used in Chapter 2.

Animal treatment

All animal experiments were approved by the Kobe University Institutional Animal Care and Use Committee (Permission # 1-27-05-09) and carried out according to the guidelines for animal experiments at Kobe University Animal Experimentation Regulation. Male ICR mice (4 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and acclimatized for 7 days with free access to a standard mouse diet (Research Diets, Tokyo, Japan) and distilled water. Before the experiment, mice were fasted for 18 h.

For the OGTT test, mice were orally administered quercetin, isoquercitrin, rutin EMIQ (0.001, 0.01, 0.1, 1 and 10 mg/ kg body weight) or water alone 60 min before oral glucose loading (1 g/kg body weight). Tail vein blood was collected in heparinized tubes at 0 (before administration), 15, 30, 60 and 120 min after the glucose load [Yamashita, Y., *et al.*, 2016]. Plasma was collected and glucose levels were measured using the commercial kit (Labassay® Glucose Wako kit).

To identify the underlying mechanisms, another 45 mice were orally administered quercetin, isoquercitrin, rutin and EMIQ (0.001 mg or 1 mg /kg body weight) or water alone. Mice were sacrificed under anesthesia, 60 min after administration. The plasma

membrane fraction and tissue lysate of the soleus muscle were prepared [Nishiumi, S., *et al.*, 2007].

Immunoblotting

Immunoblotting was carried out as described in Chapter 2.

Statistical analysis

Data are represented as the means and standard error (SE). The statistical significance of experimental observations was determined using the Dunnett test. The level of significance was set at $p < 0.05$.

Results

The effects of quercetin and its glycosides on acute hyperglycemia

OGTT was performed to examine the antihyperglycemic effects of quercetin and its glycosides. Plasma glucose level increased 15 min after the glucose loading and then decreased with time in the control group (Fig 3-1A). Pre-administration of EMIQ, isoquercitrin and rutin suppressed post-prandial hyperglycemia. The peak blood glucose levels were significantly decreased by 0.1 and 1 mg/kg BW EMIQ and 1 and 10 µg/ kg BW rutin. Isoquercitrin at 1 µg to 10 mg/kg showed a similar decreasing tendency. On the other hand, quercetin in all dose failed to affect the plasma glucose level (Fig. 3-1A). As shown in Fig. 3-1B, the area under the curve (AUC) was significantly lower in isoquercitrin (10 mg/ kg BW) and EMIQ (1 mg/kg BW) groups. Furthermore, plasma glucose levels of 1 µg and 1mg/kg BW groups at 15 min after the glucose load was shown in Fig. 3-1C. These results revealed that isoquercitrin was the most effective compound on preventing acute hyperglycemia among these quercetin glucosides. Moreover, low-dose range of rutin, and high-dose range of EMIQ showed the inhibition effects, as well. These results indicated that only quercetin glycosides

contain anti-hyperglycemic effect after a single oral administration.

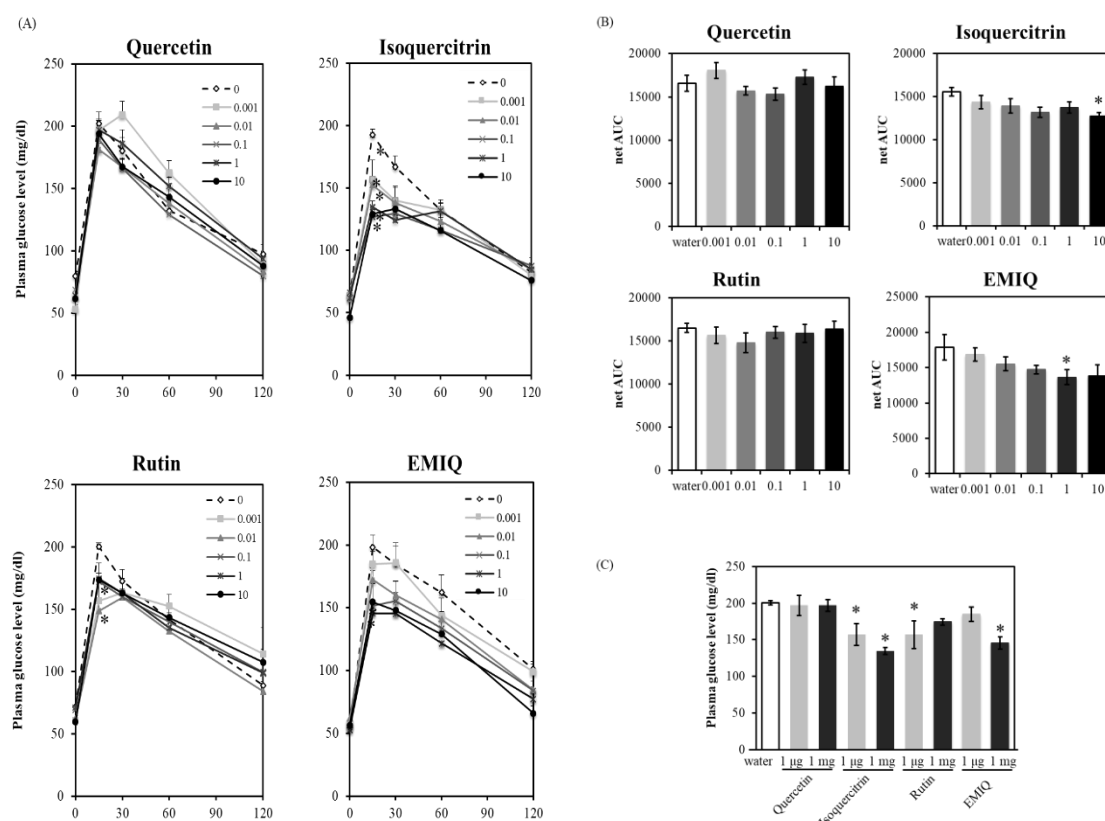


Figure 3-1. The effects of quercetin and its glucosides on the plasma glucose level in an oral glucose tolerance test (OGTT). (A) ICR mice were dose orally 0.001, 0.01, 0.1, 1, and 10 mg/kg body weight of quercetin, isoquercitrin, rutin, and EMIQ, water as a vehicle control. The mice were given orally glucose solution (1g/kg body weight) 60 minutes after the administration, and the plasma glucose levels were examined at the indicated time points. (B) AUC value was calculated. (C) The plasma glucose levels were measured 15 min after the administration. Data were shown as the mean $\pm$ SE (n = 5).

* $p < 0.05$ vs control group by Dunnett's multiple comparison test.

Quercetin glucosides promoted GLUT4 translocation to the plasma membrane in muscle

The effects of quercetin and its glycosides on GLUT4 translocation to the plasma membrane in the soleus muscle were investigated by the author. The author found that

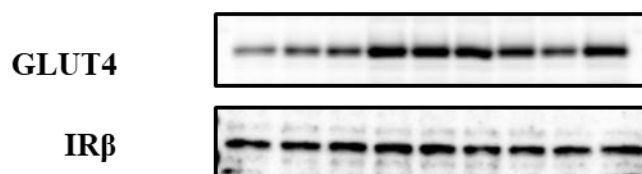
rutin and isoquercitrin at 1 µg/ kg BW significantly increased GLUT4 translocation to the plasma membrane. Rutin, isoquercitrin and EMIQ at 1 mg/ kg BW also increased GLUT4 translocation to the plasma membrane. On the other hand, the expression level of GLUT4 in the tissue lysate was not affected by these compounds (Fig. 3-2B).

Quercetin glucosides induced GLUT4 translocation by activating AMPK-signaling pathway in skeletal muscle of mice

As shown in Fig. 3-3, EMIQ, isoquercitrin and rutin increased AMPK phosphorylation after the single administration of these compounds at 1µg and 1 mg/ kg BW (Fig 3-3); Isoquercitrin and rutin both at 1µg and 1 mg/ kg BW, in particular isoquercitrin, significantly promoted AMPK and ACC phosphorylation in soleus muscle of mice. Furthermore, EMIQ at 1 mg/kg BW also activated AMPK and ACC phosphorylation. These ameliorative effects were consistent with the results of OGTT (Fig. 3-1) and GLUT4 translocation (Fig. 3-2).

The author, next examined phosphorylation of representative factors in insulin-pathway, as IRS1, Akt, and PI3K. As a result, these four compounds did not affect phosphorylation of IRS1, Akt (ser473 and thr308) and PI3K in muscle of mice (Fig. 3-4). The author next investigated phosphorylation of JAK2 and STAT3 in the same animals. However, not only quercetin, but also its glucosides, failed to increase phosphorylation of JAK and STAT3 *in vivo*, (Fig. 3-5). These results indicated that quercetin glycosides, but not quercetin, activated the AMPK signaling pathway to induce GLUT4 translocation to the plasma membrane in soleus muscle.

(A) Plasma membrane



(B) Tissue lysate

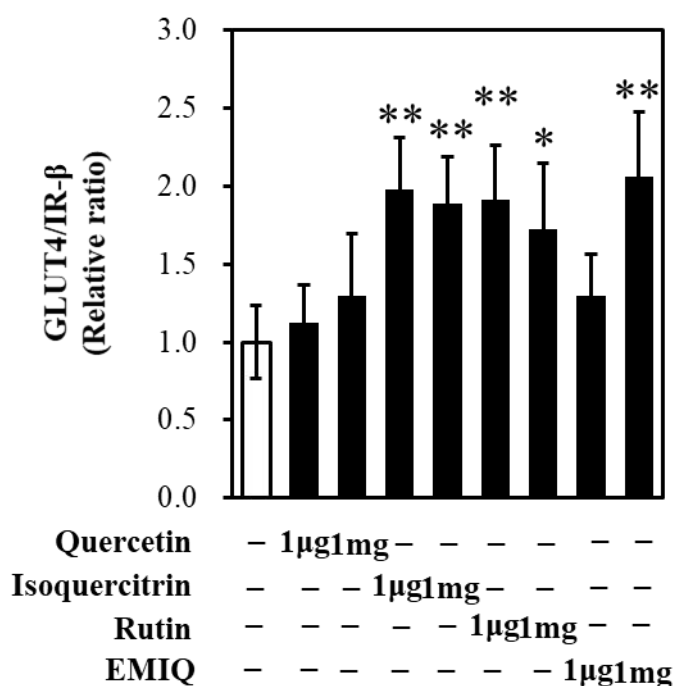
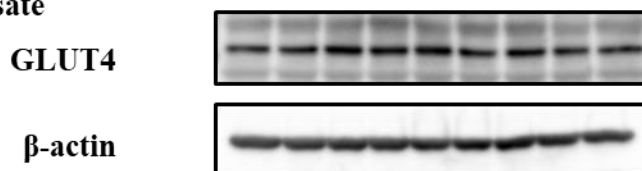
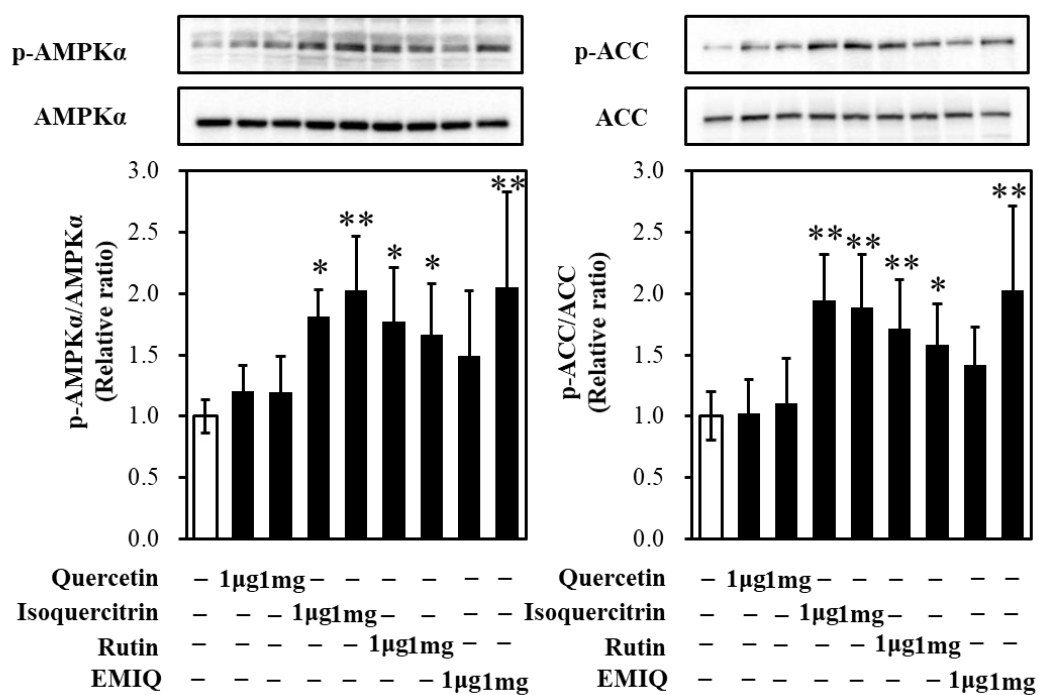


Figure 3-2. The effects of quercetin, isoquercitrin, rutin, and EMIQ on the translocation of GLUT4 in skeletal muscle. GLUT4, IR- β and β -actin protein expression in the (A) plasma membrane fraction and (B) tissue lysate of skeletal muscle were determined by western blot analysis. The density of each GLUT4 band was analyzed and normalized to IR- β band. Data were shown as the mean $\pm$ SE (n = 5). * p < 0.05 and ** p < 0.01 vs control group by Dunnett's multiple comparison test.

(B) ACC phosphorylation in muscle



The expression of (A) phosphorylated-AMPK α , AMPK α , (B) phosphorylated-ACC and ACC in the muscle lysate were determined by western blot analysis. Data were shown as the mean $\pm$ SE (n = 5).

* $p < 0.05$ and ** $p < 0.01$ vs control group by Dunnett's multiple comparison test.

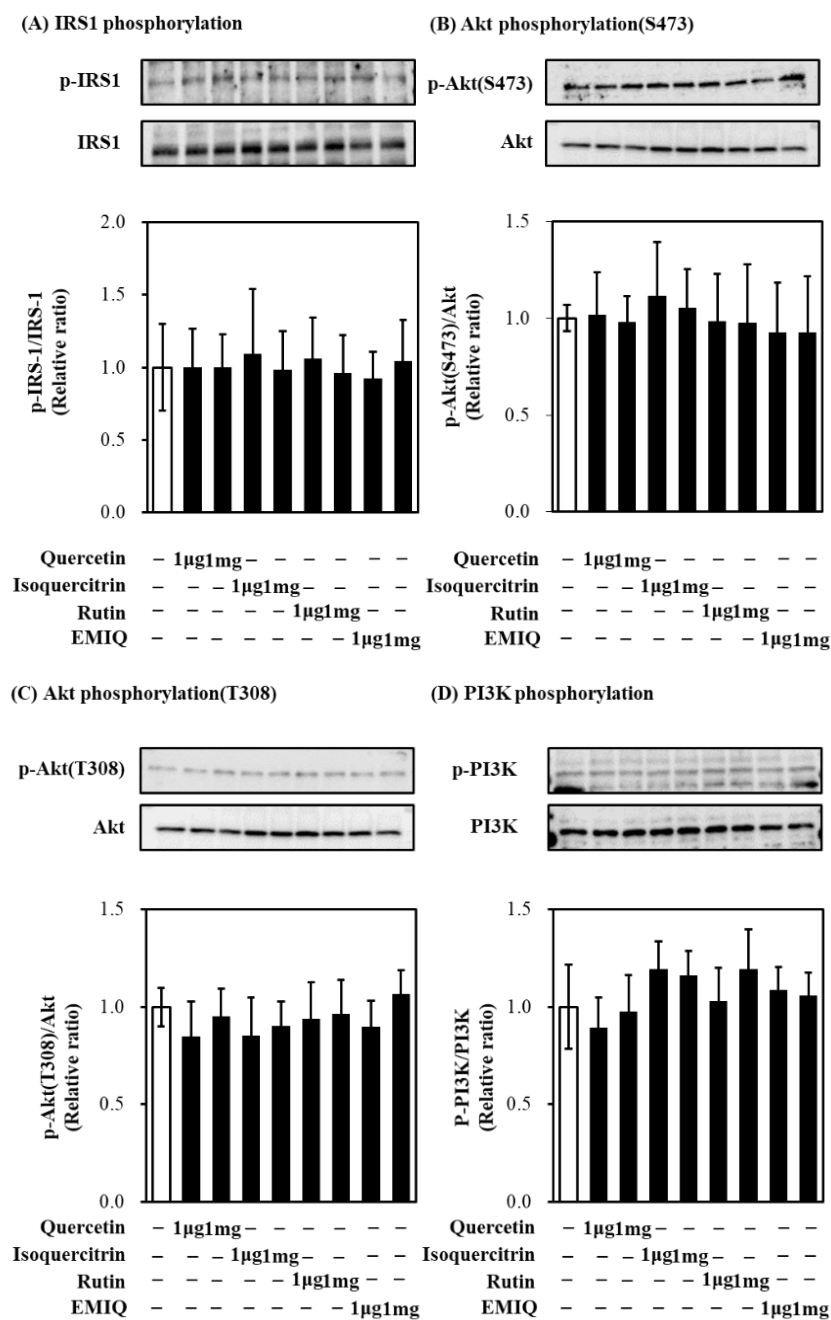


Figure 3-4. The effects of quercetin, isoquercitrin, rutin, and EMIQ on insulin-pathway in skeletal muscle. The expression of (A) phosphorylated-IRS1 and IRS1, (B) phosphorylated-Akt (S473) and Akt, (C) phosphorylated-Akt (T308) and Akt, (D) phosphorylated-PI3K and PI3K in the muscle lysate were determined by western blot analysis. Data were shown as the mean $\pm$ SE (n = 5).

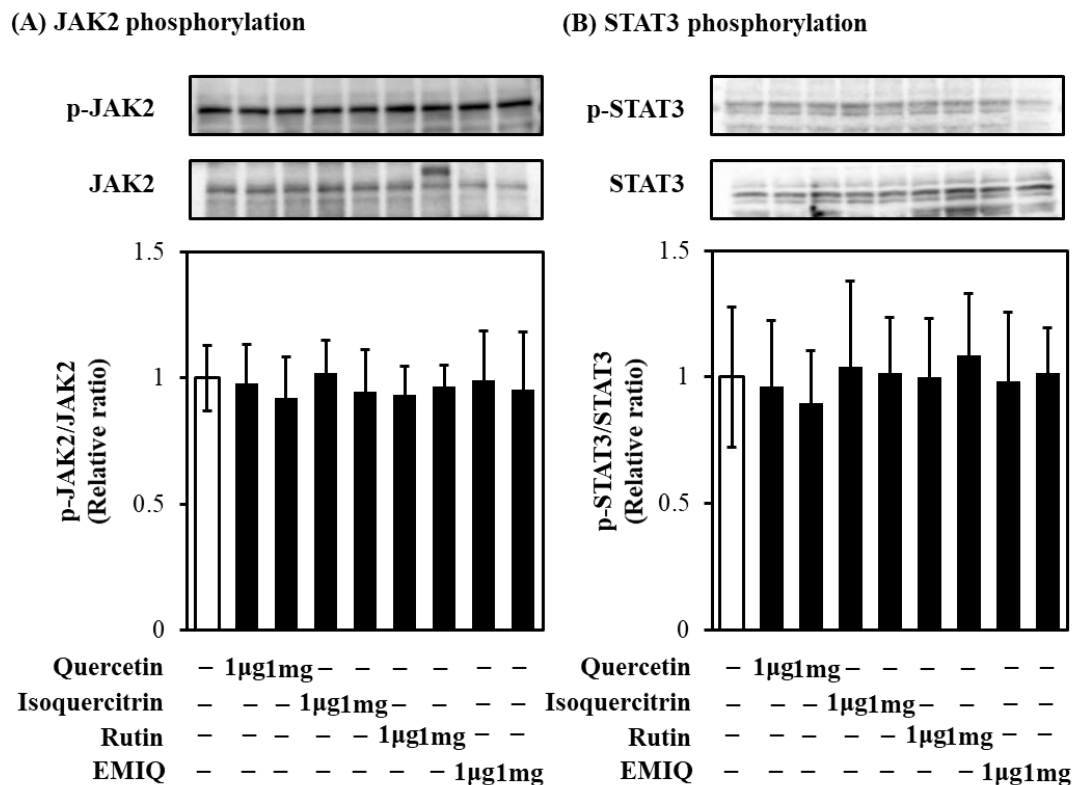


Figure 3-5. The effects of quercetin, isoquercitrin, rutin, and EMIQ on JAK/STAT-pathway in skeletal muscle. The expression of (A) phosphorylated-JAK2 and JAK2, (B) phosphohorylated-STAT3 and STAT3 in the muscle lysate were determined by western blot analysis. Data were shown as the mean $\pm$ SE (n = 5).

Discussion

Nowadays, acute hyperglycemia, which is characterized by extremely high glucose level, is a medical emergency and produces serious complications, as diabetic ketoacidosis and hyperosmolar hyperglycemic state [Bhurosy, T., *et al.*, 2014; Loewen, S.L., *et al.*, 1991]. It has been reported that the bioactive polyphenols in food contribute to ameliorate hyperglycemic effects [Ueda-Wakagi, M., *et al.*, 2018; Yamashita, Y., *et al.*, 2016; Maeda, A., *et al.*, 2018; Kawabata, K., *et al.*, 2011]. It has been reported that the daily intake of quercetin is between 25 and 50 mg/day in human [Li, Y., *et al.*, 2016],

and quercetin at high concentration range promotes glucose metabolism and prevents hyperglycemia [Stewart, LK., *et al.*, 2008; Vessal, M., *et al.*, 2003]. However, due to its poor bioavailability, the effect of quercetin at a dietary physiological concentration on anti-hyperglycemia is not understood yet. Hence, in this chapter, the author investigated whether quercetin and its glycosides prevent hyperglycemia and their signaling pathways in ICR mice after a single oral administration of them.

The author demonstrated that ICR mice were induced acute hyperglycemia after glucose load, and quercetin glycosides, but not quercetin, prevented transient hyperglycemia induced by glucose (Fig. 3-1). Particularly, isoquercitrin at all dose, revealed the anti-hyperglycemic effect. Furthermore, rutin at 1 µg/ kg BW and EMIQ at 1 mg/ kg BW showed the similar anti-hyperglycemic effect. These results were extremely related to difference of bioavailability of quercetin and its glucosides. Quercetin is poorly absorbed from the small intestine, which has been reported in animal and human studies [Murota, K., *et al.*, 2010; Nakamura, T., *et al.*, 2018]. It has been reported that microbial enzymes and LPH present on the out surface of the intestinal brush border are involved in the deglycosylation of isoquercitrin and EMIQ [Day, AJ., *et al.*, 2000]. In human and rat intestine, isoquercitrin and EMIQ were reported to be hydrolyzed and liberated quercetin in enterocytes by cytosolic β-glucosidase [Cermak, R., *et al.*, 2003]. In addition, Murota *et al.* reported that the plasma levels of quercetin metabolites were increased by EMIQ and isoquercitrin in human subjects after oral administration of quercetin and its glucosides [Murota, K., *et al.*, 2010]. In contrast, after consumption of rutin and quercetin, the plasma levels of metabolites were found in lower levels [Murota, K., *et al.*, 2010]. Moreover, a similar comparison of bioavailability was performed in rats fed quercetin and isoquercitrin. In isoquercitrin given rats, quercetin metabolites were mainly found in the liver, kidney,

and plasma after 8 days feeding [Paulke, A., *et al.*, 2012]. These evidences supported current results in this chapter that transient hyperglycemia was ameliorated by quercetin glucosides, rather than quercetin, because of their different bioavailabilities.

In this chapter, the author demonstrated that compared with isoquercitrin at all dose showed the anti-hyperglycemic effect, EMIQ only at 1 mg/ kg BW showed the similar effect. The effective concentration of quercetin aglycone may be one possible explanation. EMIQ and isoquercitrin were rapidly deglycosylated to quercetin in the intestinal epithelium [Murota, K., *et al.*, 2010]. However, the relative molecular mass of EMIQ is greater than isoquercitrin, due to EMIQ containing not only isoquercitrin, but also another 2–7 additional glucose moieties in quercetin structure. Hence, at the same weight, the concentration of EMIQ degraded to quercetin was less than that of isoquercitrin. This speculation has been confirmed that the degradation of EMIQ to quercetin aglycone transiently increases in the concentration of isoquercitrin, indicating that isoquercitrin is an intermediate of EMIQ degradation [Makino, T., *et al.*, 2009]. These results strongly suggested that the glucose moieties of EMIQ had to be hydrolyzed during its metabolism. Thus, there are some differences between quercetin mono- and oligo-glycosides on the metabolite efficiency. Hence, in order to possess the similar anti-hyperglycemic effect, EMIQ needed a higher concentration than isoquercitrin.

Controlling the postprandial blood glucose excursions is an important method for preventing hyperglycemia. GLUT4 is associated with glucose homeostasis by regulating cellular glucose uptake [Bryant, NJ., *et al.*, 2002; Kotani, K., *et al.*, 2004]. In this chapter, the author investigated that isoquercitrin and rutin at 1 µg/ kg BW and 1 mg/ kg BW, and EMIQ at 1 mg/ kg BW significantly increased GLUT4 translocation to the plasma membrane through activating AMPK-pathway rather than insulin- and

JAK/STAT-pathways (Fig. 3-2 to 3-5). On the other hand, quercetin affected neither GLUT4 translocation nor AMPK phosphorylation. These results coincide with the data of OGTT (Fig. 3-1). However, in Chapter 2, the author demonstrated that quercetin, activated not only AMPK-, but also insulin-pathways in L6 myotubes. Nonetheless, in this chapter, EMIQ only activated AMPK phosphorylation. This discrepancy was due to the difference of EMIQ concentrations. In Chapter 2, the plasma concentration of quercetin aglycone form was 4.95 nM after administration of EMIQ at 10 mg/kg BW. Hence, the concentration of quercetin aglycone form should be much lower than 4.95 nM in the plasma of mice orally given EMIQ in this chapter. It is, therefore, reasonable that EMIQ at 1mg/kg body weight only activated AMPK-pathway *in vivo*. Indeed, in Chapter 2, AMPK phosphorylation was activated by 0.1 and 1 nM quercetin in L6 myotubes. The author attempted to detect quercetin aglycone and conjugate forms by HPLC, but the author could not detect them in the plasma of mice orally given 1µg or 1 mg/ kg BW of quercetin and its glucosides. The possible reason may be related to the limit of detection and the limit of quantification. In future, the author will explore a more precise method to verify this point.

Next, in this chapter, the author found that rutin at 0.001 or 1mg/kg body weight induced GLUT4 translocation to the plasma membrane; nevertheless, only 0.001 mg/kg body weight rutin revealed the anti-hyperglycemic effect in mice. The similar phenomenon was observed in Chapter 2: in L6 myotubes, 10 nM quercetin did not increase glucose uptake, but induced GLUT4 translocation to the plasma membrane. As explained in Chapter 2, MAPK phosphorylation and PI(3,4,5)P₃ were needed for an increasing glucose uptake as the potential reasons [Niu, W., *et al.*, 2003; Shisheva, A., *et al.*, 2003]. Furthermore, there were some reports demonstrated that SOCS3, SHP1, SHP2, and IRS2 were involved in the promotion of GLUT4 translocation without

increasing glucose uptake [Bartoe, JL., and Nathanson, NM., 2002; Ijuin, T., and Takenawa, T., 2003; Sasaki-Suzuki, N., *et al.*, 2009]. This issue will be explored in future.

In conclusion, the results of this chapter indicated that a single oral administration of quercetin glycosides, but not quercetin, were significantly ameliorated the postprandial hyperglycemia through promoting GLUT4 translocation to the plasma membrane by activating AMPK signaling pathways in mice. Therefore, quercetin glycosides are beneficial for the treatment of hyperglycemia and potentially diabetes mellitus.

Chapter 4: Enzymatically modified isoquercitrin promotes energy metabolism through activating AMPK α in mice

Introduction

Metabolic disorder attenuates glucose uptake and decreases mitochondrial content and the activity of electron transporter chain in muscle and adipose tissues, causing a deterioration in insulin sensitivity that induces the pathogenesis of T2DM [Saltiel, AR., *et al.*, 2001]. Metabolic disorder also induces hypertrophy of adipocytes and increases the secretion of inflammatory adipocytokines [Jung, UJ., and Choi, MS., 2014]. These adipocytokines decrease glucose uptake by attenuating insulin signaling [Jung, UJ., and Choi, MS., 2014]. AMPK is a pivotal molecular regulator for energy metabolism. AMPK promotes lipid accumulation and gene transcription [Long, YC., and Zierath, JR., 2006]. AMPK activation suppresses many anabolic processes and activates several catabolic processes to regulate energy metabolism and adipogenesis [Cho, BY., *et al.*, 2017; Wang, S., *et al.*, 2015]. CPT1, ACC, SREBP1, PPAR γ , C/EBPs, which are key factors in energy metabolism and adipogenesis, are modulated by AMPK activation in WAT [Cho, BY., *et al.*, 2017; Moseti, D., *et al.*, 2016; Wang, S., *et al.*, 2015]. In addition, WAT browning is a potential approach for anti-obesity therapy, which modulates thermogenic capability through regulating AMPK downstream targets, including UCP1, PGC-1 α , PRDM16, and SIRT1 [Chi, J., *et al.*, 2016; Puigserver, P., *et al.*, 2003; Son, MJ., *et al.*, 2016; Zhang, T., *et al.*, 2015].

Glucose metabolism and insulin sensitivity are reported to be regulated by AMPK phosphorylation [Long, YC., and Zierath, JR., 2006]. AMPK is a potential target for treating diabetes mellitus by inducing GLUT4 translocation to the plasma membrane in

muscle [Carling, D., *et al.*, 2012]. Not only insulin stimulation, but also muscle contraction have been reported to induce GLUT4 translocation to facilitate the glucose diffusion [Sawada, K., *et al.*, 2014].

Liver is also a metabolic organ that maintains the energy level and structural stability of the body, which is involved in carbohydrate, protein, amino acid, and lipid metabolism [Rui, L., *et al.*, 2014]. Several polyphenols have been reported to attenuate hepatic lipid accumulation through activating some targets, as AMPK, CPT1 and PPAR α [Li, S., *et al.*, 2018; Srivastava, RA., *et al.*, 2012]. In addition, hepatic glucose metabolism is also regulated by AMPK phosphorylation. Gluconeogenic enzyme expression and the enzymatic activity of gluconeogenesis, such as PEPCK and G6Pase have been reported to be suppressed by FXR and CAR activation in metabolic disorder mice, which are downstream factors of AMPK [Miao, J., *et al.*, 2006; Wagner, M., *et al.*, 2005; Kulkarni, SR., *et al.*, 2013; Mo, L., *et al.*, 2016].

In Chapter 2 and 3, the author found that quercetin induced GLUT4 translocation to the plasma membrane in myotubes, but, a single oral administration of quercetin failed to prevent hyperglycemia in mice. The author also found that EMIQ revealed the anti-hyperglycemia effect accompanied by the promotion of GLUT4 translocation to the plasma membrane in a single oral administration study. These findings indicated that EMIQ has a potential to activate AMPK-signaling pathway for regulating energy metabolism. However, there is no report on energy metabolism modulated by EMIQ and its related signaling pathways. In this chapter, the effects of EMIQ on lipid accumulation and energy metabolism are investigated in the WAT, liver, and muscle of mice after two weeks feeding comparing with quercetin.

Materials and methods

Materials

Most of the materials used were mentioned before in Chapter 2. Inosine 5'-diphosphate (IDP) sodium salt, phospho(enol)pyruvic acid (PEP) monopotassium salt, and glucose dehydrogenase were supplied by Sigma-Aldrich (St. Louis, MO, USA). Mutarotase, β -nicotinamide adenine dinucleotide, oxidized form (NAD^+), β -nicotinamide-adenine dinucleotide, and reduced form (NADH) disodium salt were from Oriental Yeast Co. Ltd (Tokyo, Japan). Glucose-6-phosphate (G6P) was from Roche Diagnostics (Indianapolis, IN, USA). Commercial assay kits (Lab assay™ Glucose Wako Kit) was obtained from Wako Pure Chemical Industries (Osaka, Japan). For western blot analysis, most of the antibodies used were mentioned before in Chapter 2. Anti-FXR rabbit mAb antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Purified anti-UCP2 rabbit IgG was from BioLegend (San Diego, CA, USA). Anti-PGC-1 α mouse mAb, anti-PRDM16 rabbit IgG, and anti-SIRT1 rabbit IgG antibodies were from Millipore (Tokyo, Japan). Anti-CAR (E-1) mouse mAb, C/EBP α (14AA) rabbit IgG, C/EBP β (C-19) rabbit IgG, C/EBP δ (C-22) rabbit IgG, PPAR α (H-98) rabbit IgG, PPAR γ (H-100) rabbit IgG, and UCP1 (M-17) goat IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-CPT1 mouse mAb and anti-SREBP1 rabbit IgG antibodies were from Abcam (Cambridge, MA, USA).

Animal treatment

All animal experiments were performed according to the guidelines for animal experiments at Kobe University Animal Experimentation Regulation and approved by the Institutional Animal Care and Use Committee of Kobe University (Permission 29-05-02). Male C57BL/6 mice (4 weeks old, n=28) were obtained from Japan SLC (Shizuoka, Japan) and acclimatized for 1 week. Diets with different doses of EMIQ or

quercetin (0.02%, 0.1% and 0.5% (w/w)) were fed to mice for two weeks. After 18 h of fasting, mice were sacrificed under anesthesia and blood samples, WAT (epididymal and mesenteric), liver, and skeletal muscle were collected.

Measurement of plasma parameters

Plasma glucose concentration was detected as described in Chapter 3.

Preparation of the plasma membrane fraction and tissue lysate

For the measurement of related signal pathways, the plasma membrane fraction and tissue lysate fraction from WAT, liver, and muscle were prepared as previously described [Nishiumi, S., *et al.*, 2007].

Immunoblotting

Immunoblotting was carried out as described in Chapter 2.

Assays for hepatic gluconeogenesis enzyme activity

The activity of PEPCK and G6Pase were detected as previous description by the method of Bentle and Alegre with slight modifications, respectively [Bentle, LA., *et al.*, 1976; Alegre, M., *et al.*, 1988]. The enzyme activities of PEPCE and G6Pase were monitored by a decrease or an increase in absorbance at 340 nm for 3.5 min at 37°C.

Statistical analysis

Data are represented as the means and standard error (SE). The statistical analysis was performed using Dunnett's multiple-comparison test. $p < 0.05$ was considered statistically significant using JMP 11.2.0 (SAS, Cary, USA).

Results

The effects of EMIQ and quercetin on body weight, tissue weight, and blood parameters in mice

After two weeks, the body weight of mice, food and energy intake did not show

any significant difference among the groups (Table 4-1). However, the WAT (mesenteric and epididymal) weight gain was downregulated by diet containing 0.1% EMIQ. Meanwhile, supplementation with 0.02% and 0.1% EMIQ significantly decreased the plasma glucose level. These results indicated that EMIQ reduced the deposition of lipids in WAT and the plasma glucose level.

Table 4-1. Effect of EMIQ and quercetin on body weight, total energy intake, adipose tissue weight, and glucose level in the plasma.

	Control		EMIQ			Quercetin	
(%)		0.02	0.1	0.5	0.02	0.1	0.5
Body weight (g)	24.4±0.8	24.6±0.6	23.5±1.0	23.3±1.1	25.1±0.8	25.0±0.6	23.6±0.6
Food intake (kcal/day)	14.2	13.5	13.4	13.7	12.8	15.0	15.8
Plasma glucose (mg/dl)	134±25	84±13*	68±30*	95±35	95±21	108±6	94±7
White adipose tissue weight (g/100 g body weight)							
Mesenteric	1.02±0.01	0.98±0.06	0.53±0.04*	0.78±0.16	0.84±0.10	0.77±0.13	0.85±0.10
Epididymal	2.25±0.22	1.91±0.18	1.07±0.15*	1.76±0.21	1.66±0.07	1.60±0.10	1.58±0.24

Mice were fed a diet supplemented with different doses of EMIQ or quercetin (0.02%, 0.1% and 0.5% (w/w)) for two weeks. At the end of the experiment, body weight, total energy intake, adipose tissue weight, and glucose level in the plasma were measured after 18 h of fasting. Values are the mean ± SE (n=4). * indicates a significant difference from the control group (standard-diet) by Dunnett's multiple comparison test (* $p < 0.05$).

The effects of EMIQ and quercetin on AMPK phosphorylation in the adipose

tissues, liver, and skeletal muscle

The author confirmed that in the adipose tissues, liver, and skeletal muscle, AMPK phosphorylation, but not the expression of AMPK, was promoted in the 0.02% and 0.1% EMIQ groups (Fig. 4-1), which indicated that EMIQ at lower concentrations had the potential to involve in ameliorating energy consumption in the skeletal muscle and reducing lipid accumulation in the adipose tissues and liver by activating AMPK phosphorylation.

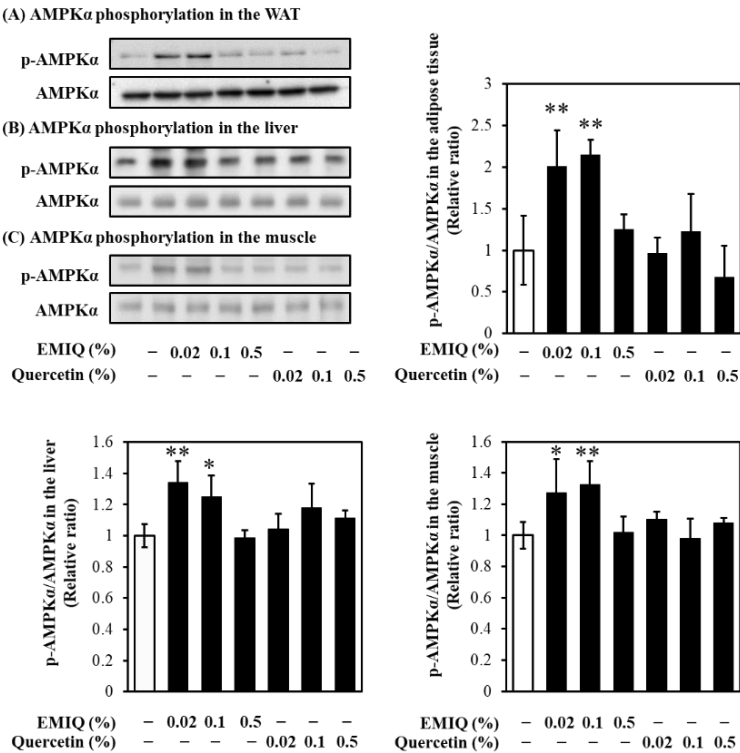


Figure 4-1. EMIQ promoted AMPK phosphorylation in different tissues. The phosphorylation and expression of AMPK in the tissue lysates of (A) WAT, (B) liver, and (C) skeletal muscle were determined by western blot analysis. Each blot shows a representative result from four

animals. Data are shown as the mean $\pm$ SE ($n = 4$). $*p < 0.05$ and $**p < 0.01$ vs control group by Dunnett's multiple comparison test.

EMIQ modulated lipolysis- and lipogenesis-related proteins expression levels in the adipose tissue

Next, the author detected the related proteins expression of lipolysis and lipogenesis in the adipose tissue. As expected, ACC phosphorylation was promoted in

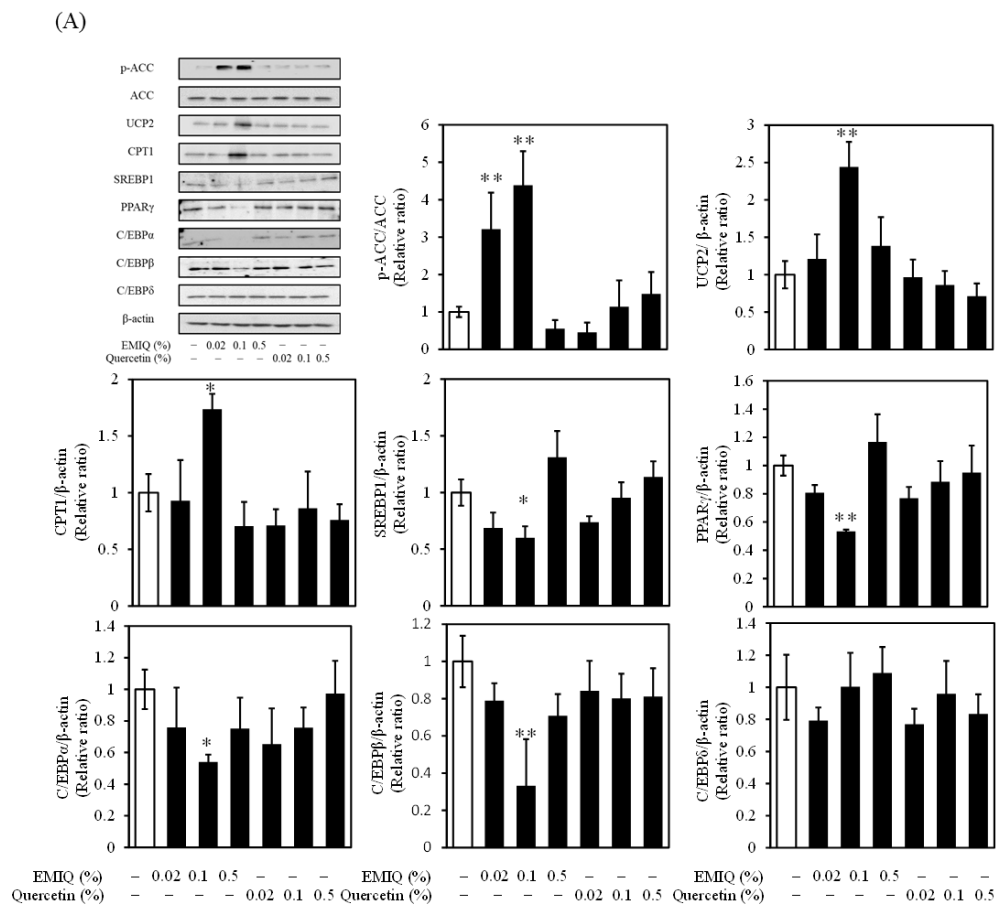
0.02% and 0.1% EMIQ groups, which was concomitant with phosphorylation of AMPK (Fig. 4-2A). Compared to the control group, diet with 0.1% EMIQ significantly upregulated UCP2 and CPT1 expression levels, which are associated with lipolysis (Fig. 4-2A). Furthermore, 0.1% EMIQ supplementation downregulated the expression levels of PPAR γ , SREBP1, C/EBP α , and C/EBP β , which are involved in fatty acid metabolism and adipogenesis [Moseti, D., *et al.*, 2016; Zhang, T., *et al.*, 2014]. Meanwhile, neither EMIQ nor quercetin could affect C/EBP δ expression after two weeks feeding. As shown in Fig. 4-2B, the expression levels of PGC-1 α , UCP1, PRDM16, and SIRT1 were significantly promoted by 0.1% EMIQ supplementation. These protein targets have been reported to associate with modulating thermogenesis [Zhang, T., *et al.*, 2015; Puigserver, P., *et al.*, 2003; Chi, J., *et al.*, 2016; Son, MJ., *et al.*, 2016]. As a result, EMIQ repressed lipogenesis and promoted lipolysis by regulating the related proteins expression levels in the WAT.

EMIQ modulated energy metabolism-related proteins expression levels in the liver

In the liver, the author firstly detected phosphorylation of ACC. Diet supplementation with 0.02% and 0.1% EMIQ enhanced phosphorylation of ACC in the liver (Fig. 4-3). It is reported that PPAR α is involved in lipolysis [Zhang, T., *et al.*, 2015]. In 0.1% EMIQ group, PPAR α expression was upregulated compared to control group. While, SREBP1 and CPT1 expression levels were not affected by supplementation with EMIQ and quercetin (Fig. 4-3).

As shown in Fig. 4-4, diet with EMIQ (0.02% and 0.1%) upregulated CAR and FXR expression levels in the liver (Fig. 4-4A). CAR and FXR are reported to associate with gluconeogenesis and drug metabolism [Mo, L., *et al.*, 2016; Wagner, M., *et al.*, 2005]. Next, the enzyme activities of PEPCK and G6Pase in the liver were evaluated by the author. Compared with control group, these enzyme activities were

downregulated in EMIQ and 0.5 quercetin groups (Fig. 4-4B). From these results, the author confirmed that energy metabolism was regulated by EMIQ supplementation in the liver.



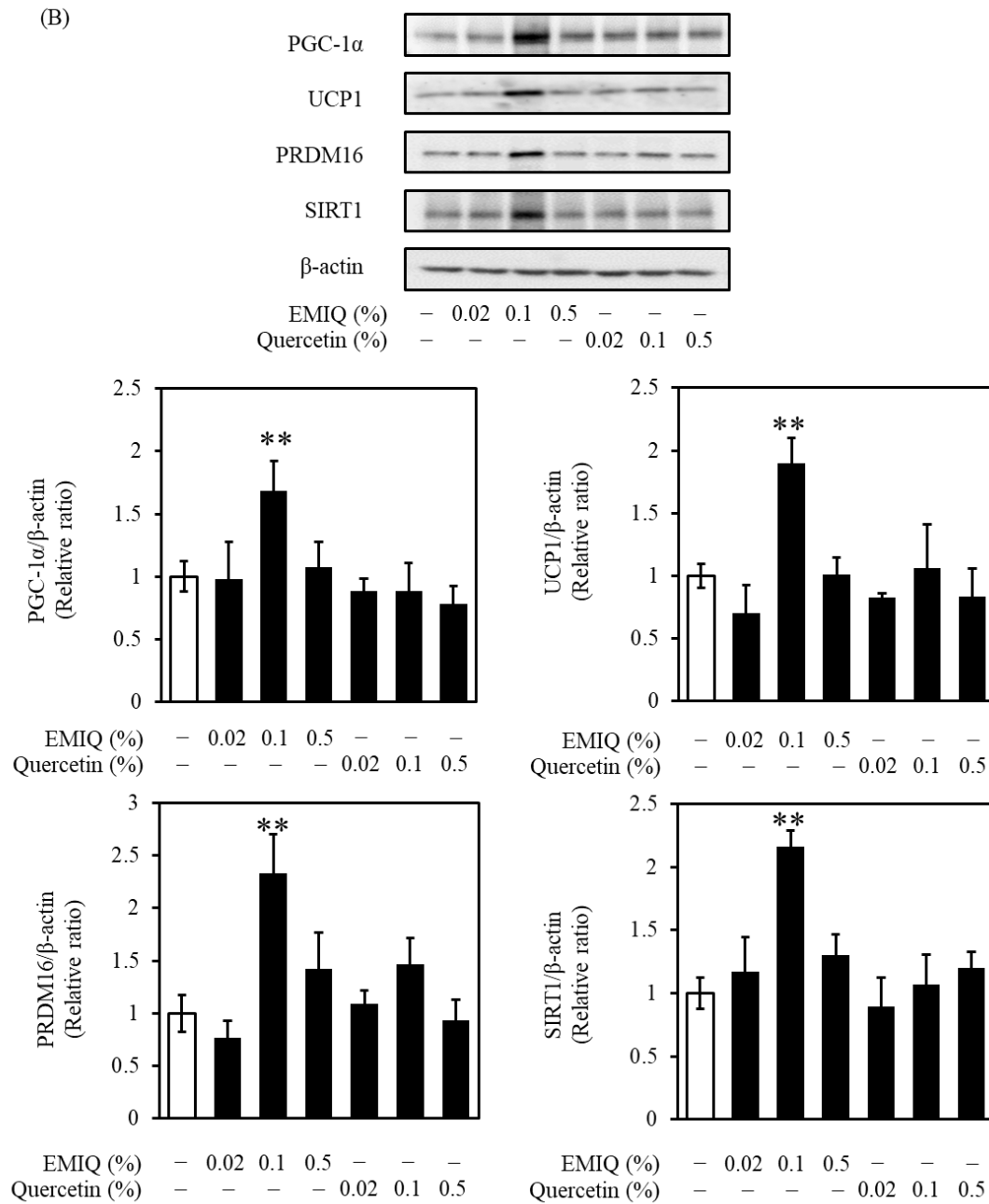


Figure 4-2. Effect of EMIQ and quercetin on the expression of adipogenesis-, energy metabolism- and thermogenesis-related proteins in WAT. The expression of (A) p-ACC, ACC, UCP2, CPT1, SREBP1, PPAR γ , C/EBP α , C/EBP β , C/EBP δ , and β -actin in the tissue lysate of WAT and the expression of (B) PGC-1 α , UCP1, PRDM16, SIRT1, and β -actin in the tissue lysate of WAT were determined by western blot analysis. Each blot shows a representative result from four animals. Data are shown as the mean $\pm$ SE (n = 4). * p < 0.05 and ** p < 0.01 vs control group by Dunnett's multiple comparison test.

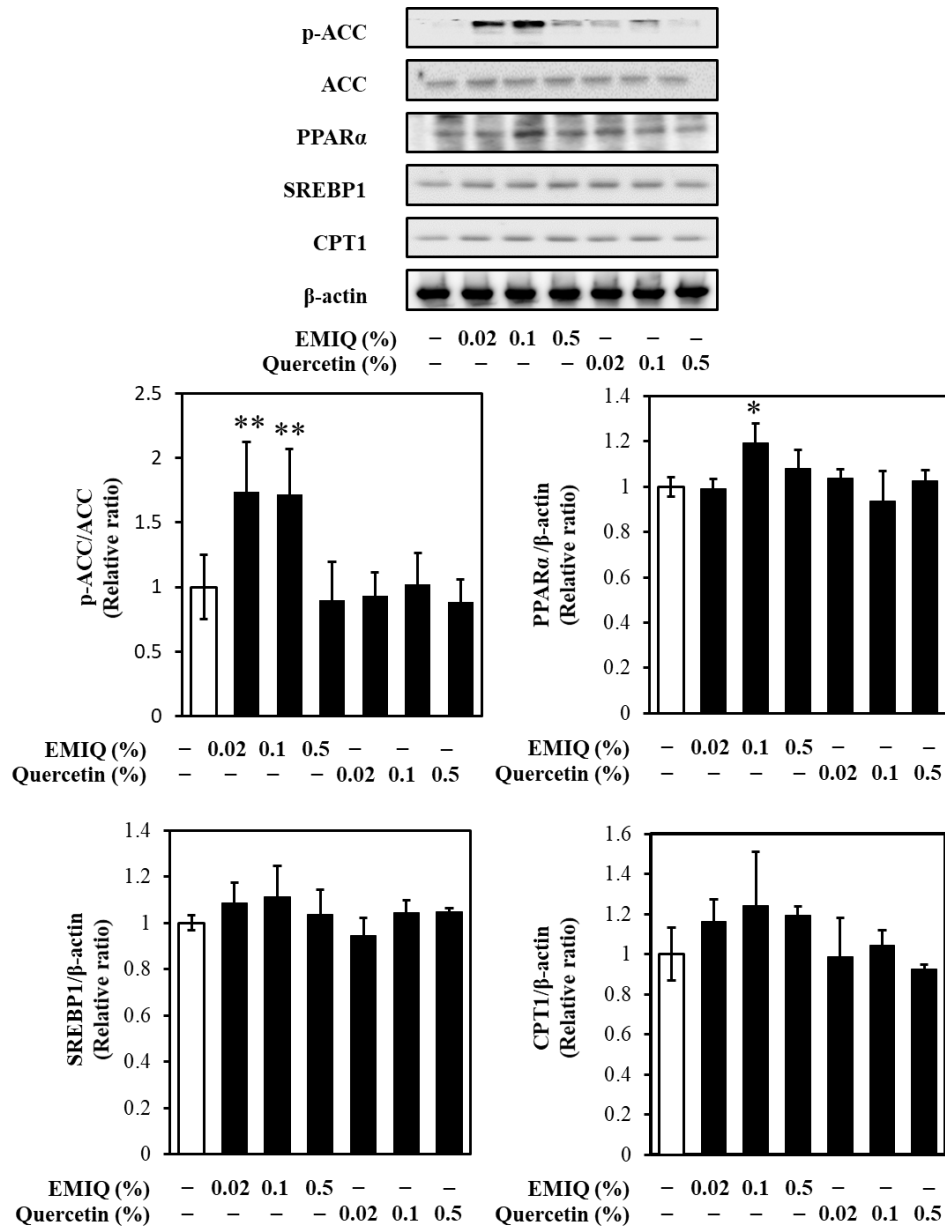
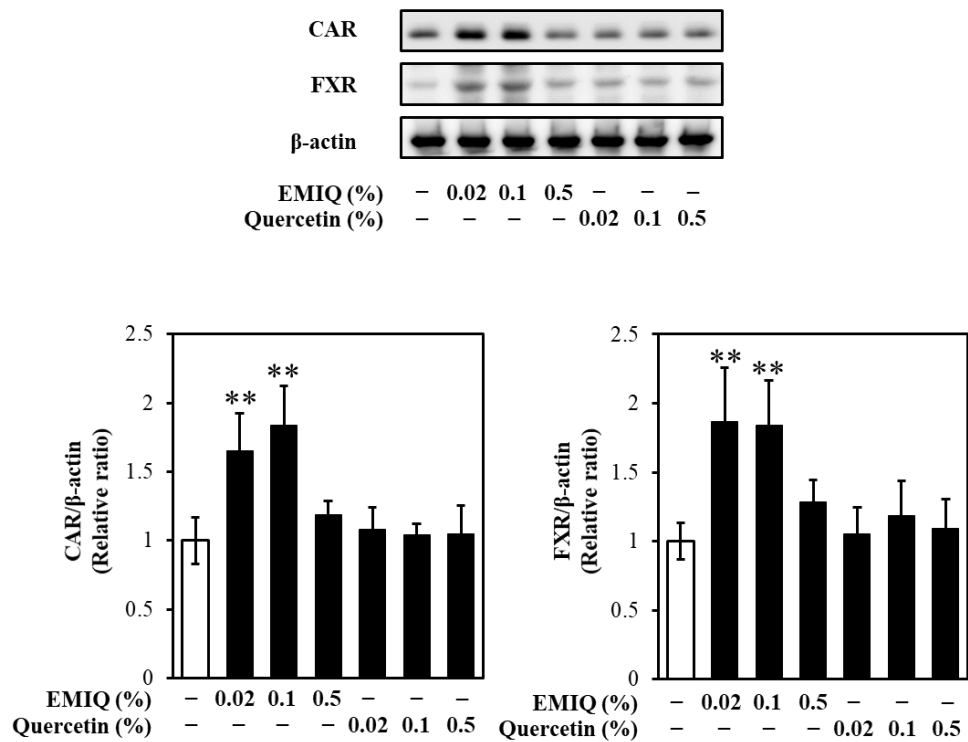


Figure 4-3. Effect of EMIQ and quercetin on the expression of adipogenesis- and energy metabolism-related proteins in the liver. The expression of p-ACC, ACC, PPAR α , SREBP1, and CPT1 in the tissue lysate of liver were determined by western blot analysis. Each blot shows a representative result from four animals. Data are shown as the mean $\pm$ SE (n = 4). * p < 0.05 and ** p < 0.01 vs control group by Dunnett's multiple comparison test.

(A)



(B)

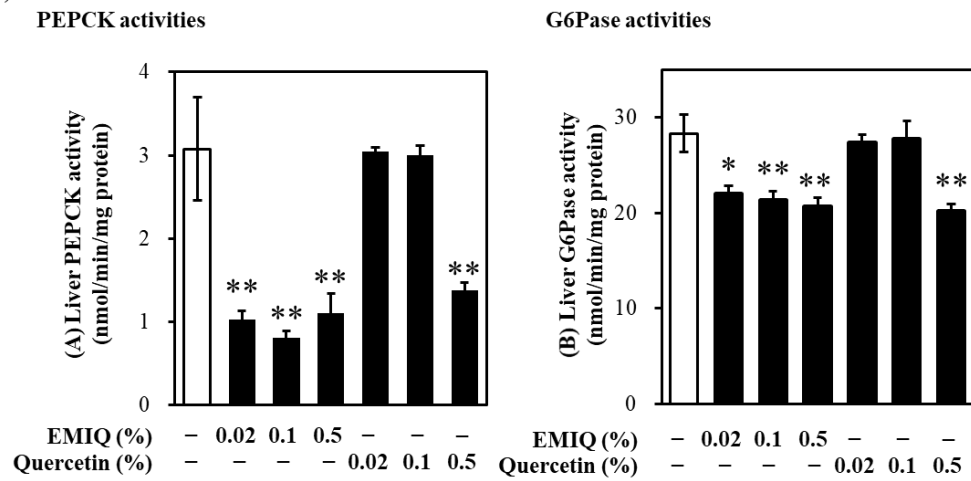


Figure 4-4. Effect of EMIQ and quercetin on gluconeogenesis in the liver. (A) The expression of CAR and FXR in the tissue lysate of liver were determined by western blot analysis. Each blot shows a representative result from four animals. (B) The enzyme activities of PEPCK and G6Pase were measured in the liver. Data are shown as the mean $\pm$ SE ($n = 4$). * $p < 0.05$ and ** $p < 0.01$ vs control group by Dunnett's multiple comparison test.

The effects of EMIQ and quercetin on translocation of GLUT4 in the muscle

GLUT4 translocation and its mechanism were evaluated by the author in the plasma membrane fraction and muscle lysate. GLUT4 translocation to the plasma membrane was increased by 0.02% and 0.1% EMIQ (Fig. 4-5). Conversely, neither EMIQ nor quercetin affected GLUT4 expression in the tissue lysate (Fig. 4-5). Furthermore, as shown in Fig. 4-6, phosphorylation of IRS1 (Ser307), Akt (Ser473 nor Thr308), PI3K (Tyr458), JAK2 (Tyr1007/1008) or STAT3 (Tyr705) were not changed by EMIQ and quercetin feeding. From these results, the author confirmed that the plasma glucose level was reduced by EMIQ supplementation, through upregulation of GLUT4 translocation to the plasma membrane and activating AMPK phosphorylation.

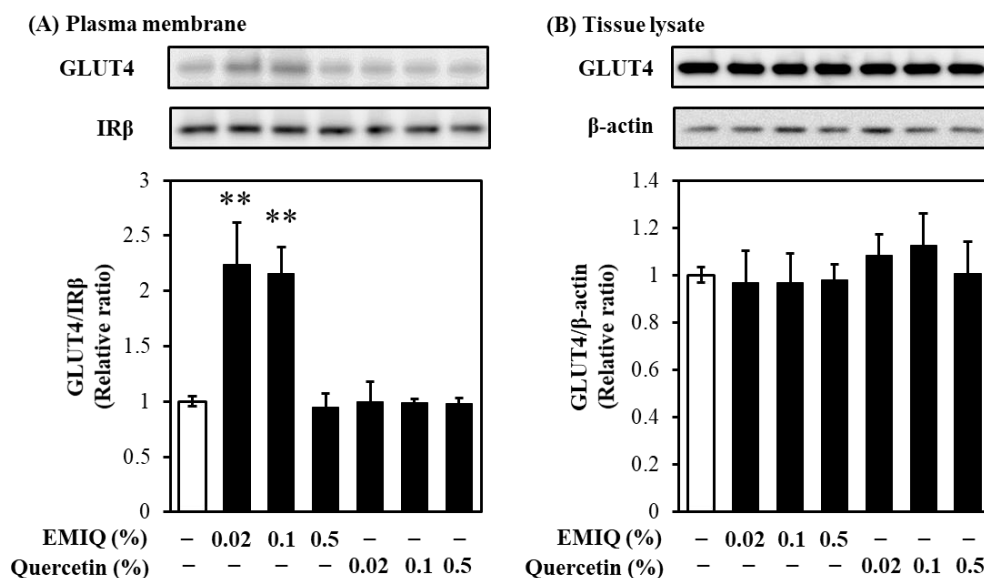


Figure 4-5. Effect of EMIQ and quercetin on the translocation and expression of GLUT4 in skeletal muscle. The expression of (A) GLUT4 and IRβ in the plasma membrane fraction and (B) GLUT4 and β-actin in the tissue lysate of skeletal muscle were determined by western blot analysis. Each blot shows a representative result from four animals. Data are shown as the mean ± SE (n = 4). * $p < 0.05$ and ** $p < 0.01$ vs control group by Dunnett's multiple comparison test.

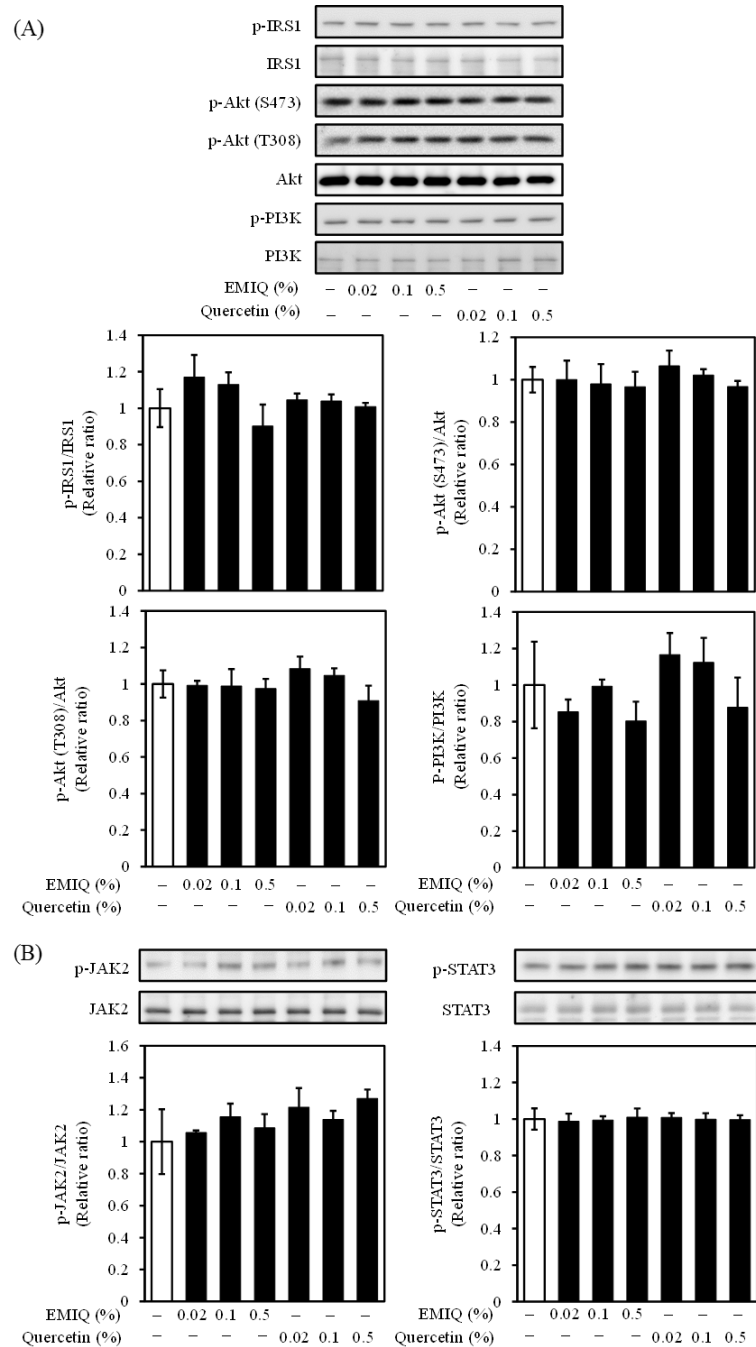


Figure 4-6. Effect of EMIQ and quercetin on factors in the insulin- and JAK/STAT-pathways in skeletal muscle. The expression of (A) p-IRS1, IRS1, p-Akt (S473), p-Akt (T308), Akt, p-PI3K, and PI3K and (B) p-JAK2, JAK2, p-STAT3, and STAT3 in the tissue lysate of skeletal muscle were determined by western blot analysis. Each blot shows a representative result from four animals. Data are shown as the mean $\pm$ SE (n = 4).

Discussion

T2DM is an epidemic multifactorial metabolic disease that is highly associated with metabolic disorder, and which is characterized by chronic hyperglycemia and dyslipidemia. Prevention of hyperglycemia and adiposity by enhancing energy expenditure, including activation of glucose and lipid metabolism and thermogenesis, are non-controversial ways to reduce the onset of diabetes mellitus. In this chapter, the author confirmed that energy metabolism was promoted by diet supplementation with EMIQ in mice. The plasma glucose level and deposition of lipids in WAT were suppressed by EMIQ supplementation (Table 4-1). These phenomena were regulated by AMPK phosphorylation in the different tissues (Fig. 4-1). Supplementation with EMIQ reduced lipogenesis, increased lipolysis, and upregulated the browning makers in WAT (Fig. 4-2A and B). In the liver, gluconeogenesis and lipolysis were regulated by EMIQ supplementation (Figs. 4-3 and 4-4). GLUT4 translocation to the plasma membrane was also increased in 0.02% and 0.1% EMIQ groups (Figs. 4-5 and 4-6). From these data, the author confirmed that AMPK is a vital factor in promoting energy metabolism, which could be regulated by EMIQ supplementation.

Recently, insulin resistance has been reported to be ameliorated by many polyphenol-rich food materials, and food-derived phytochemicals through activation of AMPK, including cacao liquor procyanidin extract [Yamashita, Y., *et al.*, 2012], ashitaba extract [Zhang, T., *et al.*, 2015], and methylxanthine derivative-rich cacao extract [Yamashita, Y., *et al.*, 2018] in animal and cell culture experiments. AMPK and its downstream factors have been regarded as the therapeutic targets to modulate energy homeostasis and mitochondrial autophagy [Long, YC., *et al.*, 2006; Mihaylova, MM., *et al.*, 2011]. Yamashita *et al* reported that AMPK inhibitor blocked ashitaba extract-

reduced the expression levels of C/EBP α , C/EBP β , and PPAR γ [Yamashita, Y., *et al.*, 2015]. Besides, the author confirmed that phosphorylation of AMPK was promoted by EMIQ supplementation in a dose-independent manner, which was consistent with Chapter 2, i.e., quercetin also induced phosphorylation of AMPK in a dose-independent manner in muscle cells. Moreover, AMPK phosphorylation increase in a dose-independent manner induced by AMPK activator (MT63-78) in DU145 cells [Zadra, G., *et al.*, 2014]. The possible explanation is that this dose-independent increase in AMPK phosphorylation was associated with the different activity of their receptors. Nevertheless, researchers still explored this issue: which receptors are associated with AMPK phosphorylation regulated by polyphenols. Conversely, AMPK phosphorylation was not increased by quercetin supplementation in these tissues (Fig. 4-1). In Chapter 2, the author found that quercetin significantly increased AMPK phosphorylation in L6 myotubes. Moreover, it is reported that adipogenesis is suppressed by quercetin and quercetin-rich onion peel through activation of AMPK in mice [Dong, J., *et al.*, 2014; Moon, J., *et al.*, 2013]. Different concentrations and feeding time are the potential explanations for this significant difference. It has been confirmed that the bioavailability of quercetin is poor [Manach, C., *et al.*, 2004]. For this reason, showing quercetin's beneficial effects on human health need long-term intervention. It is, however, worth noting that the bioavailability of EMIQ is 17-fold higher than that of quercetin [Kohara, A., *et al.*, 2017; Murota, K., *et al.*, 2010]. Thus, EMIQ, but not quercetin, induced phosphorylation of AMPK after two weeks of feeding.

Regulating lipogenesis and lipolysis [Rupasinghe, HP., *et al.*, 2016; Yamashita, Y., *et al.*, 2018], and promotion of energy expenditure, including the modulation of thermogenesis and formation of beige adipocytes [Bartelt, A., *et al.*, 2014] are the accepted approaches to regulate metabolic disorder diseases. In this chapter, EMIQ

upregulated CPT1 expression in the WAT (Fig. 4-2A). Similarly, it is reported that CPT1 mRNA expression is significantly increased by quercetin-rich onion peel extract supplementation in 3T3-L1 cells and adipose tissue [Moon, J., *et al.*, 2013]. Indeed, CPT1, UCP2, and ACC are vital factors in fatty acid β -oxidation [Zhang, T., *et al.*, 2015; Rupasinghe, HP., *et al.*, 2016; Yamashita, Y., *et al.*, 2018]. Nevertheless, 0.02% EMIQ upregulated AMPK phosphorylation, but not the expression of CPT1 and UCP2 (Figs. 4-1 and 4-2A). ACOX1, one of the other important factor in the lipolysis system is a possible explanation [de Boer, VC., *et al.*, 2006]. ACOX1 may be increased by 0.02% EMIQ. It is reported that after long-term intervention of quercetin, ACOX1 gene expression is increased compared with the control group [de Boer, VC., *et al.*, 2006]. Another potential explanation is that the feeding period is short. Hence, this issue should be clarified in the future.

In this chapter, the author confirmed that 0.1% EMIQ weakened PPAR γ , SREBP1, C/EBP α , and C/EBP β expression levels, but not C/EBP δ expression in WAT (Fig. 4-2A). It is reported that SREBP1, PPAR γ , and C/EBPs are associated with lipogenesis and adipocyte differentiation [Cho, BY., *et al.*, 2017; Moseti, D., *et al.*, 2016; Brun, RP., *et al.*, 1996]. Moreover, C/EBP α and PPAR γ expression levels were increased by activation of C/EBP β and C/EBP δ in the early stages of differentiation [Zhang, T., *et al.*, 2014; Mitani, T., *et al.*, 2018]. The results in this chapter were matched with previous report that the expression levels of C/EBP α and C/EBP β were downregulated by quercetin in 3T3-L1 cells [Seo, MJ., *et al.*, 2015]. The possible explanation for this phenomenon is that polyphenols regulate lipogenesis and differentiation by weakening C/EBP β expression, but not C/EBP δ expression in the WAT. In addition, C/EBP β expression was downregulated by cacao extract, chalcones cardamonin, and theobromine, while these polyphenols did not regulate C/EBP δ expression in the WAT

[Yamashita, Y., *et al.*, 2018; Mitani, T., *et al.*, 2017; Zhang, T., *et al.*, 2014; Liu, Y., *et al.*, 2009].

Next, not only EMIQ, but also 0.5% quercetin have been confirmed to regulate the enzyme activities of PEPCK and G6Pase (Fig. 4-4B). While, CAR and FXR expression levels were only upregulated in 0.02% and 0.1% EMIQ groups (Fig. 4-4A). This disagreement could be explained by different upstream factors. Both enzyme activities are not only regulated by activation of CAR and FXR, but also modulated by other factors [Rudraiah, S., *et al.*, 2016]. It is reported that the activities of PEPCK and G6Pase are suppressed by acute exercise [De Souza, CT., *et al.*, 2010], Akt phosphorylation [De Souza, CT., *et al.*, 2010], Foxo1 phosphorylation [De Souza, CT., *et al.*, 2010], and protein levels of HNF-4 α [Yasui, K., *et al.*, 2011] in the liver. Epigallocatechin-3-gallate has been reported to ameliorate diet-induced obesity and downregulate the enzyme activities of G6Pase and PEPCK [Yasui, K., *et al.*, 2011; Li, X., *et al.*, 2018]. The mechanisms by which EMIQ and quercetin suppress the enzyme activities of PEPCK and G6Pase needs further investigation in the future.

In conclusion, the author found that EMIQ had potent protective effects against lipid and glucose metabolism by activating AMPK phosphorylation. Activated AMPK not only inhibited adipogenesis, promoted energy expenditure and regulated thermogenesis-related proteins in WAT, but also regulated glucose homeostasis in the liver and muscle by decreasing hepatic gluconeogenesis and increasing GLUT4 translocation, respectively. Taken together, compared with quercetin, EMIQ is a promising functional quercetin glucoside for regulating energy metabolism via activation of AMPK phosphorylation.

Chapter 5: Prevention effects of quercetin derivatives on glucose and lipid metabolism in high-fat diet-fed mice after long-term feeding

Introduction

The prevalence of obesity is increasing alarmingly over the past 25 years, which is closely related to the genetics and unhealthy lifestyles, such as low physical activity and consumption of excess high-fat (HF) diet [Bhurosy, T., *et al.*, 2014]. In human and animal studies, a significant linear relationship has been verified between the amounts of dietary energy intake from fat and the proportion of the population who are overweight and glucose tolerance [Han, JC., *et al.*, 2008; Wang, CY., *et al.*, 2012]. The mechanisms by which obesity induced insulin resistance are clarified that the utilization of glucose in the muscle is decreased and glycogen synthase activity is reduced in the liver and muscle [Han, JC., *et al.*, 2008; Hardy, OT., *et al.*, 2012]. Inflammatory cytokines (TNF α and IL-6) disrupt the insulin signaling by activating nuclear factor- κ B (NF- κ B) and c-Jun aminoterminal kinase (JNK) to down-regulate phosphorylation of insulin receptor [de Luca, C., *et al.*, 2008; Stafeev, IS., *et al.*, 2017]. Furthermore, HF diet-induced insulin resistance inhibits phosphorylation of serine 307 residue on IRS1 and reduces GLUT4 protein expression [de Luca, C., *et al.*, 2008; Stafeev, IS., *et al.*, 2017].

Increasing evidences are reported that lifestyle interventions for moderating weight loss, reducing intake of saturated fatty acid, and increasing physical activity tend to improved insulin sensitivity among individuals with impaired glucose tolerance

[Guess, ND., *et al.*, 2018; Te Morenga, L., *et al.*, 2017].

The author investigated anti-hyperglycemia functions of quercetin and its glucosides in the previous chapters for a single administration and the short-term feeding experiments (Chapter 3 and 4). Isoquercitrin, rutin, and EMIQ contributed to prevent hyperglycemia through AMPK-signaling pathway (Chapter 3) and EMIQ showed the positive effect on energy metabolism by activating AMPK phosphorylation to suppress adipose tissue mass, inhibit adipogenesis and regulate thermogenesis-related proteins (Chapter 4). Moreover, the author could not detect any differences between body weight among the groups after feeding (Chapter 4), and quercetin failed to prevent glucose intolerance after a single oral administration and a short time feeding (Chapter 3 and 4). It has been reported that quercetin glycosides received metabolic conversion to the glucuronide- and sulfate-conjugation forms, and only the slight amounts of quercetin aglycone exists in the body after long-term supplementation with quercetin glucosides [Kawai, Y., *et al.*, 2008; Williamson, G., *et al.*, 2005; Yeh, SL., *et al.*, 2016]. The effects of quercetin and its glucosides on hyperglycemia and obesity in ICR mice fed HF diet for long term were compared. Quercetin, isoquercitrin, rutin and EMIQ were supplemented to the standard and HF diets and given to the mice for 13 weeks. The author examined the body weight gain and the condition of insulin resistance and adipokines, in addition to analyze GLUT4 translocation and its related proteins in the muscle, adipogenesis and thermogenesis-related proteins in the WAT and liver.

Materials and methods

Materials

All the materials and reagents, such as quercetin, isoquercitrin, rutin and EMIQ

were purchased the highest grade available from commercial sources and described in Chapter 3. Plasma insulin was tested using commercial ELISA kit (Mouse Insulin ELISA Kit) from Shibayagi. For the western blot analysis, used antibodies were the same as described in Chapter 2 and 4. Anti-FAS rabbit IgG was from Cell Signaling Technology (Danvers, MA).

Animal treatment

All animal experiments were approved by the Kobe University Institutional Animal Care and Use Committee (Permission # 1-27-05-09) and carried out according to the guidelines for animal experiments at Kobe University Animal Experimentation Regulation. Male ICR mice (4 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan) and maintained in the same conditions as described in Chapter 3. Mice were randomly divided into ten groups and fed a standard or HF diet containing 30% (w/w) lard for 13 weeks. The composition of diets and energy density are shown in Table 5-1. The standard and HF diets were supplemented with quercetin, isoquercitrin, rutin, and EMIQ at 0% (w/w) (C-0 and HF-0), or 0.1% (w/w). Body weight was monitored every week. At the end of the experiment, mice were sacrificed and the liver, skeletal muscle, WAT and BAT were weighted.

Measurement of plasma parameters and adipokines

The plasma was obtained as described in Chapter 3. Plasma triacylglycerol, total cholesterol, non-esterified fatty acid (NEFA) and glucose levels were measured using the corresponding commercial kit (Triacylglycerol-E test, Cholesterol-E test, NEFA-C test, and Lab assayTM Glucose Wako kit from Wako Pure Chemical Industries, Ltd. respectively). Plasma insulin and adiponectin were also measured using the corresponding commercial ELISA kit (Mouse Insulin ELISA Kit, and Mouse/Rat High

Molecular Weight Adiponectin ELISA Kit, Shibayagi, Gunma, Japan, respectively).

Insulin resistance index (HOMA-IR) was calculated using the following formula:

$\text{HOMA-IR} = \text{fasting plasma glucose (mg/dl)} \times \text{fasting plasma insulin (ng/ml)} / 405$.

Immunoblotting

Immunoblotting was carried out as described in Chapter 2. Antibodies were the same as mentioned in Chapter 2 and 4. Antibody for FAS was diluted to 1:5000 and used in this chapter.

Statistical analysis

Data are represented as the means and standard error (SE). The statistical analysis was performed using Tukey-Kramer multiple comparison test as indicated in each table and figure legend using JMP 11.2.0 (SAS, Cary, USA). $p < 0.05$ was considered statistically significant.

Table 5-1. Composition of the standard and high-fat diets

	Standard	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

Results

The effects of quercetin, isoquercitrin, rutin and EMIQ on body weight, and tissue weight in mice

The effects of quercetin, and its glucosides (isoquercitrin, rutin, and EMIQ) on preventing hyperglycemia and obesity were investigated in this chapter. As shown in Table 5-2, the body weight of the mice were significantly increased in the HF-0 group as expected. HF-diet caused increase in the body weight was decreased by supplementation with 0.1 % quercetin glucosides but not by 0.1 % quercetin. Isoquercitrin revealed on the decreasing in the body weight was more obvious than rutin and EMIQ. An intake of HF-diet significantly increased in the weight of all WAT (epididymal, mesenteric, retroperitoneal, subcutaneous adipose tissue) compared with the intake of standard diet. Quercetin glucosides significantly decreased the fat deposition in mesenteric adipose tissue. In the other parts of WAT, as epididymal, retroperitoneal, and subcutaneous, quercetin and its glucosides showed only the decreasing tendency. These results suggest that long-term feeding of quercetin glucosides have the potential to reduce HF diet-induced body weight and fat accumulation in WAT.

Table 5-2. Effect of quercetin, isoquercitrin, rutin and EMIQ on the body and tissue weights

	Standard					High-fat				
	Control	Quercetin	Isoquercitrin	Rutin	EMIQ	Control	Quercetin	Isoquercitrin	Rutin	EMIQ
Final body weight (g)	32.3 ± 1.4 ^{bc}	32.2 ± 0.4 ^{bc}	31.6 ± 0.8 ^c	33.2 ± 1.6 ^{bc}	31.0 ± 0.6 ^c	41.0 ± 2.3 ^a	36.9 ± 0.8 ^{ab}	32.1 ± 1.3 ^{bc}	32.8 ± 2.6 ^{bc}	35.6 ± 0.8 ^{bc}
Lean weight (g)	19.8 ± 1.5 ^a	19.1 ± 1.0 ^a	19.4 ± 0.7 ^a	20.2 ± 1.1 ^a	18.9 ± 1.1 ^a	19.3 ± 1.8 ^a	17.0 ± 1.0 ^a	15.3 ± 0.3 ^a	16.7 ± 1.7 ^a	16.2 ± 0.7 ^a
Liver (% of body weight)	4.7 ± 0.3 ^a	4.2 ± 0.7 ^a	4.4 ± 0.3 ^a	4.7 ± 0.3 ^a	4.6 ± 0.3 ^a	4.6 ± 0.5 ^a	3.9 ± 0.2 ^a	4.1 ± 0.3 ^a	4.1 ± 0.2 ^a	4.0 ± 0.5 ^a
Adipose tissue weight (% of body weight)										
Mesenteric	1.63 ± 0.25 ^b	1.67 ± 0.07 ^b	1.74 ± 0.08 ^b	1.57 ± 0.16 ^b	1.57 ± 0.09 ^b	2.59 ± 0.36 ^a	1.92 ± 0.07 ^{ab}	1.49 ± 0.08 ^b	1.37 ± 0.14 ^b	1.77 ± 0.12 ^b
Retroperitoneal	1.53 ± 0.21 ^c	1.70 ± 0.13 ^{bc}	1.71 ± 0.11 ^{bc}	1.55 ± 0.14 ^{bc}	1.56 ± 0.17 ^{bc}	2.71 ± 0.17 ^a	2.68 ± 0.15 ^a	2.60 ± 0.16 ^a	2.29 ± 0.11 ^{ab}	2.66 ± 0.13 ^a
Epididymal	3.71 ± 0.55 ^c	4.01 ± 0.39 ^{bc}	4.00 ± 0.18 ^{bc}	3.53 ± 0.20 ^c	3.51 ± 0.42 ^c	6.67 ± 0.23 ^a	6.53 ± 0.43 ^a	5.69 ± 0.47 ^{ab}	5.54 ± 0.29 ^{ab}	5.77 ± 0.27 ^{ab}
Subcutaneous	5.42 ± 0.71 ^c	6.33 ± 0.44 ^{bc}	5.66 ± 0.41 ^c	7.20 ± 0.49 ^{abc}	5.66 ± 0.61 ^c	8.81 ± 0.53 ^{ab}	9.19 ± 0.69 ^a	8.63 ± 0.62 ^{ab}	8.61 ± 0.84 ^{ab}	8.48 ± 0.64 ^{abc}
Brown adipose	0.45 ± 0.07 ^a	0.83 ± 0.12 ^a	0.82 ± 0.10 ^a	0.71 ± 0.09 ^a	0.87 ± 0.14 ^a	0.67 ± 0.11 ^a	0.51 ± 0.08 ^a	0.48 ± 0.03 ^a	0.63 ± 0.11 ^a	0.72 ± 0.06 ^a

Mice were fed the standard or high-fat diet containing quercetin and its glucosides for 13 weeks. At the end of the experiment, body, adipose tissue and liver weights were measured after an 18h fasting. Values are the mean ± SE (n = 5). Values without a common letter in each row differ significantly among groups ($p < 0.05$) by Tukey-Kramer multiple comparison test.

Table 5-3. Effect of quercetin, isoquercitrin, rutin and EMIQ on plasma lipids and adiponectin levels.

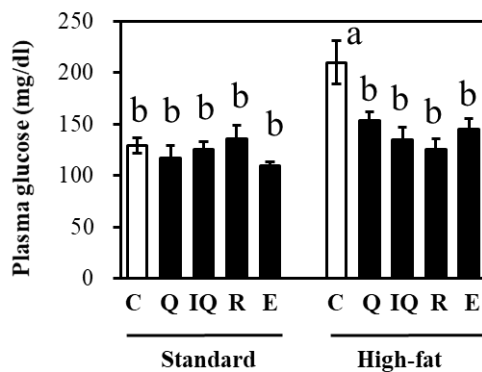
	Standard					High-fat				
	Control	Quercetin	Isoquercitrin	Rutin	EMIQ	Control	Quercetin	Isoquercitrin	Rutin	EMIQ
Cholesterol (mg/dl)	84.4 ± 4.5 ^c	82.6 ± 3.8 ^c	83.5 ± 3.1 ^c	85.7 ± 6.6 ^c	91.3 ± 4.4 ^c	142.4 ± 12.9 ^a	119.3 ± 6.6 ^{ab}	105.5 ± 3.5 ^{bc}	99.2 ± 4.4 ^{bc}	107.8 ± 6.0 ^{bc}
Triglyceride (mg/dl)	130.7 ± 20.1 ^a	139.7 ± 17.1 ^a	126.7 ± 16.8 ^a	114.2 ± 17.3 ^a	139.9 ± 15.3 ^a	128.1 ± 15.8 ^a	137.0 ± 13.7 ^a	141.1 ± 12.1 ^a	121.2 ± 5.7 ^a	124.0 ± 13.9 ^a
NEFA (meq/l)	0.76 ± 0.10 ^a	0.95 ± 0.13 ^a	0.80 ± 0.09 ^a	0.79 ± 0.08 ^a	0.89 ± 0.04 ^a	0.66 ± 0.05 ^a	0.79 ± 0.06 ^a	0.69 ± 0.05 ^a	0.74 ± 0.04 ^a	0.76 ± 0.08 ^a
Adiponectin	4.68 ± 0.46 ^a	5.42 ± 0.28 ^a	4.97 ± 0.51 ^a	4.96 ± 0.61 ^a	5.31 ± 0.34 ^a	4.83 ± 0.23 ^a	5.09 ± 0.39 ^a	6.49 ± 0.80 ^a	5.48 ± 1.13 ^a	5.82 ± 0.54 ^a
Adiponectin in dex	3.54 ± 0.63 ^{abc}	4.06 ± 0.31 ^{ab}	3.52 ± 0.67 ^{abc}	3.42 ± 0.92 ^{abc}	4.48 ± 0.54 ^a	2.43 ± 0.14 ^c	2.51 ± 0.27 ^c	3.18 ± 0.50 ^{bc}	3.04 ± 1.28 ^{bc}	3.20 ± 0.60 ^{abc}

Mice were fed the standard or high-fat diet containing quercetin and its glucosides for 13 weeks. At the end of the experiment, plasma levels of total cholesterol (T-CHL), triglyceride, NEFA and adiponectin were measured after an 18h fasting. Values are the mean ± SE (n = 5). Values without a common letter in each row differ significantly among groups ($p < 0.05$) by Tukey-Kramer multiple comparison test.

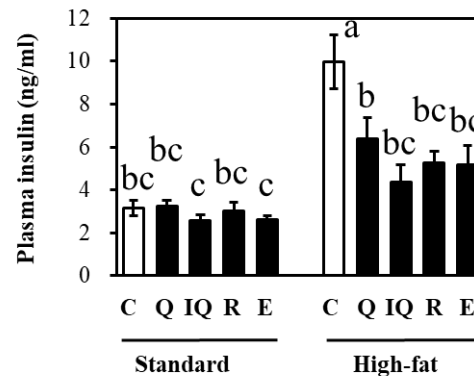
The effects of quercetin, isoquercitrin, rutin and EMIQ on plasma glucose and lipid levels

After 13 weeks, the plasma glucose level and insulin level in the C-0 group were significantly lower than those in the HF-0 group, indicating that chronic hyperglycemia and hyperinsulinemia were induced by feeding of HF diet for a long time (Fig. 5-1A). Supplementation with quercetin, isoquercitrin, rutin and EMIQ drastically reduced HF diet-induced hyperglycemia and hyperinsulinemia. Quercetin and its glucosides normalized the plasma glucose levels to the standard diet-fed groups (Fig. 5-1A). Regarding the plasma insulin level, these compounds significantly decreased it in the HF diet-fed groups (Fig. 5-1B). The HOMA-IR index in the HF-0 group was significantly higher than that in the C-0 group. Quercetin and its glucosides significantly decreased HOMA-IR index in the HF diet-fed groups. The total plasma cholesterol level in the HF-0 group increased significantly (Table. 5-3). As shown in Table 5-3, supplementation with isoquercitrin, rutin and EMIQ significantly reduced HF diet-increased plasma cholesterol level, although quercetin did not show a significant change. Obesity causes a decrease trend in circulating adiponectin index [Nishiumi, S., *et al.*, 2010]. Although the plasma adiponectin levels were not reduced, the adiponectin index shew a decreasing tendency by the intake of HF diet. The decreased adiponectin index tended to increase in the quercetin and its glucosides groups. These data indicated that not only quercetin glucosides, but also quercetin have the potential to prevent insulin resistance and only quercetin glucosides show the potential to regulate lipid metabolism.

(A) Plasma glucose levels



(B) Plasma insulin levels



(C) HOMA-IR

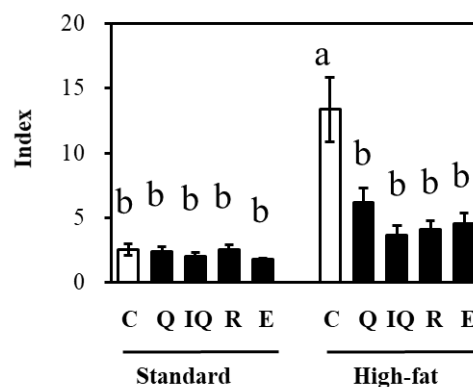


Figure 5-1. The effects of quercetin, isoquercitrin, rutin, and EMIQ on the plasma glucose, and insulin levels. The levels of (A) plasma glucose and (B) insulin were measured and (C) the HOMA-IR was calculated. Values were shown as the mean $\pm$ SE (n = 5). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ($p < 0.05$).

Quercetin and its glucosides promote AMPK phosphorylation in the skeletal muscle, adipose tissues, and liver

In Chapter 3 and 4, the author has illustrated that AMPK is a pivotal molecule for regulating energy consumption and glucose metabolism, and quercetin glucosides increase GLUT4 translocation to the plasma membrane. Furthermore, the intake of HF diet has been reported to repress the AMPK activation in WAT [Kim, S.J., *et al.*, 2008; Pang, J., *et al.*, 2008]. Whether AMPK phosphorylation and its expression levels are

involved in glucose and lipid homeostasis following the supplementation with quercetin and its glucosides was detected by the author. Dietary supplementation with quercetin and its glucosides increased AMPK phosphorylation in the skeletal muscle, adipose tissue, and liver. On the other hand, the expression level of AMPK was not affected by quercetin and its glucosides in these tissues, although the AMPK expression level tended to decrease in the HF-0 group. These results suggest that quercetin and its glucosides prevented HF diet-induced hyperglycemia and lipid accumulation through promoting phosphorylation of AMPK in the skeletal muscle, adipose tissue, and liver (Fig. 5-2).

Quercetin and its glucosides induced GLUT4 translocation in skeletal muscle

The author, next, investigated the underlying mechanism of quercetin- and its glycosides-prevented insulin resistance. As shown in Fig. 5-3, the author found that the amount of GLUT4 in the plasma membrane fraction and tissue lysate were drastically decreased in the H-0 group, which is consistent with the previous report: HF diet-induced insulin resistance accompanied by a decrease in the expression level of GLUT4 in skeletal muscle [Nishiumi, S., *et al.*, 2010]. Quercetin, isoquercitrin, rutin, and EMIQ significantly increased GLUT4 translocation to the plasma membrane of skeletal muscle in both standard and HF diet groups (Fig. 5-3). These data indicated that quercetin and its glucosides promoted GLUT4 translocation to the plasma membrane, resulting in normalization of the blood glucose level. The author investigated the activation of the insulin-, AMPK-, and JAK/STAT-signaling pathways. In both standard and HF diet groups, quercetin, isoquercitrin, rutin, and EMIQ increased AMPK and ACC phosphorylation in the skeletal muscle (Fig. 5-2 and 5-3B). As to the insulin-pathway, all compounds failed to promote phosphorylation of IRS1, Akt (ser473 and thr308), and PI3K and their expression levels (Fig. 5-4). Meanwhile, neither JAK2 nor

STAT3 phosphorylation were observed by the supplementation with quercetin and its glucoside (Fig. 5-5). These results indicated that after long-term supplementation with quercetin, isoquercitrin, rutin, and EMIQ induced GLUT4 translocation to the plasma membrane through AMPK-pathway, but not by insulin- and JAK/STAT-pathways in skeletal muscle, resulting in normalization of HF diet-induced insulin resistance.

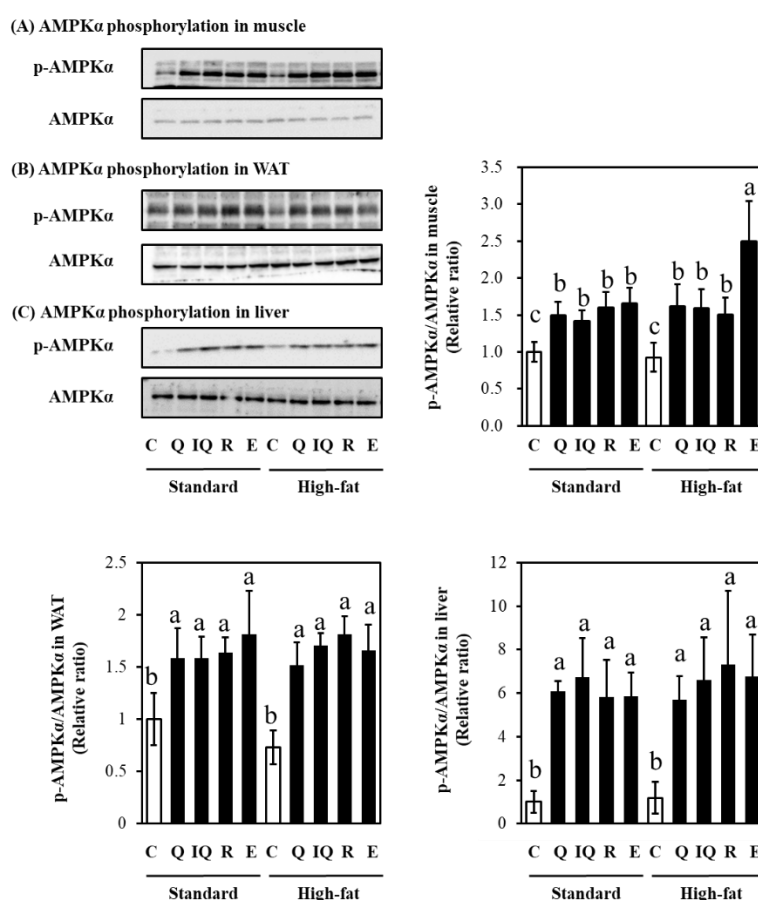


Figure 5-2. Effect of quercetin, isoquercitrin, rutin, and EMIQ on AMPK phosphorylation in the muscle, WAT, and liver. The phosphorylation and expression of AMPK in the tissue lysates of (A) skeletal muscle, (B) WAT, and (C) liver were determined by western blot analysis. Each panel shows a typical blot from five animals. Values were shown as the mean $\pm$ SE ($n = 5$). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ($p < 0.05$).

Quercetin and its glucosides suppressed adipogenesis by ameliorating the expression of energy metabolism- and thermogenesis-related proteins in the adipose tissue

The author investigated the underlying mechanism of EMIQ on energy metabolism for 2 week-feeding in Chapter 4. In this chapter, the author mainly compared the differences between quercetin and its four kinds of glucosides on the regulation of lipid metabolism and thermogenesis in the WAT for 13 week-feeding. As shown in Fig. 5-6A, quercetin and its glucosides supplementation increased ACC phosphorylation, concomitant with AMPK phosphorylation in both standard- and HF diet groups. CPT1 and UCP2 are associated with mitochondrial fatty acid oxidation [Cho, BY., *et al.*, 2017; Zhang, T., *et al.*, 2015]. The expression levels of UCP2 and CPT1 were significantly increased in the quercetin and its glucosides groups compared with the C-0 and HF-0 groups (Fig. 5-6A). PPAR γ , SREBP1, and C/EBPs are downstream targets for AMPK and involved in the adipogenesis and adipocyte differentiation [Moseti, D., *et al.*, 2016]. The expression levels of PPAR γ , SREBP1, C/EBP α and C/EBP β were significantly increased in the HF-0 group compared with the standard diet groups (Fig. 5-6B). In the HF diet groups, supplementation with quercetin and its glucosides significantly weakened the expression of these proteins. Next, UCP1, PGC-1 α and PRDM16 are reported to be associated with lipid metabolism, thermogenesis and browning [Puigserver, P., *et al.*, 2003; Chi, J., *et al.*, 2016; Son, MJ., *et al.*, 2016]. Therefore, the author determined the expression of these protein in WAT. As shown in Fig. 5-6C, dietary supplementation with quercetin and its glucosides could considerably increase the expression levels of these proteins in WAT compared with the C-0 and HF-0 groups. Based on these results, quercetin and its glucosides have the ability to suppress HF diet-induced body weight and WAT weight gain by increasing

energy expenditure due to browning of WAT and suppressing the adipocyte differentiation through promoting AMPK phosphorylation and the expression levels of UCP1, PGC-1 α and PRDM16 and weakening the expression levels of PPAR γ , SREBP1, C/EBP α and C/EBP β in the WAT.

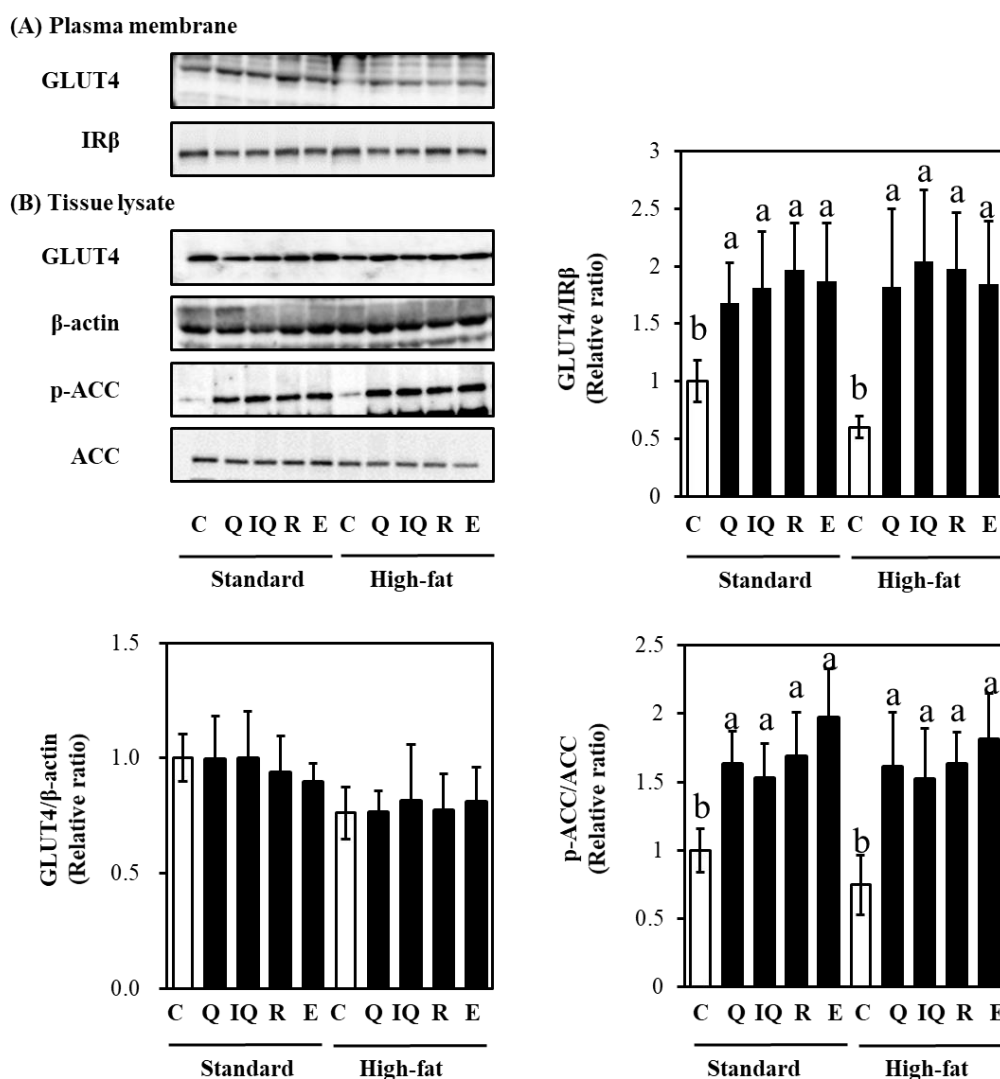


Figure 5-3. Effect of quercetin, isoquercitrin, rutin, and EMIQ on the translocation of GLUT4 in skeletal muscle. GLUT4, IR- β , β -actin, phosphorylated-ACC and ACC protein expression in (A) the plasma membrane fraction and (B) tissue lysate of skeletal muscle were determined by western blot analysis. Each panel shows a typical blot from five animals. Values were shown as the mean $\pm$ SE ($n = 5$). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ($p < 0.05$).

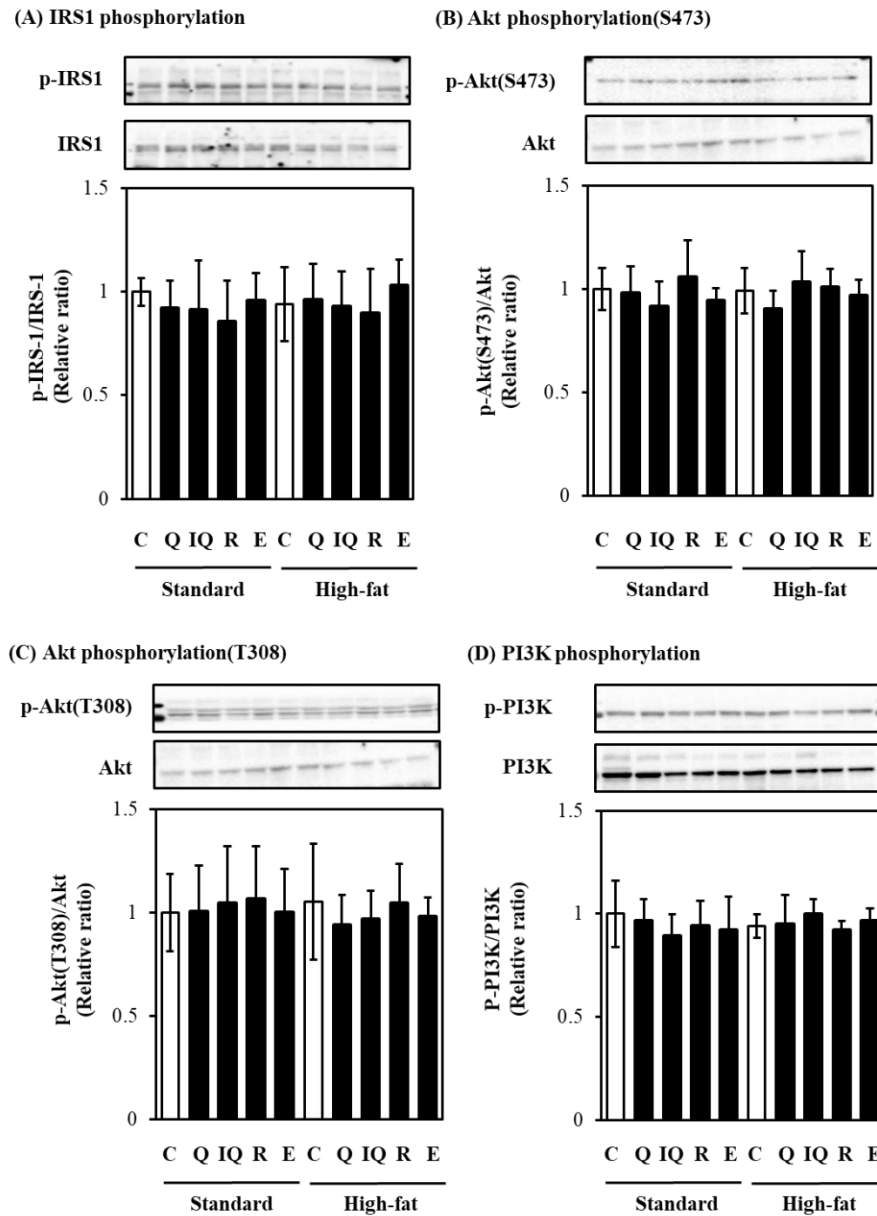


Figure 5-4. Effect of quercetin, isoquercitrin, rutin, and EMIQ on insulin-pathway in skeletal muscle. (A) phosphorylated-IRS1 and IRS1, (B) phosphorylated-Akt (S473) and Akt, (C) phosphorylated-Akt (T308) and Akt, (D) phosphorylated-PI3K and PI3K protein expression in the tissue lysate of skeletal muscle were determined by western blot analysis. Each panel shows a typical blot from five animals. Values were shown as the mean $\pm$ SE ($n = 5$). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ($p < 0.05$).

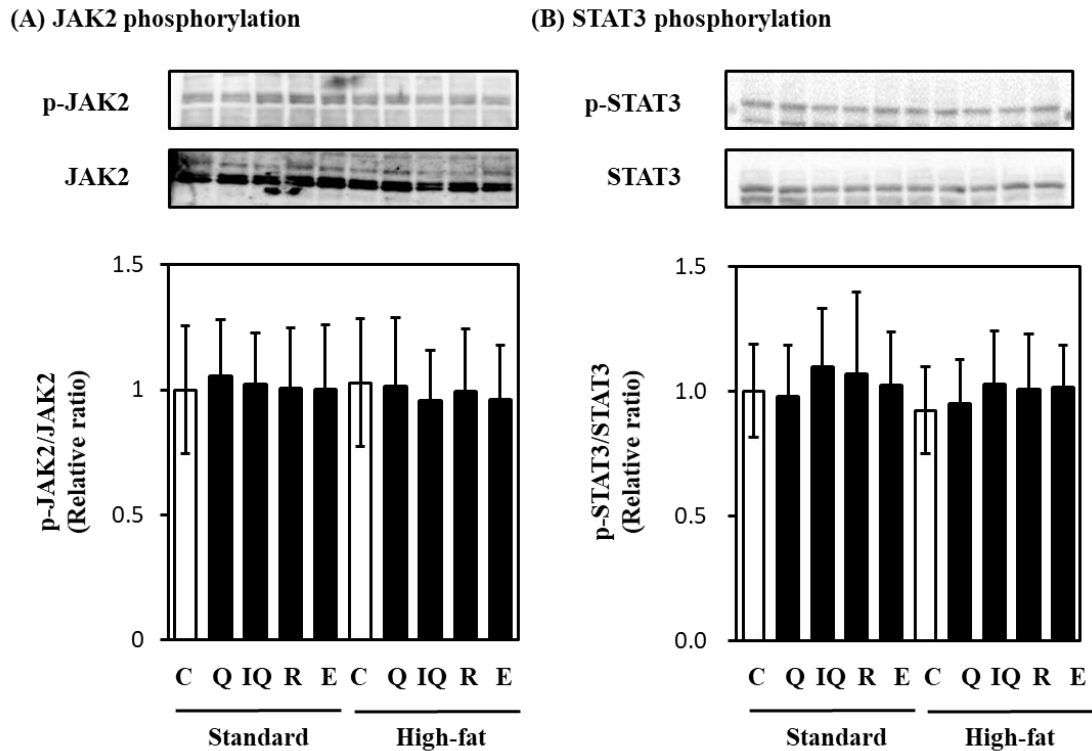
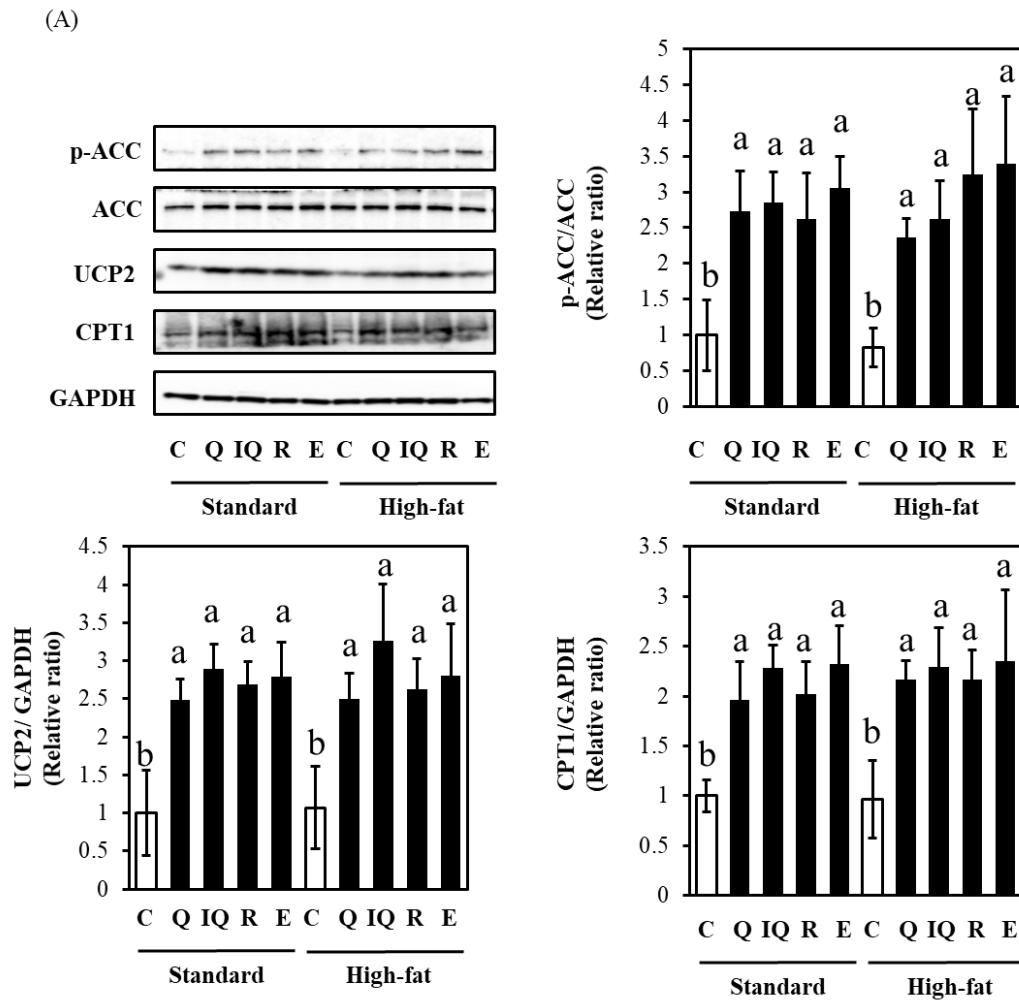


Figure 5-5. Effect of quercetin, isoquercitrin, rutin, and EMIQ on JAK/STAT-pathway in skeletal muscle. (A) phosphorylated-JAK2 and JAK2, (B) phosphorylated-STAT3 and STAT3 protein expression in the tissue lysate of skeletal muscle were determined by western blot analysis. Each panel shows a typical blot from five animals. Values were shown as the mean $\pm$ SE ($n = 5$). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ($p < 0.05$).

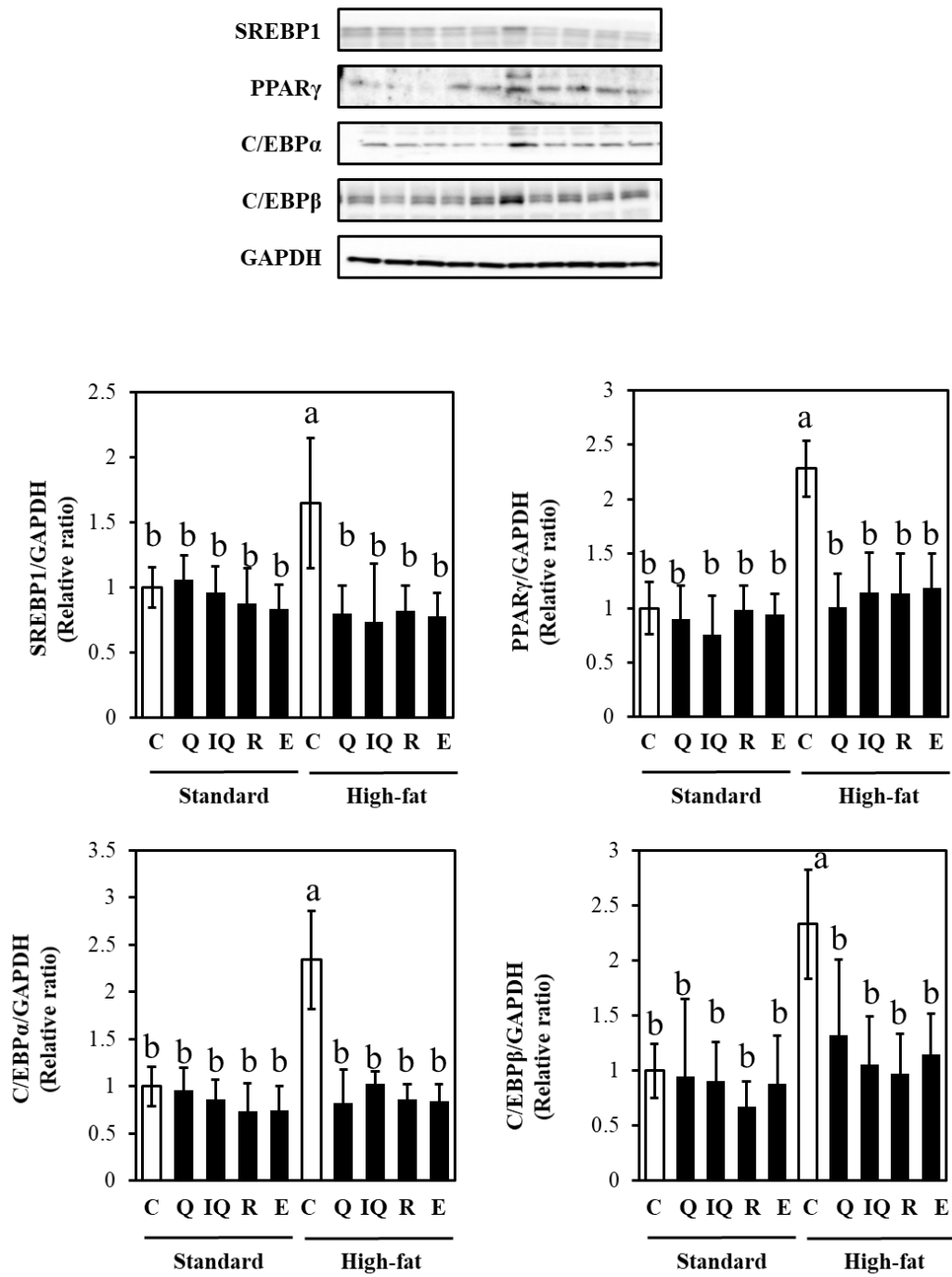
Quercetin and its glucosides modulated the expression of lipid metabolism-related proteins in the liver

The author also examined the expression of lipid metabolism-related proteins in the liver. The expression levels of SREBP1 and FAS were increased by the HF diet and supplementation with quercetin, isoquercitrin, rutin and EMIQ significantly decreased the expression levels of SREBP1 and FAS. Supplementation of HF diet with quercetin and its glucosides significantly increased ACC phosphorylation and the expression

levels of CPT1 and PPAR α in the liver. These results demonstrated that quercetin and its glucosides reduced fat mass by modulating lipid metabolism-related proteins expression in the liver.



(B)



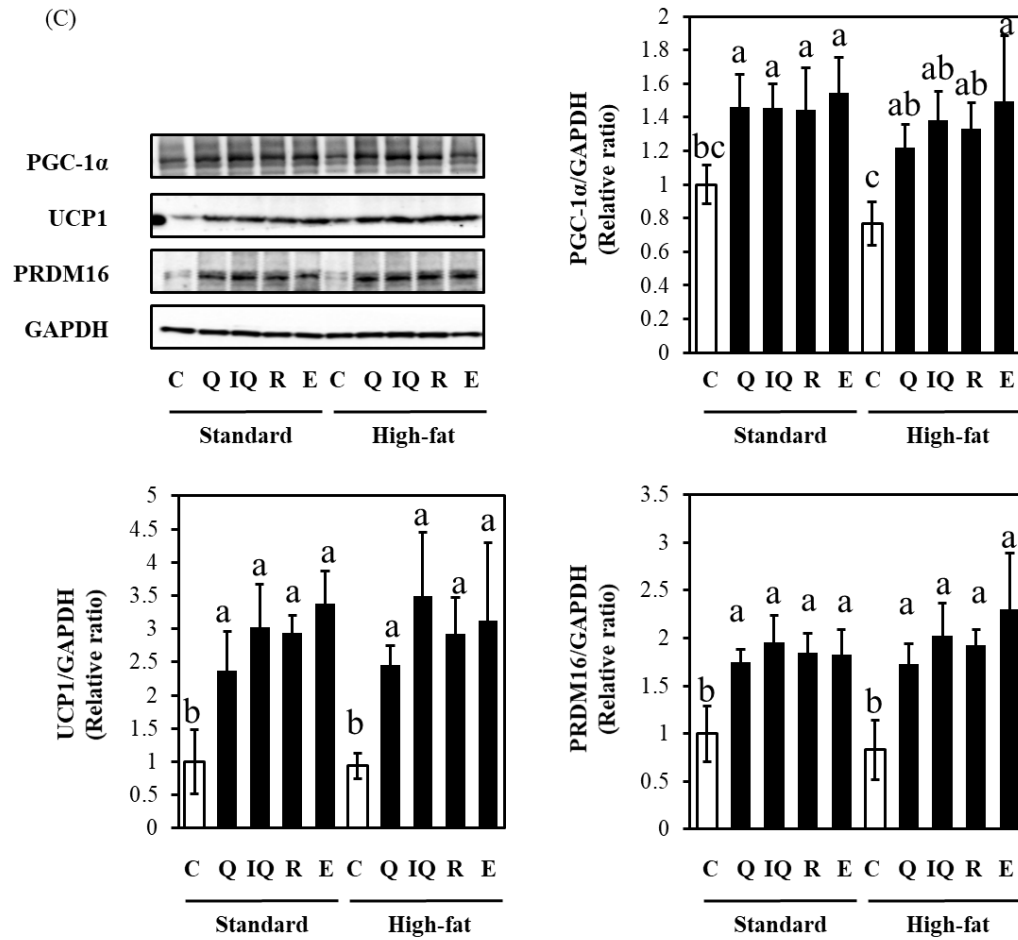


Figure 5-6. Effect of quercetin, isoquercitrin, rutin, and EMIQ on the expression of adipogenesis-, energy metabolism- and thermogenesis-related proteins in WAT. The expression of (A) p-ACC, ACC, UCP2, CPT1, and GADPH in the tissue lysate of WAT, the expression of (B) SREBP1, PPAR γ , C/EBP α , C/EBP β , and GADPH in the tissue lysate of WAT and the expression of (C) PGC-1 α , UCP1, PRDM16, SIRT1, and GADPH in the tissue lysate of WAT were determined by western blot analysis. Each panel shows a typical blot from five animals. Values were shown as the mean $\pm$ SE (n = 5). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ($p < 0.05$).

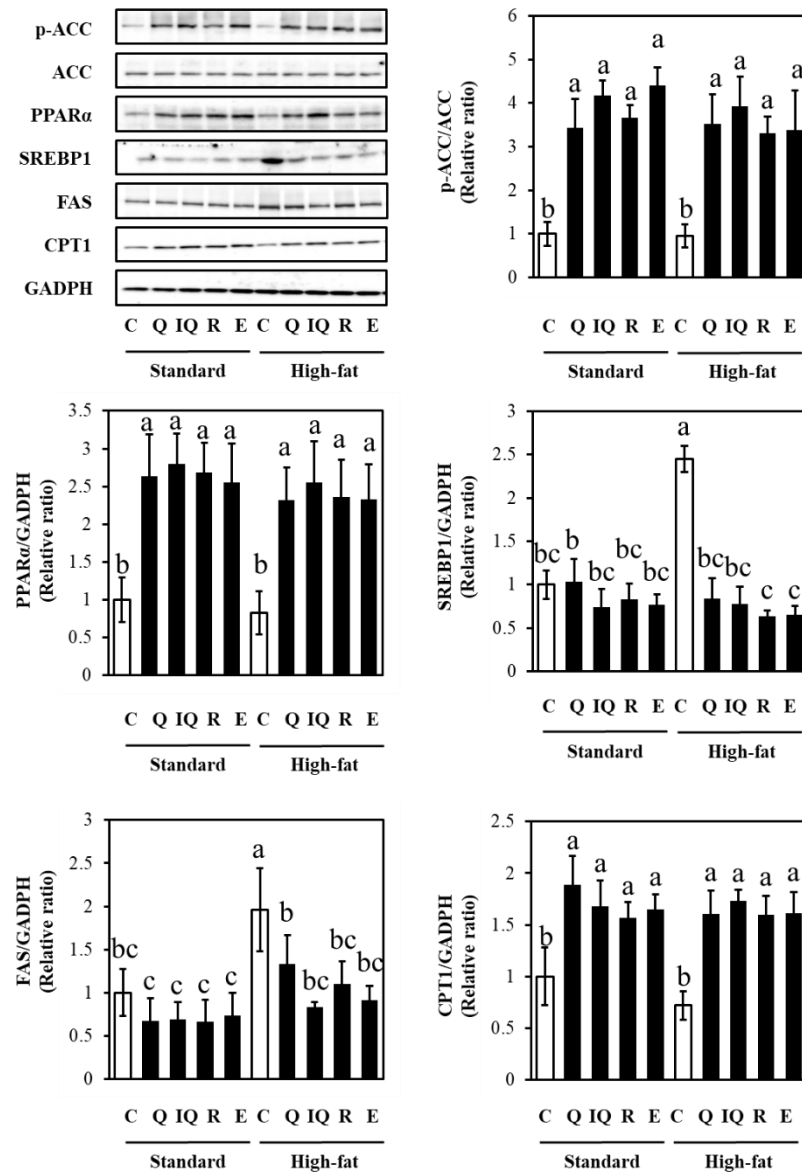


Figure 5-7. Effect of quercetin, isoquercitrin, rutin, and EMIQ on the expression of adipogenesis- and energy metabolism-related proteins in the liver. The expression of p-ACC, ACC, PPAR α , SREBP1, FAS, and CPT1 in the tissue lysate of liver were determined by western blot analysis. Each panel shows a typical blot from five animals. Values were shown as the mean $\pm$ SE ($n = 5$). Different superscripted letters indicate significant differences between the groups by Tukey-Kramer multiple comparison test ($p < 0.05$).

Discussion

Obesity usually causes by excessive energy intake accompanied by disrupting energy metabolism [Cao, L., *et al.*, 2016; Hubert, K. and Stephan, M., 2017]. Obesity induced T2DM is considered to be a risk factor for the development of various diseases. The pathophysiology of T2DM is chronic hyperglycemia, which causes a deterioration in insulin sensitivity [Benhalima, K., *et al.*, 2011]. Despite the growing knowledge of the pathophysiology of T2DM, current therapeutics include injection of insulin and increasing endogenous insulin production, only provide the transient antihyperglycemic effect and failed to prevent these abnormalities [Cryer, PE., *et al.*, 2009]. In this chapter, the effects of quercetin and its glucosides on HF diet induced obesity and hyperglycemia were evaluated and the underlying mechanisms were investigated. The author demonstrated that both quercetin and its glucosides prevented chronic hyperglycemia and obesity.

In the present study, mice were fed the standard and HF diets containing 0.1% quercetin and its glucosides for 13 weeks. The author found that AMPK phosphorylation was increased by quercetin and its glucosides in the skeletal muscle, WAT, and liver and induced GLUT4 translocation to the plasma membrane of skeletal muscle (Fig. 5-2, and 5-3). However, in Chapter 3, only quercetin glucosides, rather than quercetin, induced GLUT4 translocation and promoted AMPK phosphorylation in skeletal muscle of ICR mice. Similar results were observed in Chapter 4, quercetin also could not affect the AMPK phosphorylation in mice after two weeks feeding. These discrepancies were caused by the bioavailability of quercetin. The bioavailability of quercetin is poor. Hence, quercetin needs continuous intake for long-term can reveal its anti-hyperglycemic effect. It has been reported that 60 min after administration of radioactively labeled quercetin (630 mg /kg BW), most of the radioactivity was detected in the gastrointestinal tract, liver and kidney. Only low levels of radioactivity were

detected in the lungs [Ueno, I., *et al.*, 1983]. In short-term study, high concentrations of quercetin metabolites but not aglycone form were detected in the kidney, liver, and heart of rats [Morrice, PC., *et al.*, 2000]. However, after 11 weeks oral administration of 0.1% quercetin to the rats, certain concentration of aglycone form of quercetin was detected in muscle (0.12 nmol/g) [de Boer, VC., *et al.*, 2005]. In the current study, the concentrations of aglycone form of quercetin were 0.15 and 0.16 nmol/g in the muscle of standard- and HF-diets given mice, respectively. These data indicated that the slight amounts of quercetin aglycone are found in the muscle after long-term feeding. Another possible explanation is the internal metabolic conversion. It has been confirmed that the concentrations of quercetin metabolites are accumulated in certain organs [de Boer, VC., *et al.*, 2005]. The speculation was that conjugated forms of quercetin may be reconverted to the quercetin aglycone in the lung, liver and kidney, due to these tissues possess a high deconjugation activity, probably by β -glucuronidase [de Boer, VC., *et al.*, 2005]. Furthermore, β -glucuronidase has reported to convert conjugated forms of quercetin to aglycone in an *ex vivo* experiment [Murota, K., *et al.*, 2010]. Hence, *in vivo*, β -glucuronidase may also convert conjugated quercetin to aglycone. β -Glucuronidase could be released under certain physiologic conditions, such as inflammation and neoplasm or microsomal β -glucuronidase enzymes could directly convert glucuronides [Yuan, L., *et al.*, 2003; Shimoi, K., *et al.*, 2001]. Furthermore, obesity is associated with inflammation [de Luca, C., *et al.*, 2008; Stafeev, IS., *et al.*, 2017]. Quercetin 3-O- β -glucuronide and quercetin 7-O- β -glucuronide were deconjugated and further metabolized to quercetin 3'-O-sulfate and quercetin aglycone in hepatocytes [O'Leary, KA., *et al.*, 2003].

In this chapter, the plasma glucose and insulin levels and body weight were significantly increased by the intake of HF diet (Fig. 5-1 and Table. 5-2), which

confirmed that long-term feeding of HF diet caused chronic hyperglycemia (Fig. 5-3). Phosphorylation of AMPK might be attenuated hyperglycemia and hyperinsulinemia by dietary supplementation with quercetin and its glucosides (Fig. 5-1). These results were consistent with previous reports. For example, dietary supplementation with quercetin prevented HF diet-induced hyperglycemia after 8-weeks feeding [Stewart, LK., *et al.*, 2009]. Isoquercitrin ameliorated hyperglycemia and regulated the key enzymes for glucose metabolism in diabetic rats [Jayachandran, M., *et al.*, 2018]. Similarly, the antihyperglycemic effect was also observed after oral administration of rutin (50 mg/kg BW) [Ahmed, OM., *et al.*, 2010]. Moreover, quercetin did not affect the HF diet-increased body weight, tissue weight, and the level of plasma cholesterol in this chapter, indicating that quercetin was more effective for suppressing hyperglycemia than regulating body weight gain and fat accumulation (Table. 5-2). This result was consistent with the following previous reports: consumption of quercetin at 2 mg/kg BW attenuated HF diet-induced hyperglycemia, rather than affected the body weight in obese Zucker rats for 10 weeks [Rivera, L., *et al.*, 2008]. Contrarily, supplementation with 0.05% quercetin decreased HF diet-induced body weight gain after 20 weeks feeding [Kobori, M., *et al.*, 2011]. The author deduced that due to the poor bioavailability, quercetin preferred to attenuate HF diet increased plasma glucose level, rather than decrease the body weight gain, and quercetin needs longer time to decrease HF diet-increased body weight and hypercholesteremia.

Fat accumulation and lipid metabolism are tightly controlled by the adipose tissue and liver [Stem, JH., *et al.*, 2016]. AMPK is a key molecule to maintain energy balance. Upon activation, AMPK suppresses adipocyte differentiation and gluconeogenesis and activates fatty acid oxidation through the regulation of the downstream targets, such as C/EBPs, SREBP1, CPT1, PGC-1 α and so on [Cantó, C., *et al.*, 2009]. The author

revealed that quercetin and its glucosides significantly decreased the expression levels of PPAR γ , SREBP1, C/EBP α and C/EBP β in HF-diet fed mice (Fig. 5-6B). C/EBPs are critical transcription factors of lipogenesis and morphological modifications [Cho, BY., *et al.*, 2017; Moseti, D., *et al.*, 2016]. C/EBP β is the first transcription factor to be involved in directing the differentiation process. The transcription and expression of C/EBP β were increased in preadipocytes after treatment with inducers for differentiation [Darlington, GJ., *et al.*, 1998]. Furthermore, it has been reported that hormones could not induce differentiation in embryonic fibroblasts lacking C/EBP β . Meanwhile, C/EBP α , PPAR γ and adipocyte markers such as 422/aP2 and PEPCK also did not express in these cells [Tanaka, T., *et al.*, 1997; Yeh, WC., *et al.*, 1995]. These results suggested that the adipogenesis was blocked by the absence of C/EBP β . C/EBP α is not only involved in adipogenesis of mature adipocytes, but also solidified the correlative link to adipose-specific genes, such as *GLUT4*, *SCD1*, *leptin*, and *422/aP2* [Hwang, CS., *et al.*, 1996]. Adipose-specific genes were not expressed and triacylglycerol accumulation was not detected in the absence of C/EBP α [Lin, FT., *et al.*, 1994]. However, it has been noted that C/EBP β could play a dual role as a stimulator of cell determination as well as differentiation [Yeh, WC., *et al.*, 1995]. It has been confirmed that C/EBP β causes preadipocytes differentiation without increasing C/EBP α expression in pluripotent NIH 3T3 cells, indicating that C/EBP β may functionally replace C/EBP α [Wu, Z., *et al.*, 1995]. Furthermore, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) decreased C/EBP α and PPAR γ expression in 3T3-L1 adipocytes and adipose tissue of animals [Ashida, H., *et al.*, 2015]. Conversely, TCDD did not affect C/EBP β and C/EBP δ expression in 3T3-L1 cells and guinea pig adipose tissue [Nishiumi, S., *et al.*, 2008]. Hence, the relative relation between quercetin and its glucosides caused downregulation of C/EBP α and C/EBP β expression

is needed to clarify in future.

In addition, multiple post-translational modifications have been reported to regulate C/EBP β , including phosphorylation, acetylation, ubiquitination and sumoylation [Ceseña, TL., *et al.*, 2007; Liu, Y., *et al.*, 2013; Tang, QQ., *et al.*, 2005]. Sumoylation has been reported to affect multiple aspects of protein functions, including transcriptional activity and protein stability [Mikkonen, L., *et al.*, 2013]. Theobromine has been reported to induce C/EBP β degradation by increasing its sumoylation at Lys133 in mice [Mitani, T., *et al.*, 2017]. Quercetin treatment increases SUMO-conjugation (both SUMO-1 and SUMO-2) in SHSY5Y cells and E18 rat cortical neurons [Lee, YJ., *et al.*, 2016]. Hence, it is also needed to clarify whether quercetin and its glucosides induced C/EBP β degradation by increasing its sumoylation in future.

Next, in this research, only mesenteric adipose tissue was significantly decreased after 13 weeks feeding, epididymal, retroperitoneal, and subcutaneous adipose tissues showed the decreased tendency (Table. 5-2). The reason is that mesenteric is visceral adipose tissue, which is more sensitive to weight reduction than the other adipose tissues [Gómez-Hernández, A., *et al.*, 2016]. It is reported that epididymal adipose tissue reveals more metabolically active than subcutaneous one [Choe, SS., *et al.*, 2016]. For example, visceral adipose tissue products higher inflammatory cytokines than subcutaneous adipose tissue [Lafontan, M., *et al.*, 2008]. Similar report indicated that green tea extract (400 mg/kg body weight/day) significantly lowered the HF diet-increased the body weight of the mice and suppressed the increase in mesenteric adipose tissue weight [Cunha, CA., *et al.*, 2013]. Furthermore, quercetin and its glucosides undergo deglycosylation by microbial enzymes or LPH. After intake of quercetin and its glucosides, quercetin glucuronides appeared mainly in mesenteric blood *in situ* intestinal perfusion of rats [Zuo, Z., *et al.*, 2006]. These data illustrated

that the possible reason why mesenteric adipose tissue weight was significantly decreased. Moreover, the intestine is an important organ for biotransformation and hydrolysis of quercetin glucosides to quercetin aglycone.

The author also found that quercetin and its glucosides not only significantly affected SREBP1 and FAS expression, but also increased ACC phosphorylation and CPT1 and PPAR α expression in the liver of HF-diet fed mice (Fig. 5-7). SREBP1 is a key lipogenic transcription factor to regulate *de novo* lipogenesis [Goldstein, JL., *et al.*, 2008]. SREBP1 also cooperates with FAS to modulate hepatic fatty acid and triglyceride synthesis [Zhou, G., *et al.*, 2001]. ACC, CPT1 and PPAR α are also involved in the process of fatty acid oxidation. In the previous chapter, the author illustrated that CPT1 is critically associated with the transport of fatty acids to the mitochondrial matrix [Bonnetfont, JP., *et al.*, 2004]. Meanwhile, lipid accumulation was downregulated by PPAR α agonist in the liver of rats [Ye, JM., *et al.*, 2003]. The data are similar to the previous results that ashitaba extract inhibited the lipid accumulation through downregulating SREBP1 and upregulating PPAR α [Zhang, T., *et al.*, 2015]. These results indicated that quercetin and its glucosides regulated lipid metabolism in the liver through increasing fatty acid oxidation and decreasing lipogenesis and adipocyte differentiation.

In conclusion, these results indicated that quercetin and its glucosides prevented HF diet-induced insulin resistance by promoting GLUT4 translocation in skeletal muscle, and also prevented obesity by activating AMPK-dependent signaling pathways in adipose tissue and liver. Therefore, quercetin and its glucosides are promising food components in the treatment of insulin resistance and obesity.

Chapter 6: General discussion

Over the past 25 years, a new term diabetes appeared and spread more and more widely. Diabetes means diabetes occurring in obese persons, which indicates a close relationship between T2DM and obesity. The pathogenesis of diabetes is insulin resistance and obesity [Cao, L., *et al.*, 2016; El-Bassossy, HM., *et al.*, 2017]. The prevalence of T2DM and obesity have increased alarmingly worldwide and across all age, gender and ethnic groups [Mayer-Davis, EJ., *et al.*, 2001]. It has been reported that certain food materials show the effects on antihyperglycemia and antiobesity [Ueda-Wakagi, M., *et al.*, 2018; Yamashita, Y., *et al.*, 2016; Cheng, DM., *et al.*, 2012; Cao, H., *et al.*, 2007]. Thus, extensive researches are struggling to distinguish the effective components and underlying molecular mechanisms of food materials.

Polyphenols contain various beneficial functions including antioxidant and antitumor effects [Kühnau, J., *et al.*, 1976; Mullie, P., *et al.*, 2007; Kawabata, K., *et al.*, 2015]. Quercetin is a major dietary polyphenol and has been reported a large number of biological studies including functions and bioavailability. Hertog illustrated that quercetin intake is varied from 2.6 to 34.6 mg/d in Japan [Hertog, MG., *et al.*, 1995]. Arai also calculated an amount of 9.3 mg/d quercetin consumed by 115 Japanese women [Arai, Y., *et al.*, 2000]. In order to improve its bioavailability, more interest has been paid to phytochemical (quercetin) combinations. The reason is that combinations of certain molecules may synergistically increase their therapeutic activity, like resveratrol can help to increase quercetin bioavailability [Lee, JA., *et al.*, 2015] and co-administration of lipids and emulsifiers increased the absorption of quercetin [Azuma, K., *et al.*, 2002]. However, this combination requires a lot of time and money to explore its rationality. In recent years, quercetin glucosides, as isoquercitrin, rutin, and EMIQ

have been attracted increasing research attention due to their higher biological activities [Zhao, L., *et al.*, 2017; Li, X., *et al.*, 2018]. The accumulated evidences have been indicated that quercetin glucosides are hydrolyzed to aglycone form of quercetin in organisms, and play similar health beneficial functions as quercetin aglycone, including antihyperglycemia and antiobesity [Ju, WT., *et al.*, 2018; Lee, CW., *et al.*, 2017]. Nonetheless, until now, the effective concentrations and the potential mechanisms of quercetin and its glucosides suppress hyperglycemia and obesity, still remains unclearly.

The author focused on the effects of quercetin and its glucosides on the regulation of glucose metabolism in the skeletal muscle and liver and variation of the expression of obesity-related proteins in the WAT and liver of mice. The author found that quercetin and its glucosides prevented hyperglycemia, insulin resistance and obesity through phosphorylation of AMPK.

In chapter 2, the author demonstrated that quercetin and isorhamnetin promoted glucose uptake and induced GLUT4 translocation through AMPK- and JAK/STAT-pathways in cultured L6 myotubes, respectively. Consistently, these effective concentrations are confirmed by the *in vivo* experiments that quercetin and its glucosides suppressed acute and clinical hyperglycemia and ameliorated insulin resistance by upregulating GLUT4 translocation to the plasma membrane and activating AMPK phosphorylation in skeletal muscle (Chapter 2, 3, 4 and 5). These data indicated that quercetin and its glucosides maintained glucose homeostasis and regulate energy metabolism in the whole body to prevent acute and chronic hyperglycemia and HF diet-caused insulin resistance.

The author also found that EMIQ induced AMPK phosphorylation to regulate fatty acid oxidation, accompanied by increasing phosphorylation of ACC and expression of CPT1 and UCP2 in WAT (Chapter 4). However, under the same experimental

conditions, neither EMIQ nor quercetin could affect the expression of CPT1 in the liver. The possible reason is that the different isoforms of CPT exist in different tissues: CPT1-A is predominantly expressed in the liver, intestine, pancreas, and brain [McGarry, JD., 1997; Brown, NF., 1997], while CPT1-B is the primary isoform in the skeletal muscle, heart, and adipose tissue [Esser, V., 1996]. Furthermore, CPT1-C predominates in the brain [Price, N., *et al.*, 2002]. Another possible explanation is that the mechanisms of materials differ the CPT expression. It has been reported that diet supplementation with bacon significant increase CPT1L mRNA level in the liver of mice, which were refeed after 24 h fasting. However, CPT1L mRNA level in the adipose tissue was not affected by the same condition [Sánchez, J., *et al.*, 2009]. Last but not least, it is possible that the veritable concentrations of quercetin in the liver and adipose tissue are different. Since the liver is associated with the metabolic conversion of quercetin, the effective concentration of quercetin and its metabolites in the liver is higher than that in the adipose tissues [Terao, J., *et al.*, 2011; de Boer, VC., *et al.*, 2005; Yeh, SL., *et al.*, 2016]. Hence, the effective concentration of quercetin affected CPT1 expression in the liver should be considered. Thus, further study is required to determine these speculations.

Recently, the author learnt about that any biological effects of any substance base on its bioavailability. Bioavailability of quercetin and its glucosides are closely associated with its absorption, disposition, metabolism and excretion (Fig. 6-1). The small intestine is believed one of the major disposition for absorption of quercetin and its glucosides, which has been confirmed that first appearance of quercetin derivatives in blood of men and rat was detected by HPLC after oral intake [Cermak, R., *et al.*, 2003]. It has been noted that the bioavailability of isoquercitrin and EMIQ are higher than that of quercetin aglycone [Kohara, A., *et al.*, 2017; Murota, K., *et al.*, 2010]. The

reason is that sugar moieties of quercetin monoglucosides elevate their hydrophilicity, which make them more easily passed through the small intestine. After that enterobacteria hydrolyze the absorbed compounds to aglycone form [Bokkenheuser, VD., *et al.*, 1987]. Next reason is that intestinal absorption of quercetin monoglucosides is prior to that of quercetin aglycone, based on the sodium-dependent glucose co-transporter (SGLT1) transports quercetin monoglucosides in the intestinal epithelial cells [Hollman, PC., *et al.*, 1995; Walgren, RA., *et al.*, 2000; Wolfram, S., *et al.*, 2002]. In the intestinal tract, quercetin glucosides are converted their aglycone form before transporting into blood, due to β -glucosidase hydrolases is existed in the small intestinal epithelium [Sesink, AL., *et al.*, 2003; Németh, K., *et al.*, 2003]. β -Glycosidic bonds of isoquercitrin and EMIQ, but not rutin were cleaved by the brush border membrane enzyme LPH. LPH is also able to hydrolyze quercetin glucosides effectively [Day, AJ., *et al.*, 2000]. Subsequently, the liberated aglycone form of quercetin passively permeated across the brush border membrane. Quercetin aglycone could be passively permeated through membrane, because the lipophilic nature of quercetin increases its passively diffuse across phospholipid bilayer of cell membranes [Bokkenheuser, VD., *et al.*, 1987; Murota, K., *et al.*, 2000]. This data is compatible with the idea that the aim of hydrolysis of quercetin glucosides is to accelerate their absorption in the gut.

On the other hand, rutin, a more complex glycosides, is harder absorbed compared with quercetin aglycone and its glucosides, because the sugar moieties of rutin containing rhamnose, which seems to sterically hinder cleavage of the β -glycosidic bond by endogenous enzymes [Erlund, I., *et al.*, 2000]. Hence, rutin is not incorporated into the small intestine, rather than in the large intestine after metabolism by intestinal microflora [Crespy, V., *et al.*, 1999]. Microbial α -rhamnosidase and β -glycosidase are concurrently needed to release the quercetin aglycone from rutin [Day, AJ., *et al.*, 2003;

Erlund, I., *et al.*, 2000]. Humans contents contain 10^{10} - 10^{12} bacteria/g in the colon [Day, AJ., *et al.*, 2003]. These microfloras could not only dispose the deglycosylation of rutin through strains of colonic *bacteroides distasonis*, but also further degrade quercetin to phenolic acid, sole carbon and energy source through *Eubacterium ramulus* [Bokkenheuser, VD., *et al.*, 1987; Blaut, M., *et al.*, 2003; Cermak, R., *et al.*, 2006]. This condition may limit the bioavailability of rutin. Hence, only a small part of free aglycone may diffuse passively through the gut wall.

In addition, quercetin glucosides are completely converted into their conjugated metabolites and aglycone form during intestinal absorption, after that quercetin aglycone and conjugated metabolites are translocated into blood capillaries and accumulated into target tissues via the portal vein (Fig. 6-1) [Terao, J., *et al.*, 2011]. Not only the small intestine, but also the liver, neutrophils and human hepatoma HepG2 cells have the ability to cleave quercetin glucuronides to the aglycone [O'Leary, KA., *et al.*, 2001; O'Leary, KA., *et al.*, 2003; Spencer, JP., *et al.*, 1999]. Quercetin and its conjugated metabolites suffer a great further metabolism in the liver [Petri, N., *et al.*, 2003]. Meanwhile, a portion of quercetin and its conjugates are excreted into bile. In rat, 6% of a perfused amount of quercetin was found in bile [Crespy, V., *et al.*, 2003]. Then, this part of conjugated quercetin re-enter the small intestine via bile, this process is called enterohepatic recirculation. Subsequently, the metabolites are hydrolyzed again by bacterial enzymes in the distal ileum and in the colon [Williamson, G., *et al.*, 2000]. The secreted conjugates quercetin are converted quercetin aglycone again. This cycling has been confirmed depend on the occurrence of secondary peaks in the plasma by time-dependent experiments. For instance, when isoquercitrin (30 $\mu\text{mol/kg p.o.}$) was administered to dog, the first peak $C_{\text{max}1}$ was detected at 0.8 h, and the second peak $C_{\text{max}2}$ for quercetin appeared at 4.1 h [Reinboth, M., *et al.*, 2010]. Hence, the presences

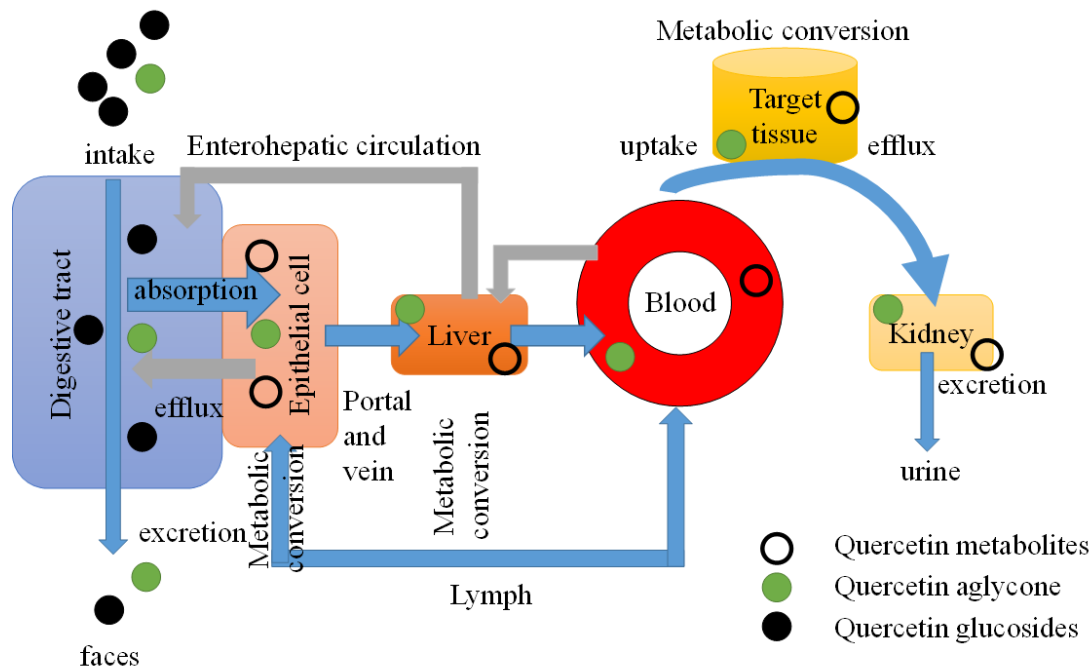


Figure 6-1. Absorption, distribution, metabolism, and excretion of dietary quercetin and its glucosides after intake. Quercetin glycosides from diet are slightly absorbed via the intestinal epithelial cells, although most of them return into the digestive tract by the action of specific transporter systems. Phase II enzymes present in the intestinal epithelial cells and the liver cells, and facilitate their metabolic conversion to glucuronide and sulfate conjugates. Accordingly, quercetin conjugates exclusively circulate in the human bloodstream and distributed to target tissues and organs. In the target tissues, quercetin conjugates convert to aglycone form under certain conditions, such as inflammatory condition (see Fig.6-2). Finally, quercetin conjugates and aglycone excreted into the urine.

of quercetin conjugates in the body were prolonged by this enterohepatic recycling, which is one possible reason of quercetin accumulate in within target tissues. Another possible explanation for this accumulation is the long elimination half-life of quercetin and its glucosides. Manach calculated that half-life of quercetin in humans reached 11-28 h in comparison to 8 h in rats [Manach, C., *et al.*, 2005; Manach, C., *et al.*, 1997]. Walle estimated a half-life of radioactively labelled quercetin metabolites in human plasma between 20-72 h [Walle, T., *et al.*, 2001]. Hence, quercetin conjugates could accumulate in target tissues after long-term repeated intake. Furthermore, β -glucuronidase could convert conjugated quercetin glucuronides to quercetin aglycone in certain conditions as inflammation (Fig. 6-2) [Terao, J., *et al.*, 2011]. These findings can explain as the main reason why quercetin only induced the AMPK phosphorylation for long-term feeding, rather than a single administration nor short-term feeding. Furthermore, chemical form, solubility, intestinal efflux transporters, food matrix, and food composition all have been reported to have a relation to the bioavailability of quercetin and its glucosides [Cermak, R., *et al.*, 2003; Azuma, K., *et al.*, 2002; Sesink, AL., *et al.*, 2005; Wang, Y., *et al.*, 2005; Ofer, M., *et al.*, 2005; Olthof, MR., *et al.*, 2000]. Hence, these interesting but complicated issues need be explored in the future.

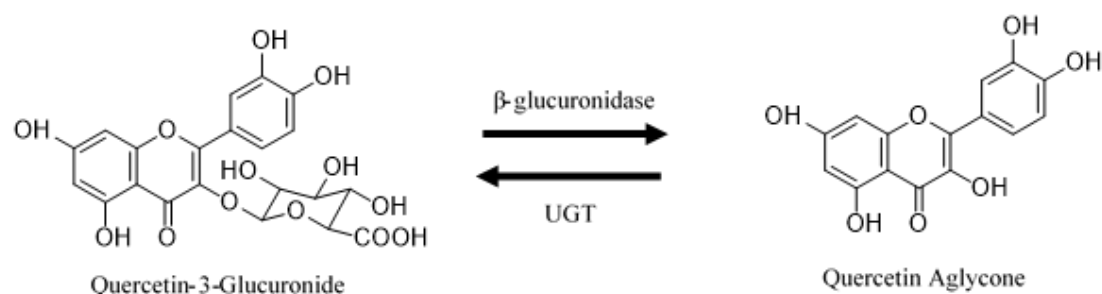


Figure 6-2. Bioconversion between quercetin 3-O- β -glucuronide and its aglycone.

Conclusion

In this dissertation, the author demonstrated the preventive mechanisms of quercetin and its glucosides on acute and chronic hyperglycemia and obesity. The author suggested that quercetin and its glucosides have the potential to suppress postprandial hyperglycemia through inducing GLUT4 translocation to the plasma membrane in the skeletal muscle and to prevent obesity through regulating lipid metabolism and suppressing the adipocytes differentiation and hypertrophy through AMPK phosphorylation. Hence, the author believed that daily consumption of quercetin and its glucosides could serve for maintaining glucose homeostasis and prevent life style diseases.

Appendix: Kaempferol promotes glucose uptake in myotubes by different pathways: involvement of insulin-, AMPK-, and JAK/STAT-pathways

Introduction

In Chapter 2, the author indicated that quercetin and its metabolite isorhamnetin promoted GLUT4 translocation to the plasma membrane and glucose uptake. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), has a similar chemistry structure with quercetin and draws great interest due to its beneficial health effects. It has been reported that administration of kaempferol ameliorated hyperglycemia in diet-induced obese mice [Alkhalidy, H., *et al.*, 2018]. However, only a few publications indicated that the relative bioavailability of kaempferol is only 2% [Barve, A., *et al.*, 2009; Zhou, Z., *et al.*, 2016]. The bioavailability of kaempferol is relatively low, due to its low liposolubility and water-solubility and characteristic of rapid metabolized in the intestine, which seriously influences bioavailability of kaempferol [Barve, A., *et al.*, 2009; Zhou, Z., *et al.*, 2016]. The concentration of kaempferol in the plasma is 1.6×10^{-7} g/ml (approximately 560 nM) at 30 min after kaempferol administration (2.5 g/kg BW) in mice [Zhang, Q., *et al.*, 2009]. Barrington showed that kaempferol underwent extensive phase 2 metabolism and generated sulfate and glucuronide conjugates in Caco-2 cells [Barrington, R., *et al.*, 2009]. Hence, only a small fraction of non-conjugated kaempferol penetrated Caco-2 cell monolayers [Barrington, R., *et al.*, 2009]. Consequently, it is reasonable to clarify the potential health benefits of kaempferol and its underlying mechanism. In this appendix, the

author clarified the antidiabetic properties of kaempferol on glucose uptake and the underlying signal transduction in the muscle cells.

Materials and methods

Materials

Most of the materials used were the same as showed in Chapter 2. Kaempferol was purchased from Extrasynthese (Genay, France). Cytochalasin B were from Wako Pure Chemical Industries (Osaka, Japan). Furthermore, 2-[³H]-deoxy-D-glucose ([³H]-2DG) was from American Radiolabeled Chemicals (St. Louis, MO). Ultima Gold XR was obtained from PerkinElmer Co. Ltd. (Yokohama, Japan). Following siRNAs 5'-ACC UAU UGC GAG GGAAAT T-3' and 5'-UUU CCC UCG CAA UAG GUT T-3 ' for PI3K and 5'-AUU CUA UCA CUA GCG UGA CUU-3' for the control were used.

Cell culture and glucose uptake assay

Differentiation of L6 myotubes were performed according to the previous report in Chapter 2. The glucose uptake assay was conducted as described in the previous report with minor modification [Ueda, M., *et al.*, 2013]. Differentiated myotubes were placed in serum-free medium for 18 h and treated with kaempferol at various concentrations, in Krebs-Ringer phosphate-HEPES (KRH) buffer for 15 min. Then, 6.5 mM [³H]-2-DG (0.5 μ Ci) was treated to the cells for 5 min at 37°C. The myotubes were washed by pre-cold KRH buffer four times. Nonspecific uptake was measured in the presence of 20 μ M cytochalasin B. NaOH (50 mM) was added to solubilize the cells. Incorporated radioactivity into the cells was measured by a liquid scintillation counter with a scintillation cocktail.

Knockdown of PI3K, AMPK α and/or JAK2 by siRNA

To knockdown PI3K, AMPK α and/or JAK2 expression, differentiated myotubes

were cultured in antibiotic-free medium. Then, siRNA for PI3K, AMPK α and/or JAK2 were transfected into cells as the same method as described in Chapter 2.

Preparation of the plasma membrane fraction and cell lysate

After serum starvation for 18 h, kaempferol was treated to myotubes at 1 and 10³ nM for 15 min. The plasma membrane fraction and cell lysate were prepared as previously described in Chapter 2.

Immunoprecipitation and immunoblot analysis

The methods of immunoprecipitation and wester blotting were performed as previously described in Chapter 2.

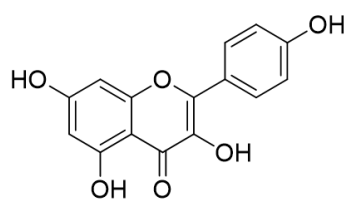
Statistical analysis

Data represent mean $\pm$ SD. Statistical analysis was performed using Dunnett, Student's *t* or Tukey–Kramer multiple-comparison tests as indicated in each figure legend using JMP 11.2.0 (SAS, Cary, USA). *p*<0.05 was considered statistically significant.

Results

Kaempferol increased glucose uptake in L6 myotubes

In this appendix, firstly, the author investigated whether kaempferol (Fig. A-1) at 10⁻² nM-10⁴ nM promotes glucose uptake in myotubes. A biphasic increase in glucose uptake was facilitated by kaempferol in L6 myotubes. The result showed that compared with the DMSO treatment, kaempferol (1 nM) significantly increased glucose at a range between 10⁻² and 1nM in L6 myotubes (Fig. A-2). At 10 nM, kaempferol had no effect on glucose uptake. On the other hand, kaempferol increased glucose uptake at 10³ nM (Fig. A-2). These data indicated that kaempferol increased glucose uptake in myotubes.



Kaempferol

Figure A-1. The chemical structure of kaempferol.

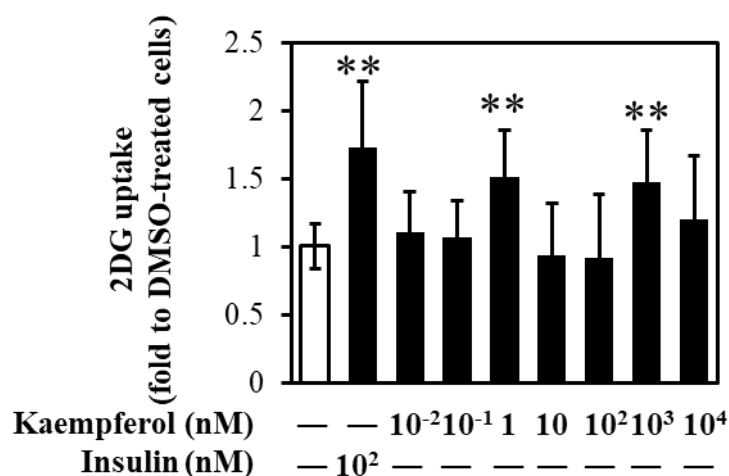


Figure A-2. Effect of kaempferol on glucose uptake in L6 myotubes. (A) Kaempferol at the indicated concentrations was treated to L6 myotubes for 15 min. After treatment, 2-DG uptake into the cells was measured. Data are shown as the mean $\pm$ SD (n = 3). $^{**}p < 0.01$ vs control group by Dunnett's multiple comparison test.

Kaempferol promoted GLUT4 translocation

As shown in Fig. A-3, kaempferol at both 1 and 10³ nM induced GLUT4 translocation to the plasma membrane fraction (Fig. A-3). Meanwhile, the expression level of GLUT4 in the post plasma membrane fraction was not affected by either kaempferol or insulin. These data indicated that kaempferol at 1 and 10³ nM had the ability to increase glucose uptake by promoting GLUT4 translocation to the plasma membrane in L6 myotubes.

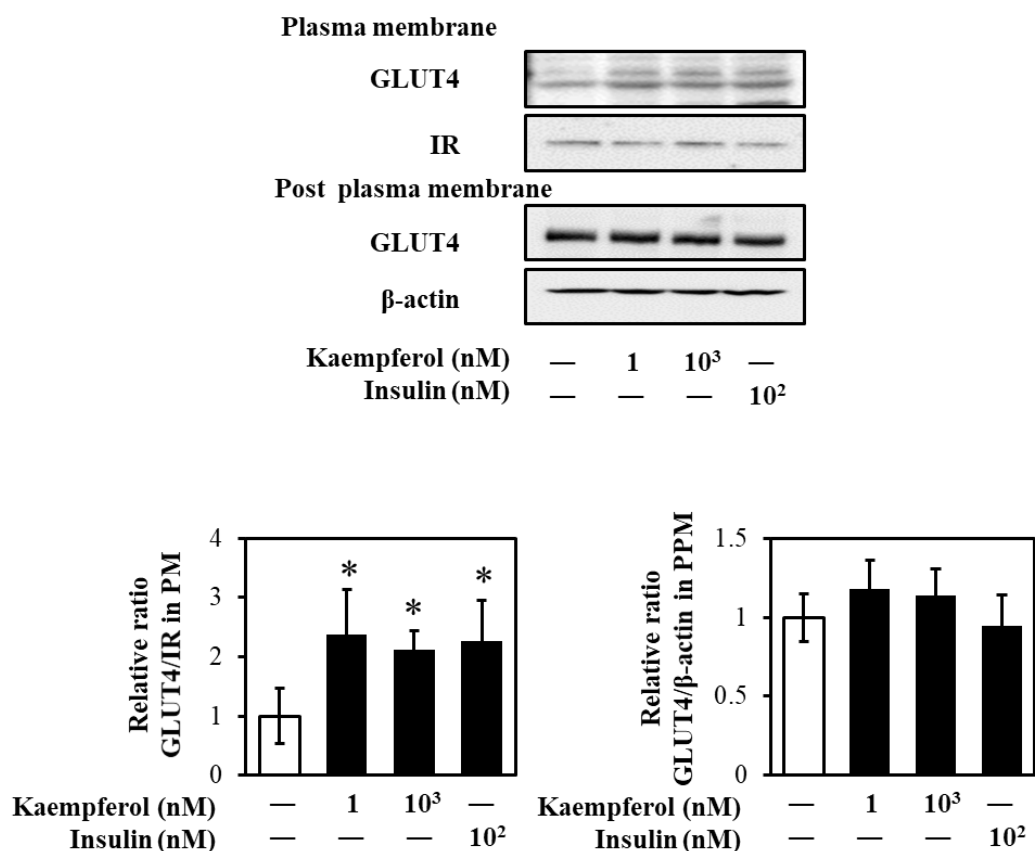


Figure A-3. Effect of kaempferol on GLUT4 translocation in L6 myotubes. (A) Kaempferol at the indicated concentrations was treated to L6 myotubes for 15 min. The expression of (A) GLUT4 and IR β in the plasma membrane fraction and (B) GLUT4 and β -actin in the cell lysate were determined by western blot analysis. Data are shown as the mean $\pm$ SD ($n = 4$). $*p < 0.05$ vs control group by Dunnett's multiple comparison test.

Effect of kaempferol on AMPK- and JAK/STAT-dependent pathways in L6 myotubes

To explore molecular mechanism of kaempferol-induced translocation of GLUT4, important targets in AMPK-dependent pathway have been examined. As shown in Fig. A-4, kaempferol at both 1 and 10³ nM and 1 mM AICAR significantly increased AMPK and ACC phosphorylation (Fig. A-4). Furthermore, JAK2 phosphorylation was

effectively promoted by kaempferol and leptin (Fig. A-5A). Meanwhile, STAT3, a downstream factor of JAK2, was phosphorylated by kaempferol at 1 and 10^3 nM (Fig. A-5B).

siRNA was introduced to confirm the involvement of AMPK α and JAK2 in kaempferol-induced translocation of GLUT4. As shown in Fig. A-6, AICAR- and leptin-induced GLUT4 translocation were completely abolished to the negative control level in the cells treated with siRNA for AMPK α and JAK2, respectively. On the other hand, kaempferol-induced translocation of GLUT4 did not affected by either siRNA for AMPK α or JAK2 (Fig. A-6). However, co-treatment with both siRNA for AMPK α and JAK2, kaempferol-induced GLUT4 translocation was almost completely abolished to the control level. From these results, the author confirmed that both AMPK α and JAK2 were involved in the kaempferol-induced GLUT4 translocation in L6 myotubes.

Effect of kaempferol on insulin-dependent pathway in L6 myotubes

The effect of kaempferol on the insulin signaling was investigated 15 min after the exposure of this compound. Kaempferol did not activate IR phosphorylation at all concentrations through immunoprecipitation. However, kaempferol at both 1 and 10^3 nM promoted tyrosine phosphorylation of the IRS1 at Ser307 (Fig. A-7A). Kaempferol at 1 and 10^3 nM also increased phosphorylation of the downstream PI3K, without affecting Akt (Thr308 and Ser473) phosphorylation, Insulin expectedly promoted phosphorylation of these proteins. siRNA was introduced to confirm the involvement of PI3K in kaempferol-induced translocation of GLUT4. siRNA for PI3K significantly decreased protein expression of PI3K (Fig. A-8). Under the same conditions, siRNA for PI3K significantly downregulated the insulin-induced translocation of GLUT4 as expected (Fig. A-8). On the other hand, siRNA for PI3K did not abolish the kaempferol-induced translocation of GLUT4, although a slight decrease was observed. Hence,

kaempferol promoted GLUT4 translocation through both AMPK- and JAK2/STAT3-signaling pathways rather than insulin-signaling pathway, although kaempferol activated part of factors in insulin-dependent pathway.

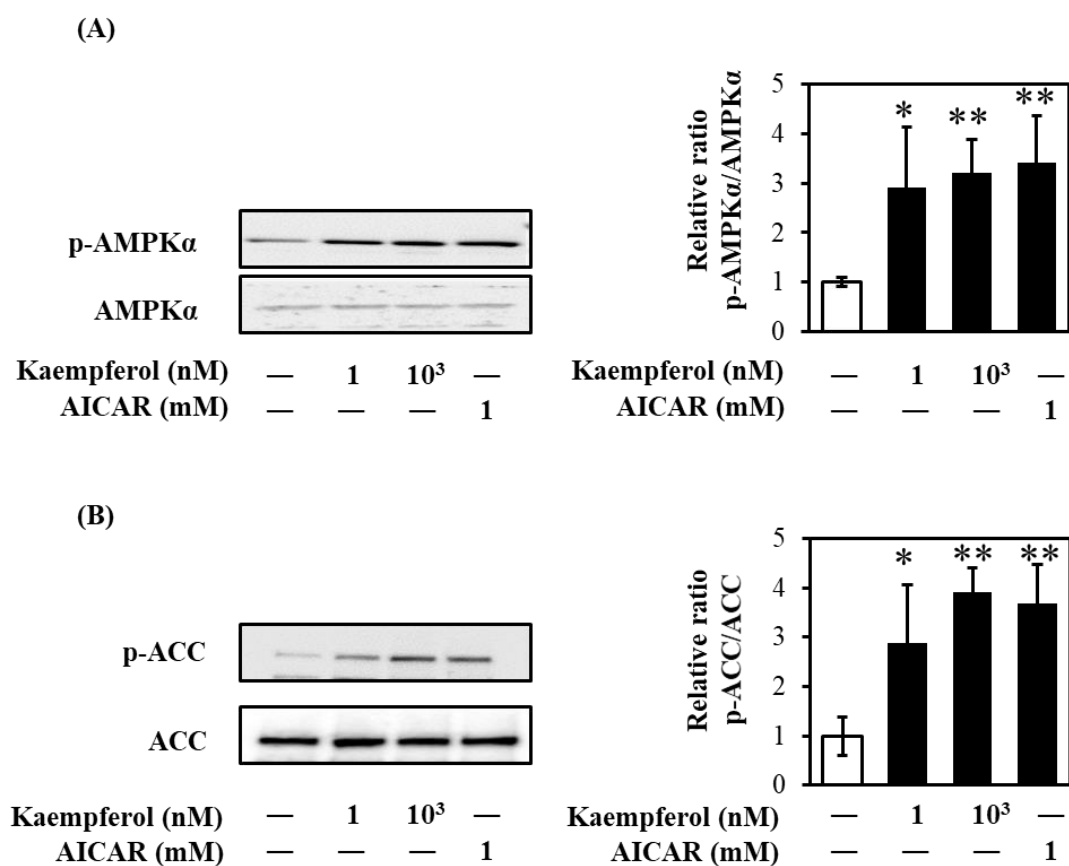


Figure A-4. Effect of kaempferol on the AMPK signaling pathway in L6 myotubes. Kaempferol at the indicated concentrations was treated to L6 myotubes for 15 min. The phosphorylation and expression of AMPK and ACC in the cell lysates were determined by western blot analysis. Data are shown as the mean $\pm$ SD (n = 3). * p < 0.05 and ** p < 0.01 vs control group by Dunnett's multiple comparison test.

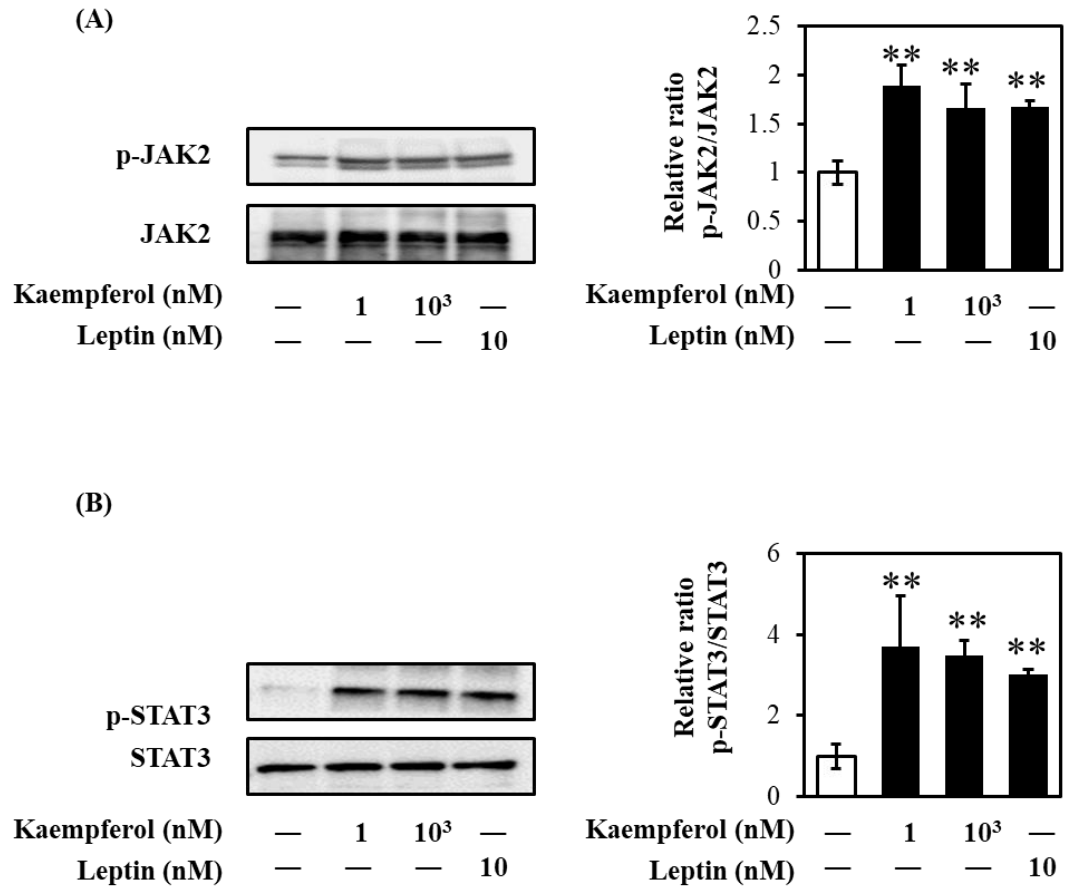


Figure A-5. Effect of kaempferol on the JAK/STAT signaling pathway in L6 myotubes. Kaempferol at the indicated concentrations was treated to L6 myotubes for 15 min. The phosphorylation and expression of JAK2 and STAT3 in the cell lysates were determined by western blot analysis. Data are shown as the mean $\pm$ SD ($n = 3$). * $p < 0.05$ and ** $p < 0.01$ vs control group by Dunnett's multiple comparison test.

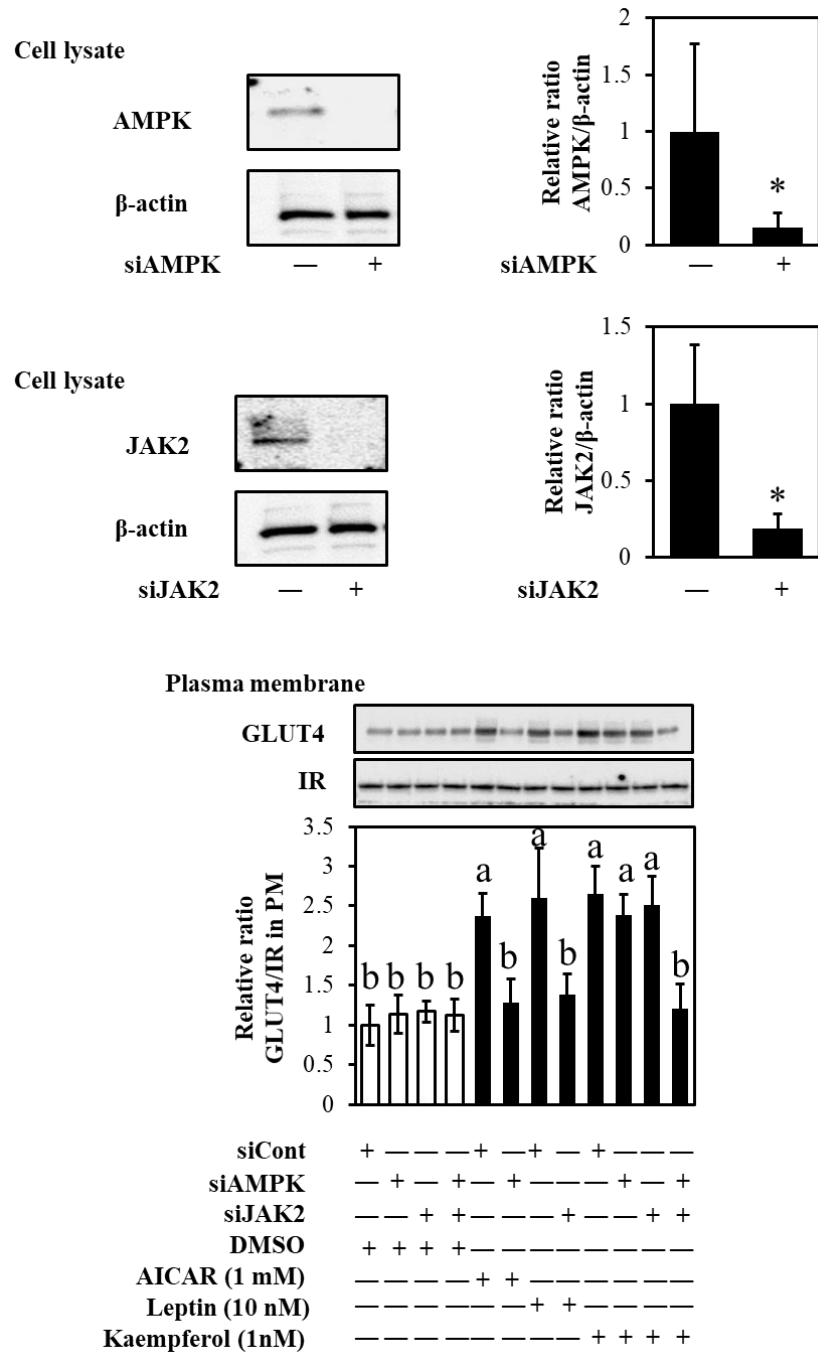


Figure A-6. Effect of kaempferol on GLUT4 translocation via AMPK- and JAK/STAT-dependent pathways. L6 myotubes were transfected with siRNAs targeting (A) AMPK α , (B) JAK2, or control siRNA and treated with 1nM kaempferol for 15 min, respectively. GLUT4 translocation and its expression were detected by western blot analysis. Data were shown as the mean $\pm$ SD ($n = 3$). $*p < 0.05$ vs control cells by student's t -test. Different superscripted letters indicate significant differences between the groups by Tukey–Kramer multiple comparison test ($p < 0.05$).

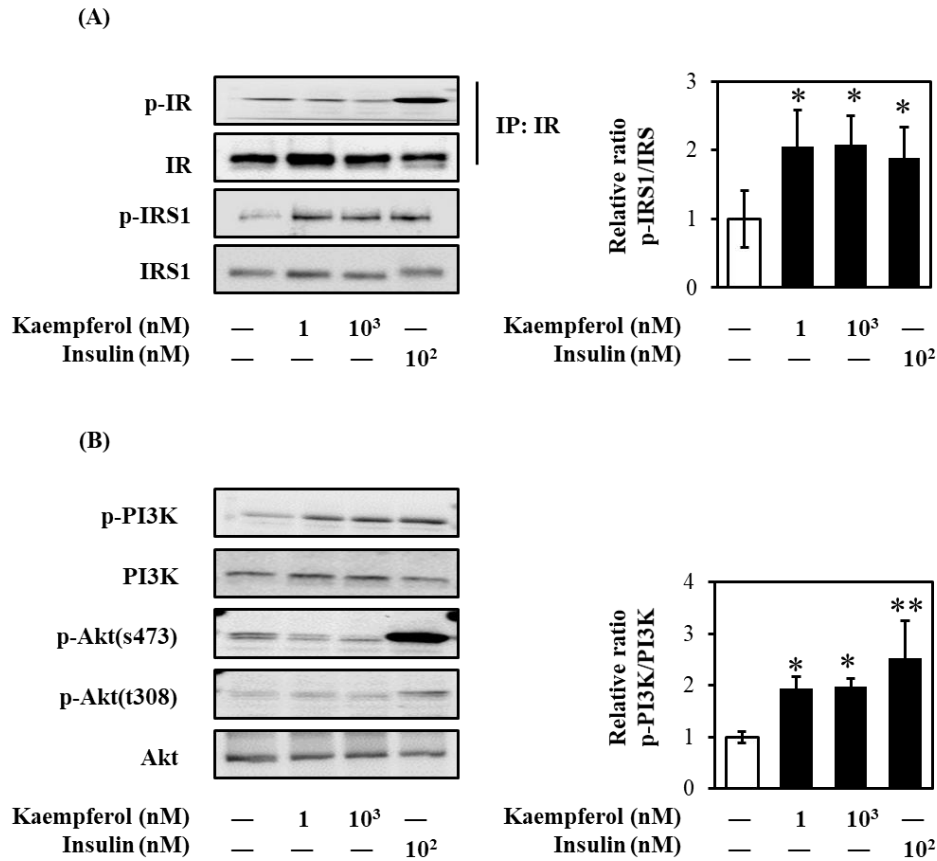


Figure A-7. Effect of kaempferol on the insulin signaling pathway in L6 myotubes. Kaempferol at the indicated concentrations was treated to L6 myotubes for 15 min. The phosphorylation and expression of (A) IR and IRS1 and (B) PI3K and Akt in the cell lysates were determined by western blot analysis. Data are shown as the mean $\pm$ SD ($n = 3$). * $p < 0.05$ and ** $p < 0.01$ vs control group by Dunnett's multiple comparison test.

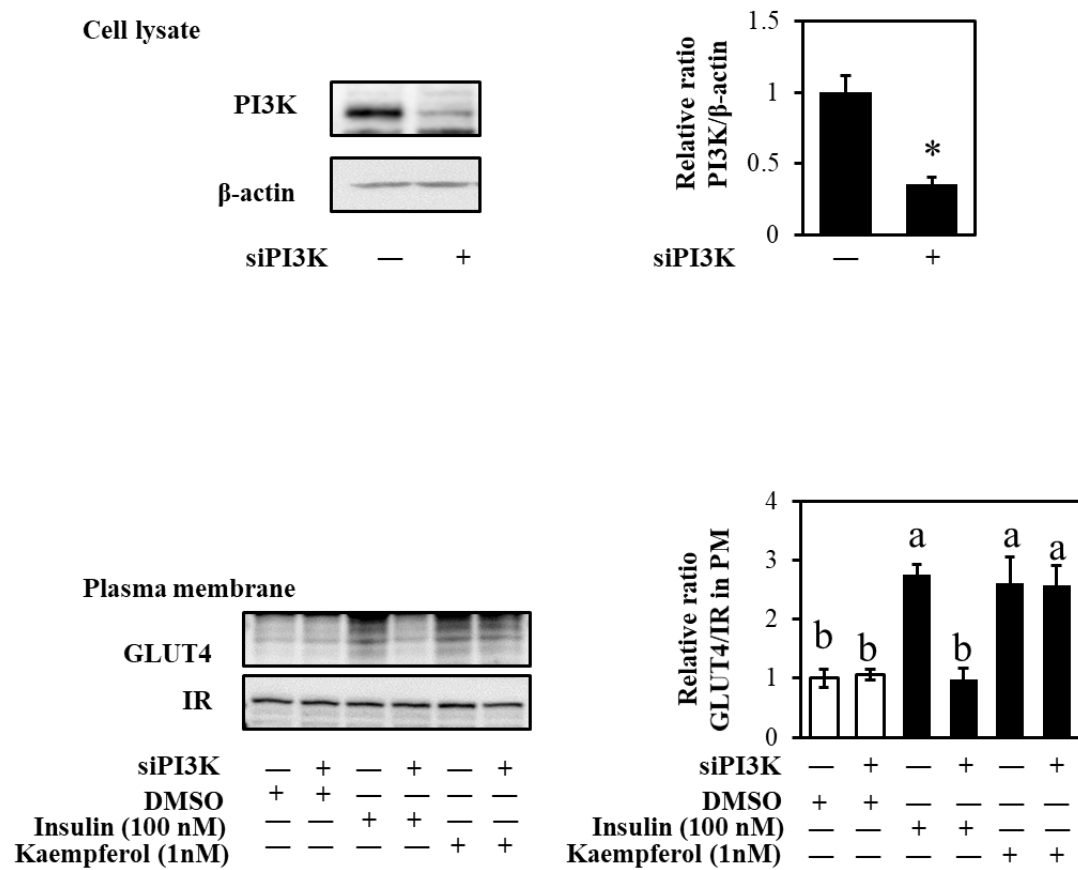


Figure A-8. Effect of kaempferol on GLUT4 translocation without PI3K phosphorylation. L6 myotubes were transfected with siRNAs targeting PI3K or control siRNA and treated with 1nM kaempferol for 15 min, respectively. GLUT4 translocation and its expression were detected by western blot analysis. Data were shown as the mean $\pm$ SD ($n = 3$). $*p < 0.05$ vs control cells by student's t -test. Different superscripted letters indicate significant differences between the groups by Tukey–Kramer multiple comparison test ($p < 0.05$).

Discussion

In this appendix chapter, the author observed that kaempferol at 1 and 10^3 nM significantly promoted glucose uptake (Fig. A-2) accompanied by GLUT4 translocation in L6 myotubes (Fig. A-3). Based on the results, the author demonstrated that kaempferol-induced GLUT4 translocation was mainly through the activation of AMPK- and JAK2/STAT3-signaling pathways (Fig. A-4, 5, and 6). Although kaempferol increased phosphorylation of IRS1 and PI3K, these phosphorylated proteins were not associated with kaempferol-induced GLUT4 translocation (Fig. A-7 and 8). This is the first report that kaempferol at physiological concentration (1 nM) promoted glucose uptake through activating AMPK- and JAK2-signaling pathways.

Kaempferol contains in many plants and has been used as traditional medicines, due to it has antioxidant [Suh, KS., *et al.*, 2009] and anticancer [Mylonis, I., *et al.*, 2010] effects. Nonetheless, poor bioavailability of kaempferol severely restricts its extensive application. Considering this concern, kaempferol elicited a biphasic increase in the glucose uptake at 1 and 10^3 nM in L6 myotubes (Fig. A-2). Similar increase was also observed in quercetin- and isorhamnetin-treated L6 myotubes in Chapter 2. Since it has reported serotonin elicited a similar biphasic increase trend by the small GTPase Rab4 serotonylation in L6 cells. Rab and/or Rho protein serotonylation is speculated to involve this increase in glucose uptake induced by kaempferol [Ai-zoairy, R., *et al.*, 2017]. Hence, further experiments are needed.

In this chapter, kaempferol increased AMPK phosphorylation on Thr172 and ACC phosphorylation at 1 and 10^3 nM without affecting their expression levels (Fig. A-4). AMPK phosphorylation is promoted through the increase in the cellular AMP:ATP ratio by exercise and muscle contraction [Kramer, HF., *et al.*, 2006; Towler, MC., *et al.*,

2007]. In addition, intracellular Ca^{2+} ion level [Gonzalez-Vicente, A., *et al.*, 2014], LKB1 [Shan, T., *et al.*, 2017] and CaMKK β [Woods, A., *et al.*, 2005] have been reported as the upstream factors for AMPK phosphorylation. Different compounds activated different upstream events of AMPK. For example, quercetin activated CaMKK β /AMPK/ACC signaling pathway in myotubes in Chapter 2. Piperine upregulated the level of intracellular Ca^{2+} ion and activated CaMKK β phosphorylation for AMPK phosphorylation in muscle [Maeda, A., *et al.*, 2018]. Epigallocatechin-3-gallate increased expression of LKB1 and phosphorylated AMPK in mice [Lu, J., *et al.*, 2018]. Hence, it need further experiments to clarify the upstream events for kaempferol-induced phosphorylation of AMPK. In addition, kaempferol activated the target receptor is still unclearly. It has been reported that norepinephrine and bradykinin also activated AMPK α 1 in the cells expressing the Gq-coupled α 1b-adrenergic receptor, because of Gq couples α 1b-adrenergic receptor triggering Ca^{2+} release [Kishi, K., *et al.*, 2000]. However, AICAR did not activated GLUT4 translocation nor stimulate glucose uptake in these cells [Kishi, K., *et al.*, 2000]. In addition, the primary target molecule of kaempferol activated the target receptor is still unclearly. Consequently, these important issues should be clarified in the future.

Kaempferol also promoted phosphorylation of JAK2 and STAT3 (Fig. A-5). The roles of AMPK α and JAK2 in kaempferol-induced GLUT4 translocation were confirmed by siRNA. As shown in Fig. A-6, both AMPK α and JAK2 are needed to the kaempferol-induced GLUT4 translocation by knock down experiment using siRNAs (Fig. A-6). It is confirmed that kaempferol-induced GLUT4 translocation is dependent on the dual-pathways. Hence, kaempferol is a more advantageous active compound than the others, due to other compounds activate either the insulin-, AMPK-, or JAK/STAT-signaling pathway. For example, glabridin induces GLUT4 translocation

through AMPK phosphorylation [Sawada, K., *et al.*, 2014]. Apple polyphenol extract induces GLUT4 translocation to the plasma membrane, via activating the insulin pathway [Manzano, M., *et al.*, 2016]. Thus, kaempferol is a potentially dual-activator to induce GLUT4 translocation.

Furthermore, phosphorylation of IRS1 and PI3K were preferentially activated by kaempferol, rather than IR and Akt (Thr308 and Ser473) (Fig. A-7 and 8). These results indicated that kaempferol stimulated IRS1 and PI3K phosphorylation through a mechanism that does not require insulin receptor. This result was matched with the previous results of quercetin in Chapter 2. The plasma membrane-bound G protein-coupled bile acid receptor is one of the conceivable substitutions to explain this condition. The G protein-coupled receptor (GPCR) regulates bile acid homeostasis, increases energy expenditure, and contributes to glucose metabolism [Fredholm, BB., *et al.*, 2001; Fredholm, BB., *et al.*, 2011; Badolato, M., *et al.*, 2017]. Indeed, kaempferol was reported to increase cellular energy expenditure via binding to the plasma membrane GPCR and subsequently activating type 2 deiodinase (D2) [da-Silva, WS., *et al.*, 2007]. Adenosine increased the GLUT4 translocation and PI3K phosphorylation through activating A1 adenosine receptor, rather than IR, in L6 myotubes [Yoshioka, Y., *et al.*, 2017]. Moreover, GLUT4 translocation does not completely associated with PI3K phosphorylation. S6K phosphorylation may be the conceivable reasons. S6K, the downstream factors of PI3K, is important in insulin-induced translocation of GLUT4 in insulin signaling. SBI-477 shown a similar result, which is a potent inhibitor of fatty acid incorporation into triacylglycerides in human skeletal myocytes. SBI-477 enhanced glucose uptake, activated phosphorylation of AMPK and reduced phosphorylation of S6K, despite partially activation of insulin signaling, as IRS1 and Akt [Ahn, B., *et al.*, 2016]. PTP1B is another possible explanation, which has the

potential to negative regulate IRS1, PI3K, and JAK2 phosphorylation. It has been reported that α -methyl artoflavanocoumarin increased glucose uptake, decreased PTP1B expression, and dose-dependently enhanced the protein levels of IRS1 and PI3K phosphorylation in insulin-resistant HepG2 cells [Jung, HJ., *et al.*, 2017]. In addition, JAK/STAT- and insulin-signaling pathways were connected by SH2B domain [Duan, C., *et al.*, 2004; Li, M., *et al.*, 2007]. Leptin activated its long form receptor and induced a JAK2/SH2B/IRS1 or IRS2 tertiary complex to regulate energy homeostasis [Duan, C., *et al.*, 2004]. Hence, it is possible that GLUT4 translocation does not completely correlate to PI3K phosphorylation.

In conclusion, this appendix demonstrate that treatment with kaempferol at 1 and 10^3 nM promoted glucose uptake by increasing GLUT4 translocation to the plasma membrane via AMPK and JAK/STAT signaling pathways in L6 myotubes. Findings in this appendix demonstrated a molecular mechanism of kaempferol may be effective for prevention and treatment of hyperglycemia and associated disorders.

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List of publications

1. Hao Jiang, Yoko Yamashita, Asuka Nakamura, Kevin Croft, Hitoshi Ashida. Quercetin and its metabolite isorhamnetin promote glucose uptake through different signaling pathways in myotubes. *Sci Rep.*, 2019, **9**, 2690-2704.
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