THE EFFECT OF RICE BRAN EXTRACT ON CARDIOVASCULAR LIPOTOXICITY IN RATS FED A HIGH-FAT DIET

BY

MR. NARONGSUK MUNKONG

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMEN OF THE REQUIREMENTS FOR THE DEGREE OF THE DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

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ABSTRACT

Metabolic syndrome is commonly associated with the development of cardiovascular diseases. Among the various pathomechanisms, the cardiovascular lipotoxicity is one of the important mechanisms for the development of cardiovascular diseases. Rice bran extract (RBE) from Khao Dawk Mali 105 rice variety (*Oryza sativa* Linn.) has shown to decrease the risk factors for cardiovascular diseases; however, the mechanisms of cardiovascular protection by RBE have not been yet elucidated. Thus, the present study aimed at verifying the protective effects of RBE on cardiovascular abnormalities in rats fed a high-fat diet (HF) so as to provide a model for prevention of metabolic syndrome and cardiovascular diseases. This study was also designed to characterize the phenolic compounds, γ-oryzanol and α-tocopherol composition of RBE and to evaluate its antioxidant activity. Male outbred Sprague-Dawley rats were randomly diveided into two groups including short- and intermediate-term groups. Short- and intermediate-term experiments were studied for 4 and 16 weeks, respectively. For short-term study, rats were subdivided into 4 groups including control (C), HF, HF + RBE (2,205 mg/kg/day, oral gavage) and HF + fenofibrate (F) (5 mg/kg/day for day 4 to day 16 and 15 mg/kg/day for the next 12 days, oral gavage) groups. HF + F group served as a positive control. For intermediate-term study, rats were subdivided into 3 groups including C, HF and HF + RBE groups. Body weight and food intake were recorded daily. After animal

sacrifice, metabolic characteristics, blood biochemical parameters and histology were determined. Left ventricular and aortic gene expressions were analyzed by real-time polymerase chain reaction and Western blot analysis. The total phenolic compounds, γ-oryzanol and and α-tocopherol contents in RBE were determined using the Folin-Ciocalteu method and high-performance liquid chromatography (HPLC), respectively. The antioxidant activity of RBE was evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay. After 4 and 16 weeks of exclusive HF feeding, elevated body weight, abdominal fat weight, adipocyte size, liver weight, liver triglyceride (TG) levels, fasting blood glucose (FBG), area under the curve of blood glucose levels (AUC-G) and serum total-cholesterol (total-C) and low-density lipoprotein-cholesterol (LDL-C) levels were found. The short-term HF group also showed elevated blood levels of TG and glucose. High-density lipoprotein-cholesterol (HDL-C) levels were significantly reduced in HF group when compared with the C group. Compared with the HF group, only those which received the 4 weeks of RBE treatment had decreased body weight, abdominal obesity, liver weight, liver TG accumulation and levels of serum total-C and blood glucose. The serum TG levels and adipocyte size were lower in all RBE-treated groups. In addition, the serum LDL-C levels and AUC-G were slightly reduced in both HF + RBE groups. Consitent with a decrease in FBG and AUC-G, the pancreatic mass was normalized after the short-term treatment with RBE. The same trends were found for F treatment. However, the HF + F group exhibited a significant increase in the liver weight and serum alanine aminotransferase (ALT) levels, suggesting an important role of F in inducing hepatotoxicity in this model. Rats fed only with intermediate-term HF also showed left ventricular hypertrophy and early signs of atherosclerotic lesions compared to the C group. In this experiment, the levels of serum aspartate aminotransferase (AST) and ALT were all significantly increased in the HF group when compared with the C group. These changes were prevented by RBE treatment. Mechanistically, expression levels of peroxisome proliferator activated receptor α (PPARα) and glucose transporter-4 (GLUT-4) were up-regulated in the hearts of both HF + RBE groups. However, down-regulated expression of fatty acid translocase/cluster of differentiation 36 (FAT/CD36) were detected in the hearts of all HF + RBE groups as compared with the HF groups. There were no significant differences in the levels of

cardiac TG, and serum creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) between three groups. Compared with the HF group, the short-term RBE-treated group had significantly increased aortic expression levels of endothelial nitric oxide synthase (eNOS), whereas the intermediate-term RBE-treated group did not have any significant changes. Interestingly, all experimental periods of RBE treatment caused significant decrease in nuclear factor-kappa B p65 (NF-κB p65) expression levels in both cardiac and aortic tissues Nonetheless, all groups showed normal aortic wall area. In conclusion, the present results suggest that the RBE consumption can prevent the derangement of cardiac energy metabolism and cardiac hypertrophy, partly by improving the expression of genes involved in fatty acid uptake (FAT/CD36), fatty acid oxidation (PPARα), glucose uptake (GLUT4) and cardiomycyte hypertrophy (NF-κB p65). In addition, it was also able to improve the expression of eNOS and NF-κB p65 in the vasculature in HF-induced metabolic stress. Collectively, all these findings indicate that RBE treatment is likely to prevent the initial development of cardiovascular diseases by regulating cardiovascular risk factors as well as their related target gene expression. In comparison with the intermediate-term study, short-term treatment with RBE is more effectively in attenuating metabolic derangements in rats fed an HF. Therefore, RBE could be a potential food supplement for the prevention of cardiovascular abnormalities in the setting of the MS. However, RBE contained low levels of total phenolic compounds, γ-oryzanol and α-tocopherol and showed low antioxidant activity. Based on previous studies, the cardiovascular protective effects of RBE are probably due to the presence of other bioactive constituents such as protein and phytic acid, which have the anti-metabolic disorder and anti-atherosclerotic effects. Thus, evaluation of the bioactive compounds in RBE will be further studied to correlate their effects on the prevention of cardiovascular disease.

Keywords: Rice bran, Metabolic syndrome, High-fat diet and Cardiovascular lipotoxicity

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Mr. Narongsuk Munkong

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
°C	Degrees Celsius
α	Alpha
β	Beta
γ	Gamma
δ	Delta
κ	Kappa
μ	Mu
μg	Microgram
μL	Microliter
μm^2	Square micrometers
μm	Micrometer
∞	Infinity
4-AAP	4-aminoantipyrine
ACC	Acetyl CoA carboxylase
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC-G	Area under the curve of blood glucose levels
BHT	Butylate hydroxytoluene
BMI	Body mass index
BSA	Bovine serum albumin
BW	Body weight
C	Control
CEPT	Cholesteryl ester transfer protein
cDNA	Complementary deoxyribonucleic acid

CK-MB	Creatine kinase-MB
CPT 1	Carnitine palmitoyltransferase 1
C_T	Threshold cycle
CT	Carnitine: acylcarnitine translocase
DAG	Diacylglycerol
DAP	Dihydroxyacetone phosphate
DBP	Diastolic blood pressure
DEPC-treated water	Diethylpyrocarbonate-treated water
DGAT	Diacylglycerol acyltransferase
dL	Deciliter
dNTPs	Deoxyribonucleotide triphosphates
DW	Distilled water
dUTPs	Deoxyuridine triphosphate
EC50	Half maximal effective concentration
EGIR	European group for the study of Insulin Resistance
eNOS	Endothelial nitric oxide synthase
ESPA	*N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine
F	Fenofibrate
FA	Fatty acid
FA^-	Fatty acid anion
FABP	Fatty acid binding protein
FACS	Fatty acyl CoA synthase
$FADH_2$	Flavin adenine dinucleotide
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FATP	Fatty acid transport protein
FFA	Free fatty acid
FOXO	Forkhead box protein O
G-1-P	Glycerol-1-phosphate
G-3-P	Glycerol-3-phosphate
G-6-P	Glycerol-6-phosphate
GAE	Gallic acid equivalents

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT-4	Glucose transporter-4
GPAT	Glycerol-3-phosphate acyltransferase
h	hour
H and E	Hematoxylin and eosin
HDL-C	High-density lipoprotein-cholesterol
HF	High-fat diet
HPLC	High performance liquid chromatography
IκB	Inhibitor of kappa B
ICAM-1	Intracellular adhesion molecule-1
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
kcal	Kilocalories
KDML 105	Khao Dawk Mali 105
kg	Kilogram
L	Liter
LDH	Lactate dehydrogenase
LDL-C	Low-density lipoprotein-cholesterol
LPL	Lipoprotein lipase
MCAD	Medium-chain acyl CoA dehydrogenase
MCD	Malonyl CoA decarboxylase
MCP-1	Monocyte chemoattractant protein-1
M-CSF-1	Macrophage-colony stimulating factor-1
mg	Milligram

MGB	Minor groove binder
MIF	Macrophage migration inhibitory factor
min	Minutes
mL	Milliliter
mm^2	Square millimeters
mmol	Millimole
mRNA	Messenger ribonucleic acid
MTE	Mitochondrial thioseterase
mU	Milliunit
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NCEP ATPIII	National Cholesterol Education Programme Adult Treatment Panel III
NF-κB p65	Nuclear factor NF-kappa B p65 subunit
NFQ	Nonfluorescent quencher
ng	Nanogram
NIK	Nuclear factor NF-kappa B inducing kinase
nm	Nanometer
NR1C	The Group C in the subfamily 1 of the superfamily of nuclear hormone receptors
NSAIDs	Nonsteroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDK-1	Phosphoinositol-dependent kinase-1
PDK 4	Pyruvate dehydrogenase kinase 4
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 3,4 bisphosphate
PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PKB	Protein kinase B
PKC-ε	Protein kinase C-epsilon
PMSF	Phenylmethylsulfonyl fluoride

PPARα	Peroxisome proliferator-activated receptor alpha
PPRE	Peroxisome proliferator response element
RBE	Rice bran water extract
RBEE	Rice bran enzymatic extract
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RQ	Relative quantitation
RT	Reverse transcription
RXR	Retinoid X receptor
s	Second
SBP	Systolic blood pressure
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S.E.M	Standard error of the means
T2D	Type 2 diabetes
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing 0.1% Tween-20
TCA	Tricarboxylic acid
TEMED	N, N, N′, N′-tetra-methyl-ethylenediamine
TG	Triglyceride
TNF-α	Tumor necrosis factor-α
Total-C	Total cholesterol
U	Unit
UCP	Uncoupling protein
UDG	Uracil-DNA glycosylase
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low-density lipoprotein-cholesterol
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Research problem

Metabolic syndrome (MS) is a common cluster of metabolic disturbances prevalent worldwide. It is composed of several vascular risk factors including abdominal (visceral) obesity, dyslipidemia, hyperglycemia, and hypertension. Obesity, particularly abdominal obesity, is a relevant predictor of chronic non-communicable diseases such as increased risk for cardiovascular diseases and type 2 diabetes mellitus (Després & Lemieux, 2006; Bray, Clearfield, Finte, & Nelinson, 2009). Indeed, obesity is accepted to be associated with the progression of MS and its consequences (Després et al., 2008; Bergman et al., 2007). Obesity has emerged as one of the most significant public health problem in the world including Thailand (Aree Kantachuvessiri, 2005; Haslam & James, 2005; Hedley et al., 2004; Hofbauer, Nicholson, & Boss, 2007; Prentice, 2006; Yach, Stuckler, & Brownell, 2006; Yoon et al., 2006). It is estimated that by 2015, 2.3 billion adults will be overweight and more than 700 million will be obese (World Health Organization, 2006).

Although the pathogenesis of MS is complex and the molecular mechanisms remain limited, unhealthy fat eating have been recognized as significant factor in the development of lipotoxicity, insulin resistance, inflammation and cardiovascular disease (Estadella et al., 2012; Lottenberg, da Silva Afonso, Lavrador, Machado, & Nakandakare, 2012; Nagao, & Yanagita, 2008; Stanley, Dabkowski, Ribeiro, & O' Connell, 2012). The appropriate diet for the prevention of body weight gain and its consequences is recommended by reduction of fat intake and increase of whole grain diet intake (Astrup, Dyerberg, Selleck, & Stender, 2008; Ignarro, Balestieri, & Napoli, 2007).

Epidemiological evidence have consistently demonstrated that intake of whole grain and its products is associated with decreased risks of several chronic non-communicable diseases such as cardiovascular disease, type 2 diabetes, some cancers, lower body mass index (BMI) and body weight gain (Giacco, Pepa, Luongo,

& Riccardi, 2011; Liu, 2007; Okarter & Liu, 2010; Sahyoun, Jacques, Zhang, Juan, & McKeown, 2006).

In 2005, Kuriyan and colleagues studied the effect of rice bran oil in hyperlipidemic patients. They found that rice bran oil reduced blood levels of total cholesterol (total-C) and triglyceride (TG). Noppamat Kandee and colleagues (2009) found that treatment with rice bran water extracted (RBE) from Khao Dawk Mali 105 or KDML 105 (*Oryza sativa* Linn.) was able to improve pre-diabetic state in rats fed a high-fat diet (HF). In addition, they also showed that the treatment with RBE decreased TG level in blood and abdominal fat weight of rats fed an HF. Consistently, fat cell size and liver steatosis were reduced by the same RBE (Chotip Chakhonpunya, Seewaboon Sireeratawong, Surat Komindr, & Nusiri Lerdvuthisopon, 2011). Many studies have also showed that both rice-bran oil and rice bran treatments have been shown to have cardiovascular protective effects including anti-atherosclerotic (Ausman, Ronga, & Nicolosi, 2005; Perez-Ternero, Herrera, Laufs, Alvarez de Sotomayor, & Werner, 2015; Wilson, Nicolosi, Woolfrey, & Kritchevsky, 2007) and anti-hypertensive effects (Ardiansyah et al., 2006; Justo et al., 2012).

Although, KDML 105 is the major variety grown and eaten among Thais, the cardiovascular protective effect of RBE from KDML 105 has not been elucidated. Therefore, it is necessary to study the effect of RBE on cardiovascular lipotoxicity in rats fed an HF. The expected outcome is to find the mechanisms of RBE in cardiovascular lipotoxicity, fat accumulation in abdominal region and liver and improve an overall energy metabolism.

1.2 Objectives

The aim of the study was to verify the effect of the RBE from KDML 105 rice bran on the prevention of cardiovascular disease in a model of MS induced by an HF. The comparison was studied between short-term of RBE feeding and intermediate-term of RBE feeding. The specific objectives of the study were as following:

1.2.1 To determine some phytochemicals composition including phenolic compounds, γ-oryzanol and α-tocopherols contents and antioxidant property in RBE

1.2.2 To investigate the effect of RBE on abdominal fat accumulation and adipocyte size

1.2.3 To investigate the effect of RBE on blood lipid profile

1.2.4 To investigate the effect of RBE on glucose homeostatic parameters

1.2.5 To investigate the effect of RBE on ectopic fat accumulation in liver and heart

1.2.6 To determine the effect of RBE on peroxisome proliferator activated receptor α (PPARα), fatty acid translocase/cluster of differentiation 36 (FAT/CD36), glucose transporter-4 (GLUT-4) and nuclear factor-kappa B (NF-κB) gene expression in the heart

1.2.7 To study the role of RBE on endothelial nitric oxide synthase (eNOS) and NF-κB gene expression in the vasculature

1.2.8 To compare the effect of RBE on metabolic homeostasis and cardiovascular lipotoxicity in short-term study with intermediate-term study

CHAPTER 2
REVIEW OF LITERATURE

2.1 MS

2.1.1 Definition and criteria of MS

MS was first defined by Gerald Reaven in 1988. MS is also known as Reaven's syndrome, syndrome X, MS X, insulin resistance syndrome and cardiometabolic syndrome. In recent years several controversies have involved the definition and criteria of MS. Many healthy organizations attempt to develop standardized criterion for identification of MS. The defining criteria of MS are used from major organizations including the World Health Organization (WHO) (Alberti, & Zimmet, 1998), the National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATPIII) (Grundy et al., 2005), International Diabetes Federation (IDF) (Zimmet et al., 2005) and the European group for the study of Insulin Resistance (EGIR) (Balkau, & Charles, 1999). For all that, the WHO, NCEP ATPIII and IDF criteria are accepted worldwide for epidemiological studies and clinical applications (Singh, Arora, Goswami, & Mallika, 2009; Thaman & Arora, 2013). The detail of diagnostic criteria of MS is summarized in table 2.1.

In fact, MS is not specific disease but rather a constellation of diverse symptoms enclosing the cardiometabolic risk factors. However, patient with MS demonstrates constant components of metabolic derangement and cardiovascular events. A cluster of metabolic and cardiovascular symptoms in MS is composed of abdominal obesity, dyslipidemia [increased level of serum TG and decreased level of serum high-density lipoprotein cholesterol (HDL-C)], abnormal glucose homeostasis (impaired fasting glucose, impaired glucose tolerance, insulin resistance, hyperglycemia and hyperinsulinemia) and hypertension. The combination of metabolic and cardiovascular symptoms is related to elevate the risk factors for cardiovascular disease and diabetes mellitus. More recently, MS has also been associated with other medical problems such as non-alcoholic fatty liver disease (NAFLD), chronic kidney disease, cancer, sleep apnea, polycystic ovarian syndrome, proinflammatory and prothrombotic state (Bruce & Byrne, 2009; Scaglione, Chiara, Cariello, & Licata, 2010). The definition of MS provides parameters to evaluate the

cardiometabolic dysfunctions and the opportunities to prevent the chronic non-communicable diseases.

2.1.2 Pathogenesis of MS

To date, pathogenesis of MS involves multiple etiology, cell types and molecular pathways. The understanding of the pathogenesis of MS remains very important for effective healthy management of the general population and patients with MS (Moller & Kaufman, 2005; Singh et al., 2009). Currently, abdominal obesity, lipotoxicity and insulin resistance are the most accepted event for the explanation of the pathogenesis and consequences of MS (Després & Lemieux, 2006; Guilherme, Virbasius, Puri, & Czech, 2008; Scaglione et al., 2010; Snel et al., 2012; Virtue & Vidal-Puig, 2010).

Recently, epidemiological studies recommend that obesity especially when characterized by intra-abdominal adiposity is associated with a higher prevalence and incidence of diabetes mellitus and cardiovascular disease (Després, 2006). Obesity can be defined as an excess of body fat accumulation and adverse contributor to human health. Although knowledge of the role of genetic and social factors in obesity is elevating, the fast increase in prevalence of overweight and obesity throughout the world indicates that environmental factors such as HF, high-sugar diet and physical inactivity are the major contributor of this epidemic (Estadella et al., 2012; Lottenberg et al., 2012; Nagao, & Yanagita, 2008; Stanley et al., 2012). HF consumption often has been considered for the elevating in adiposity. In human studies showed that diets high in fat (≥ 30% of energy from fat) can easily conduce to obesity (French & Robinson, 2003; Hill, Melanson, & Wyatt, 2000; Jequier, 2002; Schrauwen & Westerterp, 2000; Wu et al., 2010). Like humans, HF also induces obesity and averse metabolic effects in animal model (Buettner, Scholmerich, & Bollheimer, 2007; Ghibaudi et al., 2002; McDonald, Pesachuk, Don-Wauchope, Zimaity, & Holloway, 2011). Thus, HF in rat has been needed and used as the model of human MS (Hariri & Thibault, 2010; Panchal & Brown, 2011).

Table 2.1

The clinical criteria for diagnosis of MS in various organizations

Clinical measure	NCEP ATPIII (2005 revision)	WHO (1999)	EGIR (1999)	IDF (2005)
Absolutely required	None	IR[b] (IGT, IFG, T2D, or other evidence of IR)	Hyperinsulinemia (plasma insulin > 75th percentile) in non-T2D patients	Central obesity: waist circumference ≥94 cm (European men) ≥90 cm (Asian men) ≥80 cm (Women)
Criteria	Any 3 of 5 criteria below	IR or diabetes, plus 2 of 5 criteria below	Hyperinsulinemia, plus 2 of 4 criteria below	Central obesity, plus 2 of 4 criteria below
Obesity	Waist circumference >102 cm (Men) (40 inches) >88 cm (Women) (35 inches)	Waist/hip ratio >0.9 (Men) >0.85 (Women) and/or BMI >30 kg/m^2	Waist circumference ≥94 cm (Men) ≥80 cm (Women)	Central obesity already required
Dyslipidemia	TG ≥1.7 mmol/L (150 mg/dL) or Rx	TG ≥1.7 mmol/L (150 mg/dL) or HDL-C <0.9 mmol/L (Men) (35 mg/dL) <1.0 mmol/L (Women) (39 mg/dL)	TG ≥2 mmol/L (178 mg/dL) or HDL-C <1 mmol/L (39 mg/dL)	TG ≥1.7 mmol/L (150 mg/dL) or Rx
Dyslipidemia (second, separate criteria)	HDL-C <1.03 mmol/L (Men) (40 mg/dL) <1.29 mmol/L (Women) (50 mg/dL) or Rx			HDL-C <1.03 mmol/L (Men) (40 mg/dL) <1.29 mmol/L (Women) (50 mg/dL) or Rx
Dysglycemia	Fasting glucose ≥ 5.6 mmol/L (100 mg/dL) or Rx	IR already required	IR already required Fasting glucose ≥ 6.1 mmol/L (110 mg/dL), but non-diabetic	Fasting glucose ≥ 5.6 mmol/L (100 mg/dL) or Rx
Hypertension	SBP[a] >130 mmHg or DBP[a] >85 mmHg or Rx	≥140/90 mmHg	≥140/90 mmHg or Rx	SBP ≥130 mmHg or DBP ≥85 mmHg or Rx
Other criteria		Microalbuminuria[c]		

Note. Adapted from "eNOS, metabolic syndrome and cardiovascular disease," by P. L. Huang, 20 2009, *nds to Endocrinology and Metabolism, 20*, p. 296.

[a] DBP, diastolic blood pressure; SBP, systolic blood pressure

[b] IGT, impaired glucose tolerance; IFG, impaired fasting glucose; T2D, type 2 diabetes; IR, insulin resistance; other evidence includes glucose uptake during hyperinsulinaemic-euglycemic clamp in lowest quartile for population

[c] Urinary albumin excretion ≥ 20μg/minute or albumin-creatinine-ratio ≥ 30 mg/g

Pathogenesis of HF-initiated MS can be explained as a sustained disequilibrium between energy intake and energy expenditure in the body, may result from either on nutrient overload relative to utilizable capacity, a decreased in energy efflux ability to limit increased energy influx, or combination of both situations. Disruption of this balance potentially can affect increase the positive energy balance and fat deposits in adipose tissue, especially abdominal fat accumulation and non-adipose tissue (ectopic fat accumulation).

The adipose tissue is not only concerned in major source of energy storage and liporegulation but is also an endocrine organ, paracrine organ and autocrine organ. It produces and secretes a large number of bioactive substances, known as adipokines or adipocytokines, that have regulatory activities of metabolic homeostasis, feeding behavior, blood pressure, coagulation and inflammatory responses, such as free fatty acid (FFA), leptin, resistin and adiponectin (Hajer, van Haeften, & Visseren, 2008). The dysregulated generation and release of these substances, caused by excess adiposity, abnormal adipocyte structure and adipocyte dysfunction, play a role in the pathogenesis of various diseases through changed metabolic and immunoinflammatory responses or metainflammation (Galic, Oakhill, & Steinberg, 2010; Harwood, 2012; Karastergiou & Mohamed-Ali, 2010).

Under situations of chronic overnutrition that results from a combination of increased caloric intake and decreased energy expenditure conduces to expanding adipose tissue mass (adipocyte hypertrophy and hyperplasia), initiating a conditions of cellular stress and stimulation of pro-inflammatory pathways, especially NF-κB signaling, c-Jun N-terminal kinase (JNK) and endoplasmic reticulum stress. This results in up-regulated adipocyte production of pro-inflammatory adipokines such as monocyte chemoattractant protein-1 (MCP-1), macrophage-colony stimulating factor-1 (M-CSF-1) and macrophage migration inhibitory factor (MIF) that recruits monocytes-macrophages and other immune cells infiltration into the adipose tissue and aggravates the inflammatory response. These macrophages are activated to release inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1β, and induced inducible nitric oxide synthase (iNOS) which interfere the antilipolytic and liposynthetic effect of insulin. Insulin-resistant adipocytes lead to an elevated efflux of FFA from adipocytes to circulation and extraadipose tissues such as liver, muscle, pancreatic β-cell, hypothalamus, kidney,

macrophage, heart and blood vessel as well as reflect to adipose tissue. This active adipocytes-macrophages crosstalk leading to dysregulated secretion of a variety of adipokines such as low adiponectin levels and massive amounts of intracellular lipid and its toxic metabolites accumulation (lipotoxicity) result in metabolic derangements, insulin resistance, inflammation, oxidative stress and multi-organ dysfunction on numerous ectopic tissues. These systemic dysfunction that increases the risk for development of the MS (dyslipidemia and abnormal glucose homeostasis), type 2 diabetes, and cardiovascular disease (Attie & Scherer, 2008; Cusi, 2010; Gregor & Hotamisligil, 2011; Harwood, 2012; Karastergiou & Mohamed-Ali, 2010; Lionetti et al., 2009; Lottenberg et al., 2012; Maury & Brichard, 2010; Muoio & Newgard, 2006; Samuel, Petersen, & Shulman, 2010; Snel et al., 2012; Unger, Clark, Scherer, & Orci, 2010). The pathogenesis of obesity and its related disease is shown in Figure 2.1.

Dietary and physical exercise modification is the first line for preventing and treating the metabolic and cardiovascular diseases. However, maintaining a healthy body weight and reduction of other cardiometabolic risk factors are a great challenge for people living in the modern obesogenic environment that predisposes food consumption and restricts energy expenditure. More recently, foods, some nutrients and phytochemicals have received special attention for the prevention and treatment of patients with MS such as whole grain, fruits, vegetables, fiber, protein, unsaturated fatty fish, polyphenols, phytosterols, terpenoids (estradiols) and oganosulfurs (Abete, Goyenechea, Zulet, & Martínez, 2011; Gilbert, Bendsen, Tremblay, & Astrup, 2011; ; Giacco, Pepa, Luongo, & Riccardi, 2011; González-Castejón & Rodriguez-Casado, 2011; Okarter & Liu, 2010; Salas-Salvadó, Martinez-González, Bulló, & Ros, 2011; Visioli, 2011). Thus, new safer and more effective anti-MS and its-related diseases are needed. Present reports suggest that body fat distribution might be a better predictor of cardiovascular disease than obesity itself (Després, & Lemieux, 2006; Fox et al., 2007). In particular, fat distribution in abdominal or visceral region has a significant role of cardiometabolic and vascular risk factors (Després, 2006).

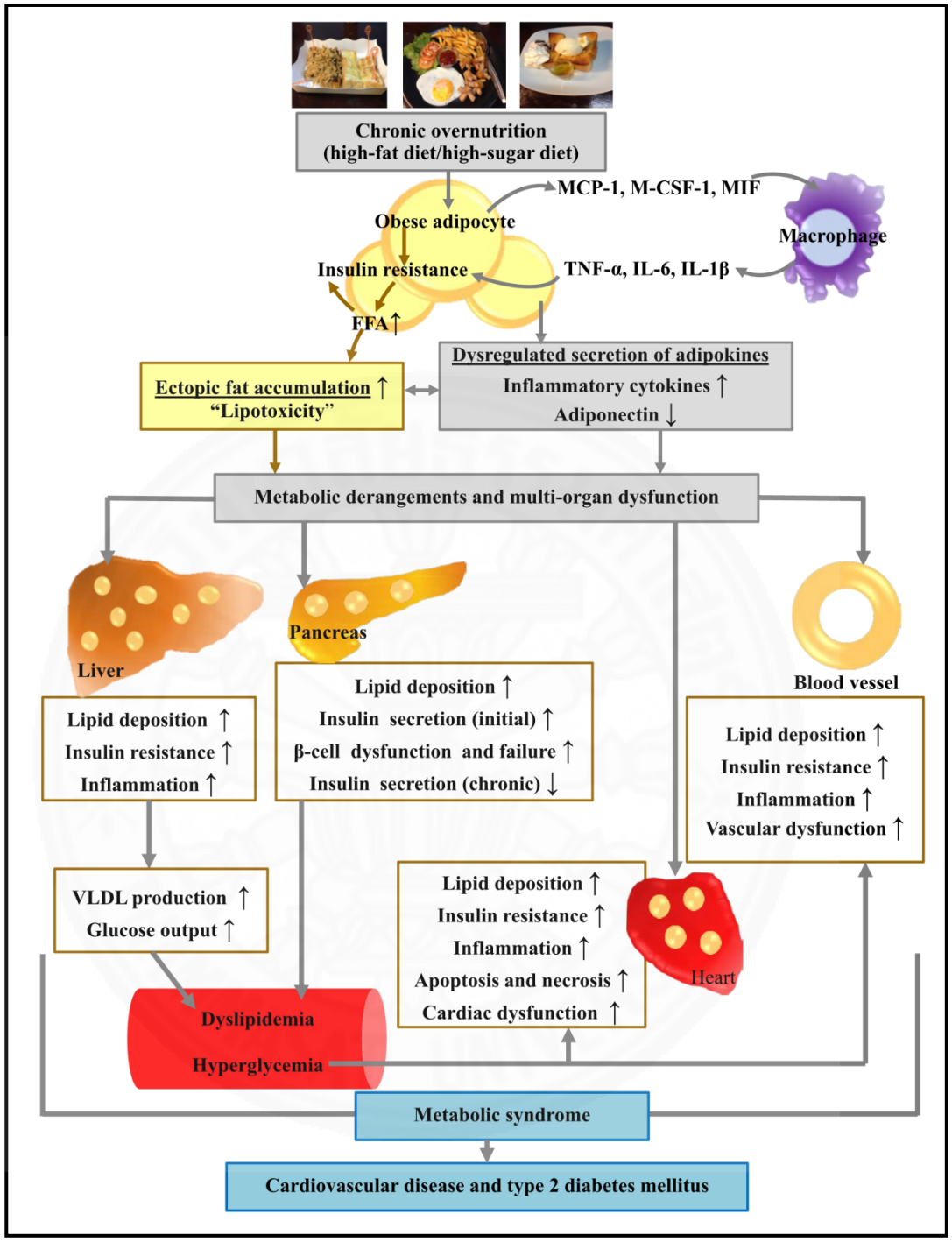

Figure 2.1 The potential pathogenesis of overnutrition-induced MS and its related complications. ↑, increased; ↓, decreased.

2.1.3 Lipotoxicity

As mentioned above, the lipotoxic hypothesis was first described by Urger in 1995. In animal and human studies, the progression of obesity is not only associated with elevated lipid accumulation in classically adipose tissue, but can also be deposited within (intracellularly) and around non-adipose tissue (intercellularly). Excess lipids may lead to the overproduction of lipotoxic intermediates that can mediate many detrimental effects, such as insulin resistance, inflammation, oxidative stress, and apoptosis. This phenomenon, known as lipotoxicity or ectopic fat accumulation or steatosis, may play an important role in the pathogenesis of MS and its related diseases. Although the precise mechanisms of lipotoxicity are not well understood, they are linked to the dysregulation of lipid metabolism. The major pathogenesis of cardiac lipid accumulation involves (DeFronzo, 2010; Schaffer, 2003):

(1) Excessive energy intake
(2) Increased circulating free FFA or TG
(3) Increased FFA uptake
(4) Impaired fatty acid (FA)oxidation (or enhancing oxidation)
(5) Increased synthesis of lipotoxic metabolites and related metabolites such as acyl-CoA, diacylglycerol (DAG), TG, and ceramide (Figure 2.2)

High circulating FFA or TG levels contribute to augmented FFA supply into non-adipose tissue, leading to intracellular lipid accumulation. Alternatively, lipid overload results from imbalance between FFA uptake and utilization. The consequences of lipotoxicity involved the insulin resistance, inflammation, oxidative stress and lipoapoptotic pathway in various organs (Li, Klett, & Coleman, 2010; Urger, 2005). Evidences from human studies and animal models suggest that excessive lipid accumulation in the heart, blood vessel, liver, skeletal muscle, pancreas, macophage, kidney and brain play an important role in the pathogenesis of MS and its related diseases (Gastaldelli, A. 2011). Insight into these mechanisms may lead to the development of more effective preventions and therapy for lipotxocity-related human disease.

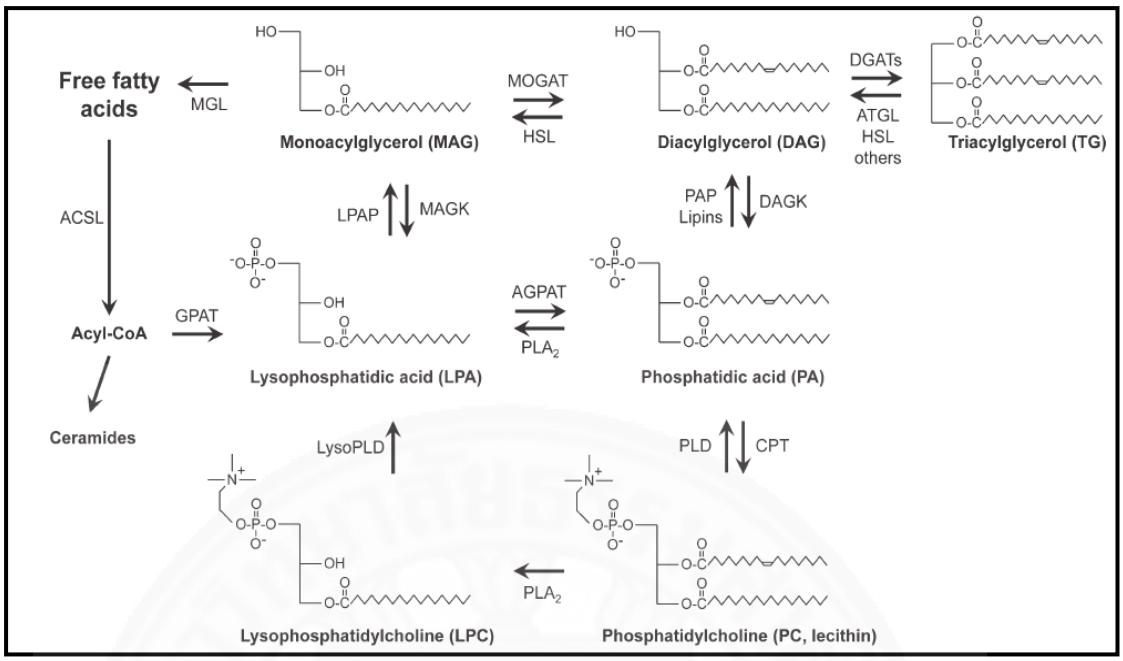

Figure 2.2 The possible metabolism of FA–derived lipotoxic metabolites. From "Hepatic lipotoxicity and the pathogenesis of non-alcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites," B. Neuschwander-Tetri, 2010, *Hepatology*, *52*, p. 776.

All of the main organs are directly impairment of structure and functions in response to chronic exposure to excess lipotoxic metabolites. However, adipose tissue and liver are the primary organs or driver organs for the initiation of metabolic fuel homeostasis in the setting of overnutrition and obesity. The failure of adipocytes and hepatocytes has adequately excess lipids, resulting in lipids redirection to other organs, especially in cardiovascular tissue (Muoio & Newgard, 2006). In addition to the abdominal fat accumulation, NAFLD appears to play an important role in the development of cardiovascular risk factors (Anstee, Targher, & Day, 2013; Hamaguchi et al., 2007). It is widely accepted that lipid overaccumulation in the hepatic tissue can cause atherogenic dyslipidemia, hyperglycemia, oxidative stress, sub-clinical inflammation and lipotoxicity, thereby inducing cardiovascular disease and type 2 diabetes mellitus (Nsier, Shalata, Marmor, & Assy, 2011). Thus, NAFLD has been considered as the independent risk factor of cardiovascular disease (Targher, Day, & Bonora, 2011).

2.1.4 Insulin resistance and hyperglycemia

Classically, insulin resistance is strongly associated with MS and plays principal role in the pathogenesis of MS, type 2 diabetes mellitus, and cardiovascular disease (Rader, 2007). Insulin resistance is defined as an inability of insulin actions to maintain its physiological effects in which higher than normal insulin levels or normal insulin levels fail to accomplish a normal metabolic responses. Insulin has many physiological activities in which regulation of glucose, lipid and protein metabolism.

Insulin receptor is a tetrameric transmembrane protein and tyrosine kinase receptor. The binding of insulin to the α-subunit of the insulin receptor activates autophosphorylation reactions whereby the intracellular part of β-subunit becomes tyrosine-phosphorylated by the intrinsic protein kinase activity of these same receptors. A phosphorylation cascade follows, initiating a protein phosphorylation cascade. Activated insulin receptor phosphorylates the insulin receptor substrate (IRS). The phosphorylated IRS leads to binding and activation of phosphatidylinositol 3-kinase (PI3K), which converts phosphatidylinositol 3,4 bisphosphate (PIP2) to phosphatidylinositol 3,4,5 trisphosphate (PIP3). These nucleotides act as anchors, binding protein kinases to the cell membrane and activating them. PIP3 bounds to the cell membrane associates with phosphoinositol-dependent kinase-1 (PDK-1), and this leads to phosphorylation and activation of protein kinase B (PKB or Akt). Activated PKB is thought to initiate many of the physiological actions of insulin in the adipose tissue, muscle, liver, pancreas, kidney and cardiovascular tissues (Figure 2.3). This contributes to: (1) translocation of glucose transporter to elevate their glucose uptake in adiopoyte, myocyte, cardiomyocyte and vascular cells; (2) suppression of glucose release from liver and kidney; (3) activation of synthesis of glycogen in liver and muscle; (4) inhibition of transport of FFA to the bloodstream; (5) activation of growth of pancreatic β cells; (6) activation of eNOS acivity in the blood vessel; (7) activation of cardiac contractility and heart rate; (8) induction of anti-appototic pathaway in the heart (Bano, Muniyappa, Montagnani, Koh, & Quon, 2007)

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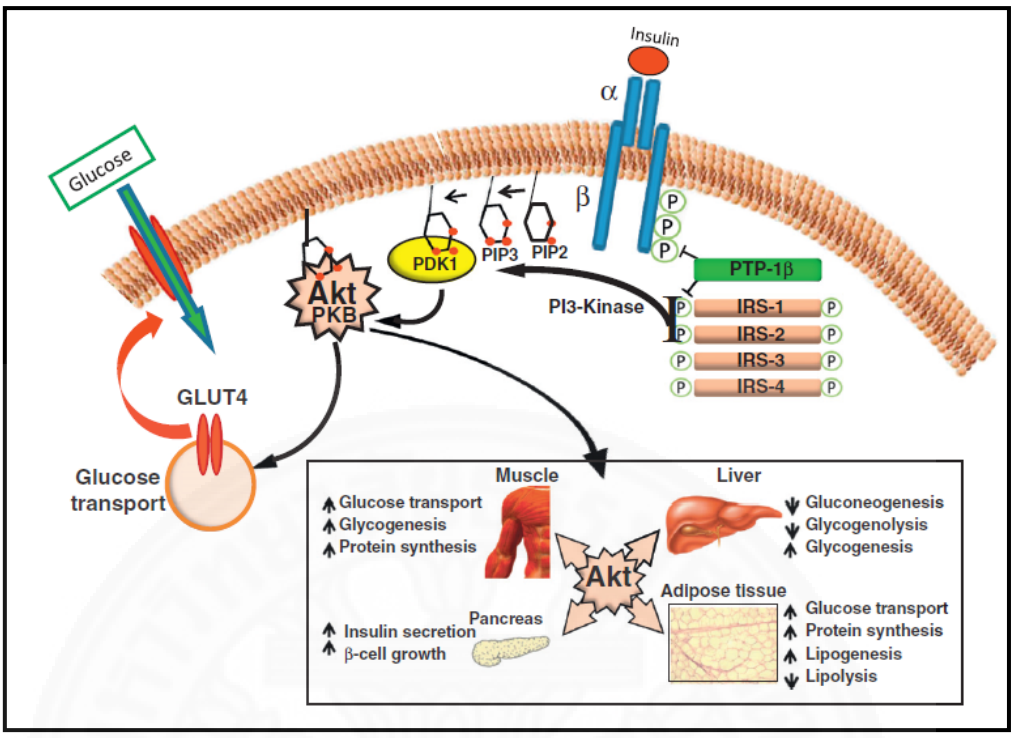

Figure 2.3 Insulin signaling pathway in various tissues. ↑, increased; ↓, decreased. From "From excess adiposity to insulin resistance: the role of free fatty acids," C. Capurso and A.Capurso, 2012, *Vascular Pharmacology*, *57*, p. 94.

Insulin resistance is manifested primary by adipose tissue. Among numerous factors described, excess of FFA and TNF-α demonstrates to suppress the insulin signaling. In adipocytes, it was suggest that cause insulin resistance though inhibition IRS and protein kinase cascade. While TNF-α induces insulin resistance in adipocytes by affecting insulin receptor, IRS, protein kinase and glucose uptake (Arner, 2003).

It is now established that insulin resistance may play a major role in the pathogenesis of NAFLD. Consistent with this hypothesis, mild insulin resistance is very common in the earliest stages of NAFLD, and more severe insulin resistance (as in type 2 diabetes) correlates with more advanced stages of NAFLD (Marchesini et al., 1999). Increased hepatic diacylglycerol levels activate protein kinase C-ε (PKC-ε) and other serine kinases, contributing to decrease insulin receptor activity and decrease IRS-2 phosphorylation, resulting in impairment of glycogenesis. Besides, diacylglycerolc cascade dephosphprylate the forkhead box protein O (FOXO), leading to increase the gluconeogenesis (Morrino, Peterson, & Shulman,

2006). The mechanisms of insulin-resistant state in adipocyte and liver are shown in Figure 2.4 and 2.5, respectively.

Clinical consequences of insulin resistance were demonstrated by hyperglycemia, dyslipidemia, hypertension and cardiovascular disease. The hyperglycemia that appears with insulin resistance can have deleterious effects on tissue and organs such as pancreas, blood vessels and heart (Jellinger, 2007). It is clearly established that the control of blood glucose and insulin sensitivity remains the major means by which the development of hyperglycemia/hyperinsulinemia-associated complications can be minimized.

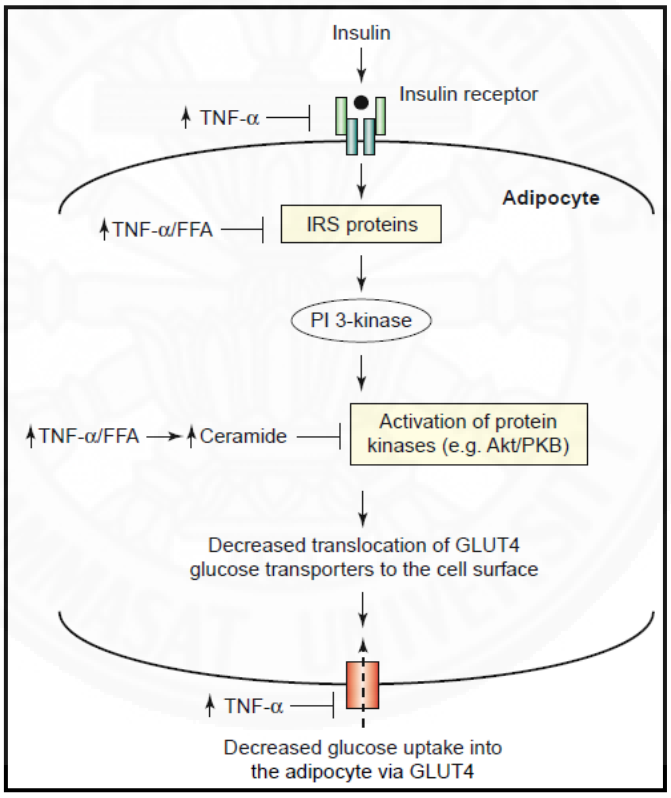

Figure 2.4 Molecular mechanisms of insulin resistance in the adipocyte. ↑, increased. Adapted from "The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones," P. Arner, 2003, *TRENDS in Endocrinology and Metabolism, 14*, p. 139.

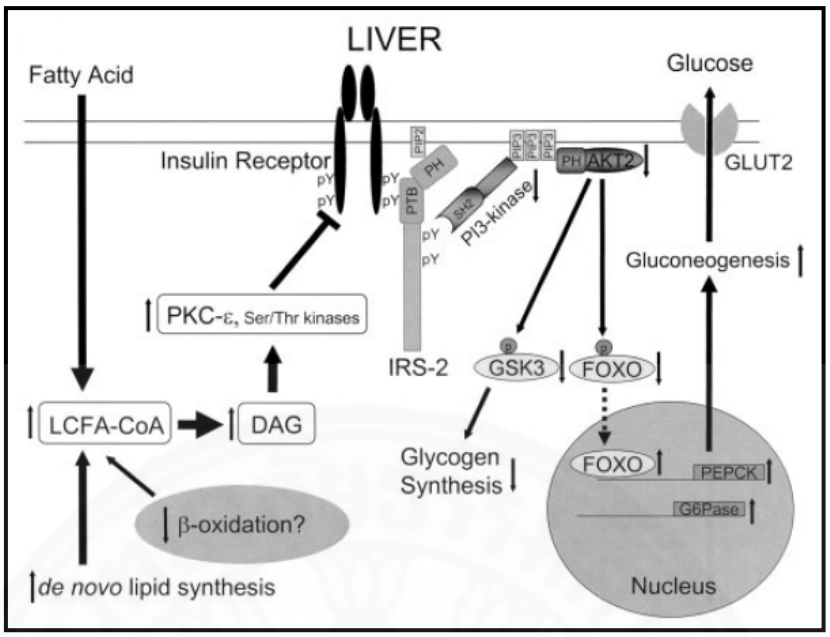

Figure 2.5 Molecular mechanisms of insulin resistance in the liver. ↑, increased; ↓, decreased. Adapted from "Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction," M. Morrino, K. F. Peterson, and G. I. Shulman, 2006, *Diabetes*, *55*, p. S10.

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2.1.5 Dyslipidemia

The major components of dyslipidemia associated with the MS is characterized by increased levels of TG, low levels of HDL-C, and small, dense low-density lipoprotein particles with normal or slightly increased low-density lipoprotein-cholesterol (LDL-C). The abnormality of lipid and lipoprotein metabolism frequently presented in insulin-resistant state in both patients with type 2 diabetes and non-diabetes. This evidence is convincing that insulin resistance is the principal underlying abnormality for contribution to the dyslipidemia (Ginberg, Zhang, & Hernandez-Ono, 2006; Rutolo & Howard, 2002).

Under normal condition, insulin suppresses hepatic glucose output, hepatic very low-density lipoprotein-cholesterol (VLDL-C) and apolipoprotein B production and secretion. In addition to insulin effects on the liver that suppresses releasing of FFA from adipocyte, glucose uptake and oxidation in muscle. In insulin-resistant state and compensatory hyperinsulinemia, increased glucose and FFA influx to the liver from insulin-resistant adipocyte, insulin-resistant muscle, de novo hepatic lipogenesis, chylomicron and VLDL remnant. This liver has been shown to

deteriorate hepatic insulin action. Consequently, hepatic insulin resistance leads to the increased TG containing VLDL-C production and secretion. An increased VLDL-C production contributes to increased blood levels that releases FFA and synthesizes its remnants as a result of hydrolysis by lipoprotein lipase (LPL). The FFA and remnants of TG-rich lipoprotein return to elevate FA pool in liver, suggesting that this interconnection acts as a positive feedback loop or vicious cycle for the development of hypertriglyceridemia.

HDL-C level is controlled by the balance between the rate of HDL-C production and the rate of HDL-C clearance. The functions of HDL mediate the transfer of cholesterol from extra-hepatic tissue to the liver. In obese subjects have been demonstrated to increase HDL-C clearance. Once in the blood circulation, liver derived VLDL particles undergo enzymatic exchanges with other lipoprotein particles such as HDL and LDL, via cholesteryl ester transfer protein (CETP). The TG-rich HDL particles are hydrolyzed by various lipases, then HDL particles may become smaller and are more prone to degradation and excretion by the kidney, resulting in low HDL-C concentrations (Bays et al., 2013) (Figure 2.6). Recognition that dyslipidemia is a risk factor has contributed to development of drugs or natural products that modify lipoprotein metabolism.

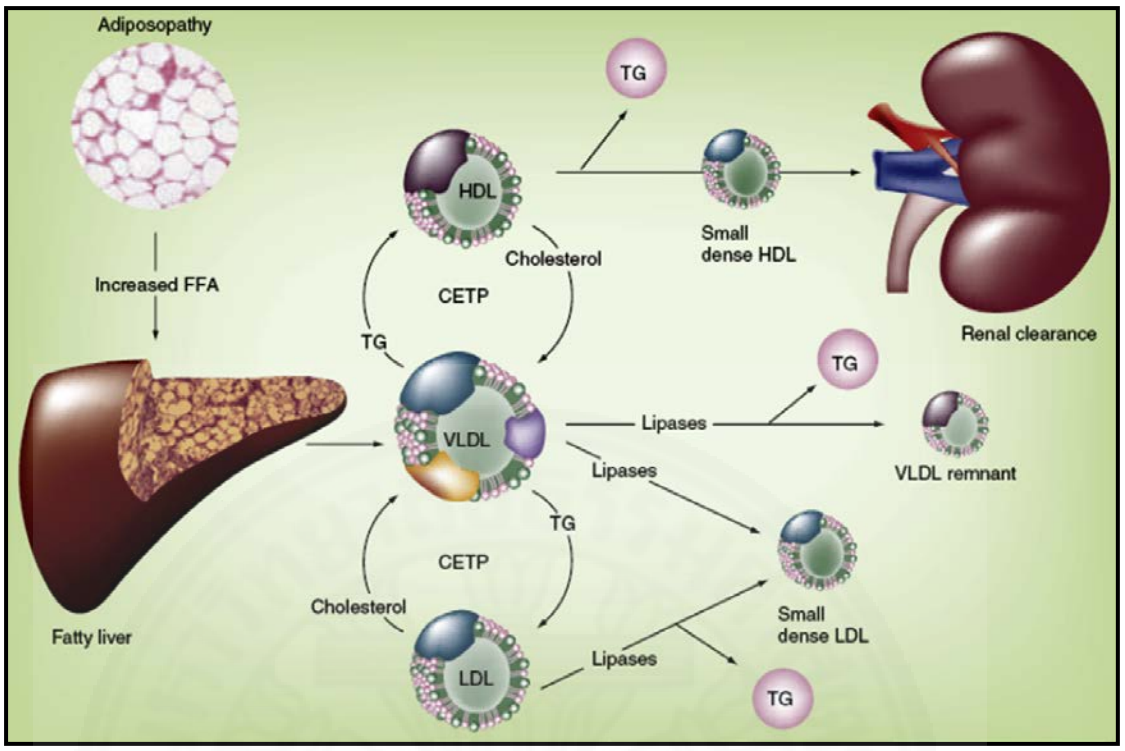

Figure 2.6 The mechanism of dyslipidemia. From "Obesity, adiposity, and dyslipidemia: a consensus statement from the National Lipid Association," H. E. Bays et al., 2013, *Journal of Clinical Lipidology*, *7*, p. 318.

2.2 Cardiolipotoxicity

Heart failure is currently a contributing cause of morbidity and mortality across the globe (Llyod-Jones et al., 2010). It is well established that dietary lipids and obesity considerably elevate the risk for heart failure (Kenchaiah et al., 2002). Appearing evidence supports the concept that interruption in myocardial energy substrate metabolism lead to the progression of cardiolipotoxicity (cardiac lipotoxicity), ventricular remodeling and cardiac dysfunction in patients with MS and heart failure (Stanley, Recchia, & Lopaschuk, 2005). Before the dysregulated cardiac energy metabolism in heart failure and MS can be completely appreciated, it is significant to have a thorough discerning of the regulatory mechanisms of energy metabolism in the normal adult heart.

2.2.1 Cardiac energy metabolism

The heart has a very high energy demand, which is associated almost fully by the mitochondrial oxidation of FA and glucose. A highly energy requirement of the heart must continually produce adenosine triphosphate (ATP) at a high rate to sustain contractile-relaxative function, basal metabolic processes, and ionic homeostasis. In the normal adult heart, almost all (greater than 95%) of ATP generation is derived from mitochondrial oxidative phosphorylation, with the remainder being derived from glycolysis and guanosine triphosphate (GTP) formation in the Krebs or tricarboxylic acid (TCA) cycle. The adult heart normally obtains 60–90% of its ATP from FA β-oxidation and 10-40% from pyruvate oxidation which itself from either glycolysis or lactate oxidation. Besides, oxidation of FFA contributes the main carbon substates to ATP generation under normal conditions at rest. To maintain adequate ATP production, the heart occupies vast metabolic flexibility by its ability to use a multiple of different carbon substrates as energy sources if convenient including glucose, lactate, ketones and amino acids.

FA is supplied to the heart fundamentally originate from either FA contained within TG-rich lipoproteins (chylomicron, VLDL-TG and their remnants) or from plasma FA bound to albumin. FA in the form TG contained within lipoprotein, which are hydrolyzed by LPL in vascular lumen. Released FA enter into cardiomyocytes occurs either by simple diffusion or by protein-mediated transport across the sarcolemma via FAT/CD36, fatty acid binding protein (FABP) or fatty acid transport protein (FATP). FAT/CD36 is the major FA transporter in the heart (Schwenk, Luiken, Bonen, & Glatz, 2008). Once intracellular component of the cardiomyocytes, FA bind to FABP and are then esterified to fatty acyl CoA by fatty acyl CoA synthase (FACS). The fatty acyl CoA can then be esterified to complex lipids such as TG for temporary storage or the acyl group transferred to carnitine via carnitine palmitoyltransferase 1 (CPT 1). The acylcarnitine is then shuttled into the mitochondria via carnitine: acylcarnitine translocase (CT), where it is converted back to fatty acyl CoA and carnitine by CPT 2. The majority of this fatty acyl CoA then enters the FA β-oxidation cycle, producing acetyl CoA, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) and subsequently produces ATP via electron transport chain and oxidative phophorylation. Under certain conditions, mitochondrial thioseterase (MTE) can cleave long-chain acyl CoA

to fatty acid anions (FA^-), which may leave the mitochondrial matrix via uncoupling protein (UCP) (Kienesberger, Pulinilkunnil, Nagendran, & Dyck, 2013)

The glucose is mostly is mostly mediated by glucose transporter protein-4 (GLUT-4), subsequently altered to glucose-6-phosphate (G-6-P), which is then utilized for the glycolysis and pyruvate oxidation or stored into glycogen (Nagoshi, Yoshimura, Rosano, Lopaschuk, & Mochizuk, 2011; Lopaschuk, Folmes & Stanley, 2007; Lopaschuk, Ussher, Folmes, Jaswal & Stanley, 2010; Rider, Cox, Tyler, Clarke, & Neubauer, 2012). Overview of cardiac energy metabolism as presented in Figure 2.2.

2.2.2 The pathogenesis of cardiolipotoxicity

MS is strongly related with cardiac metabolic derangement, cardiac hypotrophy and cardiac dysfunction that contribute to heart failure (Marfella et al., 2009; Shama et al., 2004). In physiological conditions, most TG is stored in adipocytes. The amount of TG stored in non-adipose tissue (hepatic and cardiac tissues) is minimal level and very tightly controlled. However, myocardial TG overaccumulation was observed in both obese and diabetic animal models (Zhou, 2000).

The general concept of lipid accumulation in ectopic tissue can occur by increased TG and FA in blood circulation, increased FA uptake, increased lipid synthesis or decreased FA oxidation (disposal) (Herpen & Schrauwen-Hindering, 2008). In other ectopic tissues such as liver and skeletal muscle, low rates of FA oxidation have been demonstrated in the progression of lipotoxicity and insulin resistance. However, current researches in animals and humans do not support the concept reduced FA oxidation rates lead to deposition of toxic lipid metabolites in the heart and have shown that cardiac FA oxidation are certainly raised in obesity and type 2 diabetes mellitus. Although, FA oxidation is increased, but the elevation in intramyocadial lipid accumulation exceeds the heart's ability to oxidize excess FA. Finally, this cardiolipotoxicity may lead to cardiac insulin resistance, cardiac inflammation, cardiac cells death and subsequently promote cardiac dysfunction and remodeling (Wende & Abel, 2010; Wende, Symons, & Abel, 2012 Yang & Cheng, 2005; Zhang & Ren, 2011).

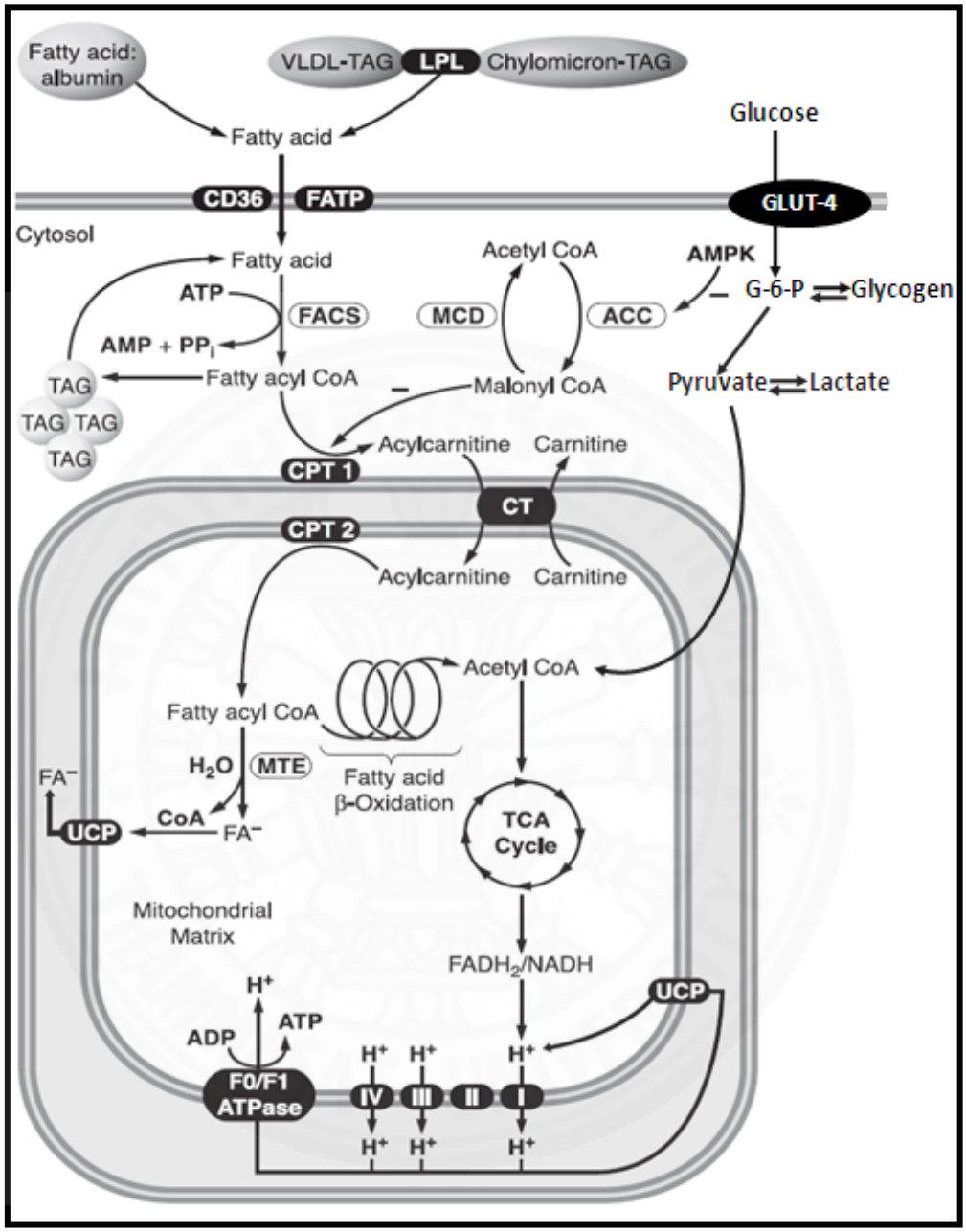

Figure 2.7 Overview of energy metabolism in the normal adult heart. Adapted from "Myocardial fatty acid metabolism in heart and disease," G. D. Lopaschuk, J. R. Ussher, C. D. Folmes, J. S. Jaswal, and W. C. Stanley, 2010, *Physiological Review*, *90*, p. 216.

Lipotoxicity is associated with altered expression of genes involved in metabolism in the models of MS such as decreased PPARα expression (Yan et al., 2009; Zhou, 2000), increased CD36 expression (Bonen et al., 2009; Yan et al., 2009), decreased GLUT-4 expression (Bonen et al., 2009) and increased NF-κB expression (Aragno et al., 2009; Sun, Pan, Tan, & Yu, 2012).The pathogensis of cardiolipotoxicity is shown in Figure 2.8.

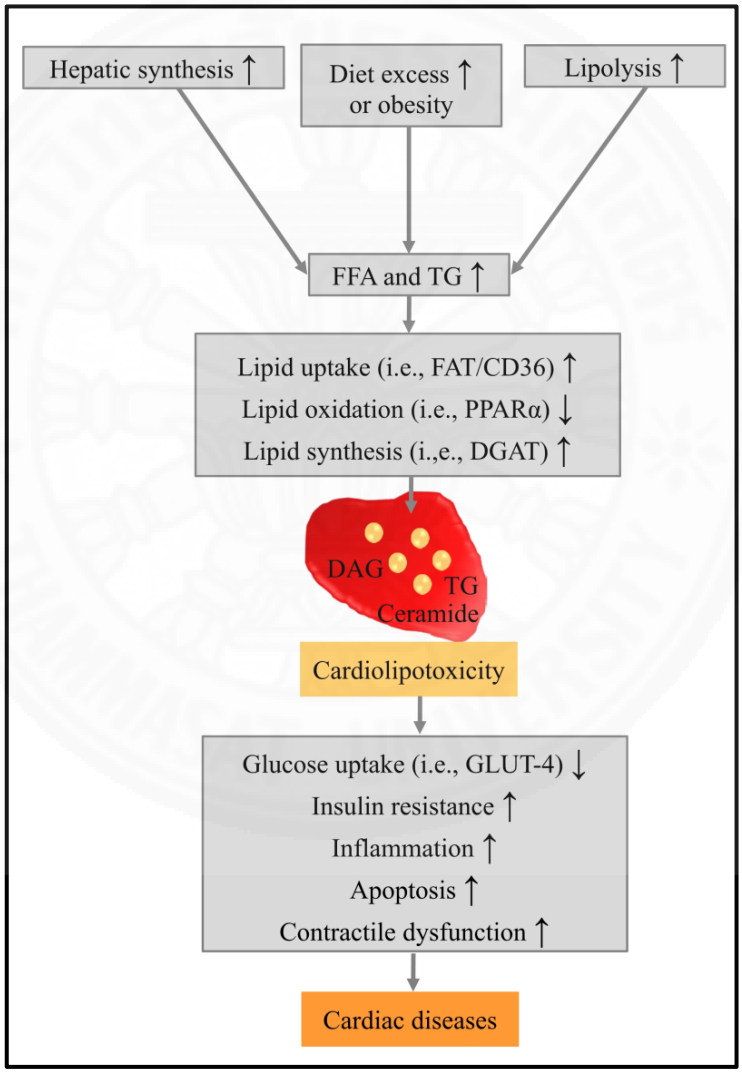

Figure 2.8 The pathogenesis of cardiolipotoxicity. ↑, increased; ↓, decreased.

2.2.2.1 PPARα

Cardiac metabolic disturbances are believed to be a consequence of impairment in energy regulation involved with several regulators. Among these, peroxisome proliferator-activated receptor α (PPARα) is a well-established member of the nuclear receptor family of transcription factors [the Group C in the subfamily 1 of the superfamily of nuclear hormone receptors (NR1C)] that is highly expressed in the myocardium (Finck & Kelly, 2002; Lopaschuk et al., 2010). PPARα functions as a transcription factor that controls the expression of wide range of genes. A variety of endogenous (natural) and synthetic ligands for PPARα have been identified. Its ligands include natural FA, FA metabolites, leukotriene B4 and 8-S-hydroxytetraenoic acid from eicosanoid metabolites, oxidized phospholipids from oxidized lipoprotein, fibrates, nonsteroidal anti-inflammatory drugs (NSAIDs), etc. Originally, PPARα was identified as being essential for lipid metabolism and energy homeostasis. Thus, synthetic PPARα agonists, such as fenofibrate (F) and gemfibrozil, are used in dyslipidemic therapy as hypolipidemic activator. The PPARα signaling transductions and activities are regulated at the level of gene expression and stability, post-translational modifications affecting receptor stability and potency, availability, ligand specificity and cofactor interaction (Barbier et al., 2002) Regulation of gene expression by PPARα requires the binding to PPARα ligands. After ligands bind to the ligand-binding domain of PPARα, PPARα heterodimerizes with retinoid X receptor (RXR) isotypes and then recruitments and/or derecruits several cofactors to stimulate (transactivation) or inhibit (transrepression) the gene transcription machinery. The PPARα/RXR regulatory complex binds to specific peroxisome proliferator response element (PPRE) located in the promoter region of its target genes and modulate transcription of various PPARα-regulated genes (Feige, Gelman, Michalik, Desvergne, & Washi, 2006; Hamblin, Chang, Fan, Zhang, & Chen, 2009; Kota, Huang, & Roufogalis, 2005).

PPARα has been well studied more than other PPAR isoforms (PPARβ/δ and PPARγ). Its target genes include those encoding a various enzymes and proteins involved in FA uptake (FAT/CD36 and FATP1), cytosolic FA binding and esterification [FABP, FACS, glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT)], malonyl CoA metabolism [malonyl CoA

decarboxylase (MCD)], mitochondrial FA uptake (CPT 1), FA β-oxidation [very-long-chain acyl CoA dehydrogenase, long-chain acyl CoA dehydrogenase and medium-chain acyl CoA dehydrogenase (MCAD)], mitochondrial uncoupling (MTE-1, UCP2 and UCP3) and glucose oxidation [pyruvate dehydrogenase kinase 4 (PDK 4)] (Finck & Kelly, 2002; Lopaschuk et al., 2010). The PPARα signaling pathway in the heart is shown in Figure 2.9. As PPARα is considered to be the main energy sensor, its down-regulation can lead to metabolic disturbances in the heart (Aroor, Mandavia, & Sowers, 2012; Geetha, Yogalakshmi, Sreeja, Bhavani, & Anuradha, 2014; Haffar, Bérubé-Simard, & Bousette, 2015; Yan et al., 2009; Zhou et al., 2000).

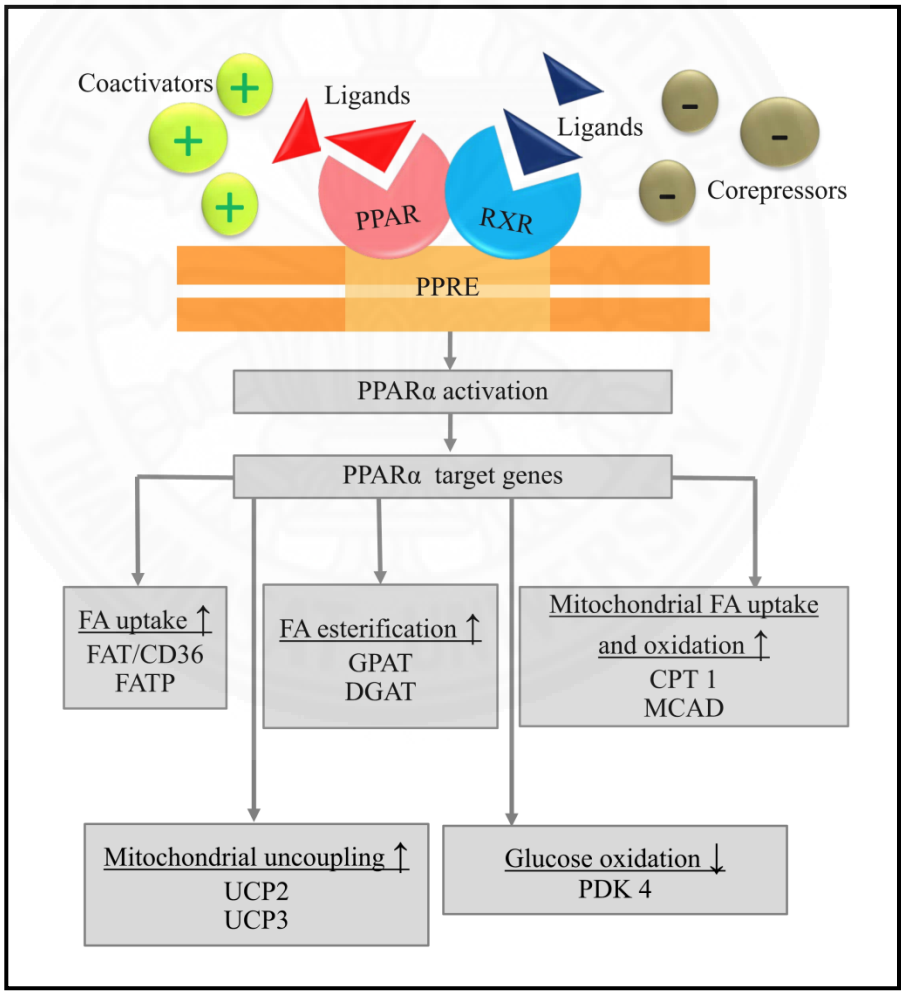

Figure 2.9 PPAR-α effects on the cardiac tissue. ↑, increased; ↓, decreased.

2.2.3.2 FAT/CD36

Among the accepted causes of cardiac TG accumulation are augmentations in the uptake of long-chain FA (Coort et al., 2004; Finck et al., 2002; Mazumder et al., 2004). In the heart, approximately 50% of long-chain FA uptake is transported by FAT/CD36 (Koonen, Glatz, Bonen, & Luiken, 2005). FAT/CD36 is an 88 kDa glycosylated integral membrane protein that belongs to the class B family of scavenger receptor (Febbraio & Silverstein, 2007). This transporter is highly expressed in tissue active in FA metabolism such as skeletal muscle, cardiac muscle, adipose tissue and intestine (Brinkmann, Abumrad, Ibrahimi, van der Vusse, & Glatz, 2002). In normal conditions, both insulin and contraction can trigger FA uptake by stimulating the translocation of FAT/CD36 from intracellular storage compartment to the sarcolemma.

Insulin resistance, obesity and type 2 diabetes mellitus are strongly associated with the dysregulation of cardiac lipid metabolism (Glatz, Bonen, & Luiken, 2007; Steinbusch et al., 2011). In the animal and human with MS, the up-regulation of cardiac FA uptake was attributable to a translocation of FAT/CD36 to the plasma membrane (Bonen et al., 2004; Coort et al., 2004). The increased FAT/CD36 expression and sarcolemmal FAT/CD36 content elevated the rate of FA uptake, led to augmented FA esterification into TG, and thus contributed to deposition of lipid in the cardiac tissue or lipotoxicity (Coort et al., 2004; Kennedy et al., 2011; Tepavcevic, 2011). A previous study in rats fed an HF implies that an augmented sarcolemmal FAT/CD36 content, and the resulting increase in FA uptake and esterification, precedes the progression of cardiac insulin resistance and subsequent contractile dysfunction in left ventricle (Ouwens et al., 2007). Conversely, the ablation of FAT/CD36 has been demonstrated to protect the induction of metabolic stress and cardiac dysfunction associated with HF and cardiac pressure overload in mice (Steinbusch et al., 2011). Also, the deletion of FAT/CD36 could prevent intramyocardial TG accumulation and cardiac dysfunction in transgenic mice with cardiac-restricted PPARα overexpression, suggesting a potential role for FAT/CD36 in the development of cardiolipotoxicity (Yang, Sambandam, et al., 2007). These novel comprehensions result in new strategies for prevention and therapy of development of cardiolipotoxicity in MS.

2.2.3.3 GLUT-4

The initial step of glucose metabolism depends on the uptake of glucose across the cell membrane. Glucose transportation exists by facilitated diffusion through selective transporter proteins of GLUT family. Although both GLUT-1 and GLUT-4 is common glucose transporter of cardiomyocyte, GLUT-4 is the major isoform in fully differentiated cardiomyocyte. GLUT-4 is mainly located in intracellular pools and translocated to the sarcolemma in response to stimuli including insulin, catecholamines, increased contraction, hypoxia and ischemia. In addition, GLUT-4 has a greater affinity for glucose (Km ≈ 4-7 mM) than GLUT-1 (Km ≈ 20-26 mM). This characteristic combined with its higher expression in the heart considers GLUT-4 responsible for the majority of glucose transport, even in resting state (Montessuit & Lerch, 2013). The reduction of cardiac GLUT-4 content is characteristic of hyperlipidemia, type 2 diabetes mellitus and insulin resistance (Armoni, Harel, Bar-Yoseph, Milo, & Karnieli, 2005; Cook et al., 2010). Ko and co-workers (2009) found that nutrient stress suppressed myocardial glucose metabolism via inhibition of AMP-activated protein kinase (AMPK) signaling, and GLUT-1 and GLUT-4 gene expressions. In line with this observation, GLUT-4 protein levels were decreased in the cardiac tissues of ob/ob diabetic mice (Cook et al., 2010) and Zucker diabetic fatty rats (Bonen et al., 2009).

Based on animal studies, a hypothetical model is demonstrated for the development of FAT/CD36-mediated lipotxicity and impaired insulin stimulated GLUT-4 translocation in the heart (Figure 2.10). The relocation of FAT/CD36 to cell surface is associated with the accumulation of TG and its metabolites, i.e. acyl-CoA, DAG and ceramides eventually contributing to the development of cardiac lipotoxicity and insulin resistance (Coort, Bonen, van der Vussue, Glatz, & Luiken, 2007; Glatz, Bonen, & Luiken, 2007). This knowledge of the mechanisms by which FA regulates GLUT-4 expression will help the development of novel therapeutic strategies for better management of cardiometabolic risk factors.

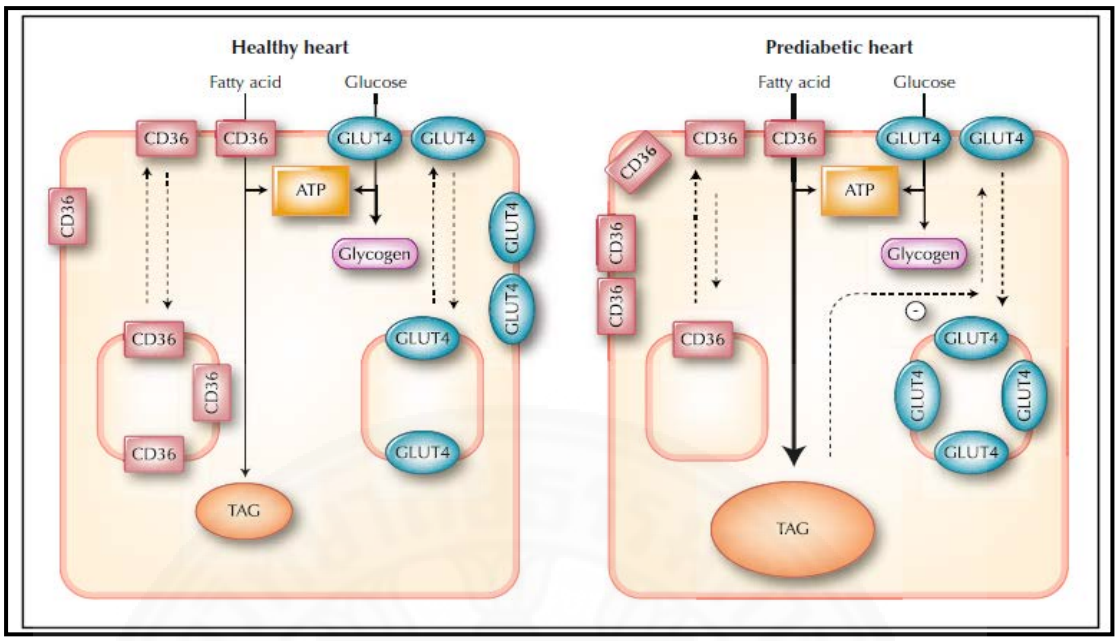

Figure 2.10 Role of FAT/CD36 and GLUT-4 in the heart under (pre-) diabetic conditions compared to physiological conditions. From "Sarcolemmal fatty acid transport in normal and diseased hearts," J.F. Glatz, A. Bonen, and J. J., 2007, *Current Hypertension Reports*, *9*, p. 451.

2.2.3.4 NF-κB

NF-κB (nuclear factor-kappa B or nuclear factor kappa-light-chain-enhancer of activated B cells) is heterodimeric or homodimeric transcription factors of Rel protein family. The NF-κB family is composed of five mammalian family members: Rel (c-Rel), RelA (p65) and RelB, and the longer precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are cleaved to create p50 and p52, respectively (Oeckinghaus, Hayden & Ghosh, 2011; Van Der Heiden, Cuhlmann, Luong, Zakkar & Evans, 2010). All NF-κB family show as a homodimer or heterodimer in cells such as p50/p50 dimer, p50/p52 dimer and p50/p65 dimer; however, p50/p65 dimer is the most common NF-κB dimer and the most studied in the cardiac cells and others mammalian cells (Gordon, Shaw & Kirshenbaum, 2011). NF-κB signaling pathway is implicated in a multiple physiological and pathobiological pathways in the heart including inflammatory response, immune response, redox homeostasis, cellular hypertrophy and apoptosis (Lorenzo et al., 2011).

Generally, NF-κB signaling pathways are divided to two main signaling pathways, including canonical or classical pathway and non-canonical or alternative pathway. The NF-κB pathway is activated by many stimuli and conditions though signal transduction such as pro-inflammatory cytokines (TNF-α, ILs and interferon γ), lipopolysaccharide (endotoxin), reactive oxygen species (ROS), hyperglycemia, hyperlipidemia, vasoactives (angiotensin II and endothelin I) and growth factors (transforming growth factor β). The canonical NF-κB pathway progresses via phosphorylation, ubiquitination and proteosomal degradation of inhibitor of kappa B (IκB) (Figure 2.11A). In unactivated conditions, NF-κB interacts with the IκB and is located in the cytoplasm. Upon activation, the phosphrylation of IκB is induced by the inhibitor of nuclear factor kappa-B kinase (IKK) complex. Phosphorylated IκB is then ubiquitinated and degraded by proteosome. Disassociated NF-κB can translocate to the nucleus and then bind to NF-κB-DNA-sites (κB sites) for regulation its target genes. The non-canonical NF-κB pathway begins thought phosphorylation, ubiquitination and proteosomal degradation of inhibitory NF-*κ*B1 (p105) and NF-*κ*B2 (p100) proteins (Figure 2.11B). In stimulated cells, IKK complex (IKKα) is phosphorylated by NF-κB-inducing kinase (NIK), allowing phosphorylated IKKα to phosphorylate the p100/p105-bound RelB. Subsequent proteolysis of the p100 or p105 produces p52 and p50, respectively, which heterodimerizes with RelB. These heterodimers then translocate into the nucleus, where it bind to κB sites and regulate gene expression. NF-κB controls the expression of more than 400 different genes, most of which regulate the expression of inflammatory genes, such as cytokines, chemokines and cell adhesion molecules (Chen & Chen, 2013; Lorenzo et al., 2011; Pasparakis, 2009). It has been proposed that activated NF-κB signaling pathway is also associated with the development of cardiac hypertrophy (Gupta, Young, & Sen, 2005).

Earlier reports have suggested that activation of NF-κB signaling pathway is related to the development of obesity, diabetes mellitus and cardiovascular disease (cardiac hypertrophy, diabetic cardiomyopathy, heart failure and coronary artery disease) (Beaker, Hayden & Ghosh, 2011; Carlsen et al., 2009; Lorenzo et al., 2011). Current studies have showed that prolonged stimulation of NF-κB cloud promote heart failure by the activation of inflammation, contribution to cardiocyte dysfunctions and death (Gordon, Shaw & Kirshenbaum, 2011). Studies in animal model of MS found that NF-κB expression and activity increased and both had a correlation with cardiac lipid deposition (Aragno et al., 2009; Sun et al., 2012). These suggested that cardiolopotoxicity affected the structure and function of the heart by elevating the expression and activity of NF-κB. Moreover, previous evidences also suggested that blockade of NF-κB in transgenic mice could lead to attenuation of cardiac hypertrophy and improvement of cardiac function and survival (Kawamura et al., 2005; Kawano et al., 2005; Kawano et al., 2006). Additionally, several oxidative stress-responsive transcription factors, including NF-κB, can also induce oxidative and nitrosative stress in the heart by producing ROS and reactive nitrogen species, respectively (Mariappan et al., 2010). Therefore, inhibition of NF-κB expression and activity may be a new protective or therapeutic strategy for cardiac disease by reducing inflammation, oxidative stress and hypertrophy.

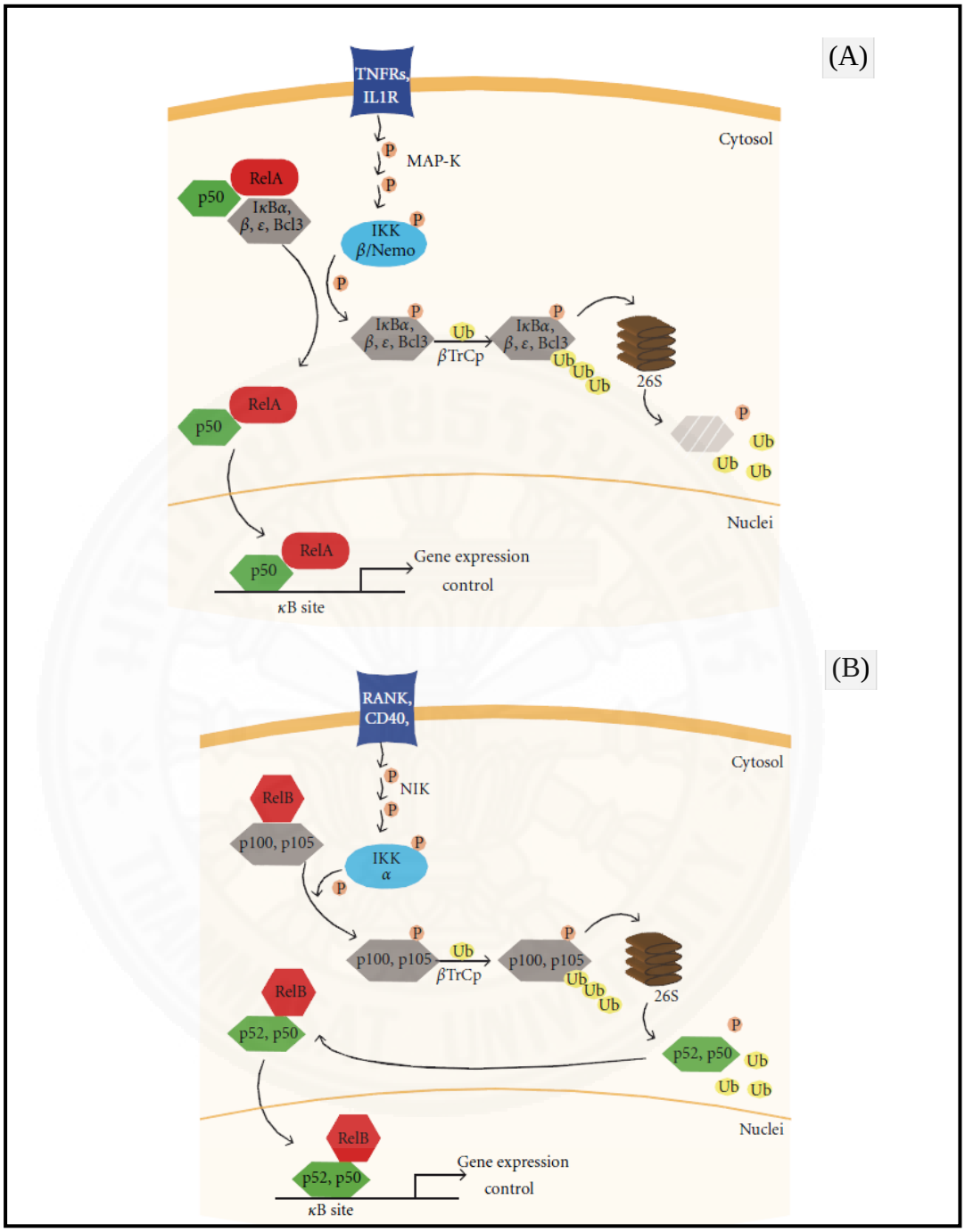

Figure 2.11 The canonical or classical (A) and non-canonical or alternative (B) pathways of NF-κB. From "Potential role of nuclear factor NF-κB in diabetic cardiomyopathy," O. Lorenzo et al., 2011, *Mediators of Inflammation*, p. 2.

2.3 Vascular lipotoxicity

Obesity and type 2 diabetes are major risk factors for the development of atherosclerosis and its complications (Booth, Kapral, Fung, & Tu, 2006; Madala et al., 2008). Vascular dysfunction is a well-established characteristic of MS. There are multiple mechanisms that impede vascular function in this prevalent state. Recent findings provide strong evidence that increased lipotoxicity may impair vascular function and structure by promoting lipid accumulation, interfering insulin signaling, activating inflammatory processes, inducing oxidative stress and disrupting mitochondrial functions. It is likely that these changes are not parallel pathways, but are synergistically interacting to trigger vascular dysfunction (Imrie, Abbas, & Kearney, 2010; Wende et al., 2012).

2.3.1 Vascular biology and dysfunction

The progression of MS and atherosclerosis starts many years before its consequent clinical manifestations with the destruction of a wide range of vascular anatomy and homeostasis. This topic will primarily explain some of the expressive structural and functional features of blood vessels so it can better comprehend the MS and atherosclerosis that affect them. The general structure and cellular composition of healthy vasculature is the same throughout the circulatory system. The vascular wall consists three distinct layers (tunics): tunica intima, tunica media and tunica adventitia. The vascular endothelium is the monolayer of endothelial cells that lines the interior surface of all blood vessels and forms an interface between circulating blood and the subendothelial tissue. Given its unique structural position, the endothelium is primordial target for biological, chemical, and physical injurious stimuli that can lead to endothelial injuries and/or death. In generality, the injured or dead endothelial cells are regenerated or repaired by endothelial progenitor cells (EPCs) and neighboring mature endothelial cells, which stimulate reendothelialization and preserve normal vasculature (Dimmeler & Zeiher, 2004; Miller-Kasprzak & Jagodzinski, 2007). Current understanding shows that the endothelium is a dynamic and multifunctional organ, modulating the circulation in response to several physiological and pathological stimuli by the synthesis of a variety of bioactive factors such as NO, endothelin and prostaglandin. The endothelial cell layer has

numerous functional roles in maintaining vascular homeostasis. These functions including regulation of permeability, vascular tone, hemostasis, vascular growth, immunity, inflammation, and repair. Under normal conditions, the endothelium has thromboresistant actions (anticoagulant and fibrinolytic functions), vasodilatory actions, anti-adhesive effects against leukocytes and platelets, growth inhibitory actions on underlying smooth muscle cells, and regenerative actions to repair the injured blood vessel. Vascular endothelial dysfunction is characterized by impaired vasodilation and increased adhesion of monocytes and platelets, augmented permeability to lipids and inflammatory cells, smooth muscle cell migration and proliferation and reduced repair mechanisms.

2.3.2 eNOS

Although no single event can absolute explain the loss of vascular homeostasis, impaired NO concentrations and bioavailability have been proposed as a major mechanism of vascular dysfunction and contributor to atherosclerosis and hypertension. Because, NO has many favorable physiologic actions (Figure 2.12) including lessening platelet adhesion and aggregation, vasodilation, attenuating leukocyte infiltration, reducing smooth muscle cell proliferation and decreasing endothelin production, inhibiting LDL oxidation, reducing inflammatory cytokines, recruitment of EPCs to the injured or dead vascular region (Van Craenenbroeck & Conraads, 2010), thus exhibiting important vasculoprotective and anti-atherosclerotic effects (Hiroaki, 1999; Adams et al., 2000; Pober, Min, & Bradley, 2009). Originally called endothelium-derived relaxation factor, it was identified as the free radical NO in 1987 by the awarding of the 1998 Nobel Prize for physiology or medicine to R. F. Furchgott, L. J. Ignarro and F. Murad. NO is a labile gaseous molecule for intercellular communication and intracellular signaling transduction because it has a short half-life. In vascular endothelial cells, NO is biosynthesized from L-arginine, molecular oxygen and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) by endothelial nitric oxide synthase (eNOS or NOS3) (Zhao, Vanhoutte, & Leung, 2015). At basal state, eNOS is constitutively expressed and has powerful biological effects in the blood vessel (Figure 2.9).

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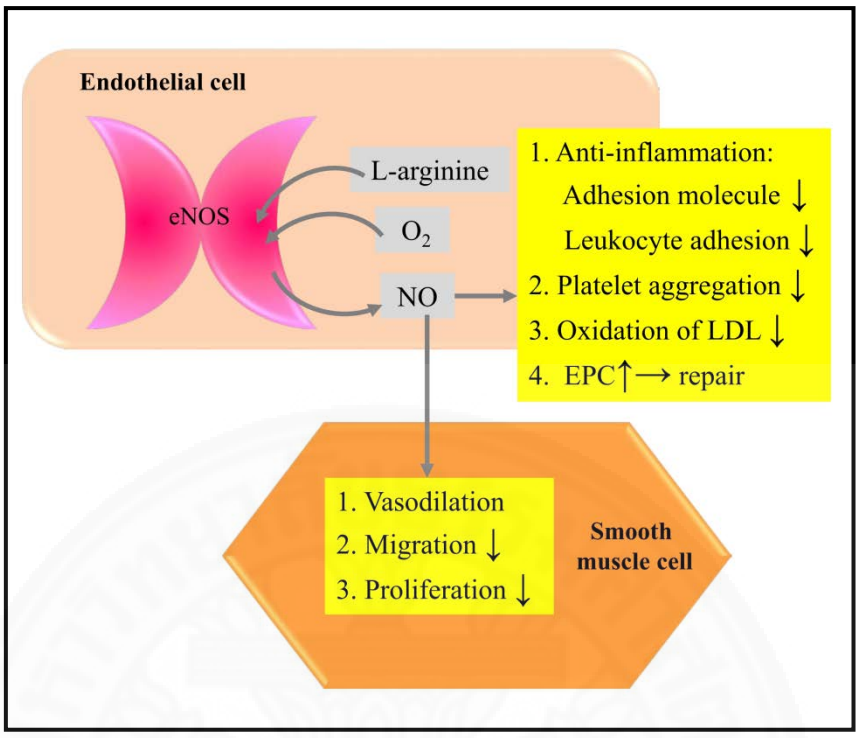

Figure 2.12 NO biosynthesis and its physiological effects in vasculature. ↑, increased; ↓, decreased.

In obesity or MS, there are many risk factors that could adversely affect vascular homeostasis, which include changes in lipid metabolism, glucose level, blood pressure, oxidative/nitrosative stress and systemic inflammation. The pathological conditions, such as high levels of FA, hypertriglyceridemia and hyperglycemia, can promote vascular dysfunctions (Arcaro et al., 2002; Monti et al., 2004; Zhang et al., 2013). These results support a pathogenic role for promotion of glucolipotoxicity in vascular disease. In addition, the reduction of eNOS expression and activity was observed in the vasculature induced by an HF, thus impairing NO synthesis (Bourgoin et al., 2008; Kobayasi et al., 2009; Yang, Ying, et al., 2007). The down-regulation of eNOS gene was also observed in the vasculature of patient with atherosclerosis (Oemar et al., 1998). Moreover, overexpression of eNOS has been revealed to protect rodents from the development of vascular disease (Zhao et al., 2008). Thus, the up-regulation of eNOS expression in the vasculature is considered to be an important preventive mechanism for vascular disease (Fleming, 2010; Förstermann & Münzel, 2006; Huang, 2009).

2.3.1 NF-κB

As mentioned above, NF-κB was originally recognized as a nuclear factor that bound the immunoglobulin-κB light chain promoter in B-lymphocytes by David Baltimore in 1986. The NF-κB and its inhibitory protein IκB constitute an important signaling that mediates inflammatory and oxidative response. In endothelial cells, activated NF-κB triggers the increased ROS levels (Maloney et al., 2009) and diminishes insulin-activated NO production (Kim et al., 2007). In addition, secretory products from adipocytes increase adhesion molecules expression, monocyte adhesion, apoptosis and dysfunction of endothelial cells via NF-κB activation (Kralisch, et al., 2008). In vivo study represented expression of the NF-κB inhibitor (IκB-α) was decreased in the aortas from obese mice as compared with the controls (Kobayasi et al., 2009). Endothelial cells from overweight and obese humans showed abundant expression of NF-κB when compared with lean adults (Silver et al., 2007). Li and co-researchers (2011) demonstrated that FFA induced NF-κB expression and vascular lipotoxicity in an animal model. Given the well-established pro-inflammatory and redox sensitive functions of NF-κB, its inhibition would be expected to have anti-inflammation, antioxidant and anti-atherosclerotic effects (de Winther, Kanters, Kraal, & Hofker, 2005).

2.4 Experimental model of MS and positive control

2.4.1 Rat model of HF-induced MS

As mentioned above, high dietary fat intake is an important cause of metabolic and cardiovascular diseases in both human and experimental animals (Lottenberg et al., 2012; Panchal & Brown, 2011). It is well-established that rodent models of MS, including the high-HF-fed rodents, are involved in the development of metabolic syndrome and cardiovascular disease (Geetha et al. 2014; Kim et al., 2000; Wu et al., 2010). In the previous studies, outbred male Sprague-Dawley rats fed an high-energy diet developed metabolic syndrome, which were characterized by obesity, ectopic fat accumulation, dyslipidemia and insulin resistance, compared with rats fed a standard diet (Archer, Rayner, Rozman, Klingenspor, & Mercer, 2003; Eu, Lim, Ton, & Kadir, 2010;). These metabolic dysfunctions may predispose to increased cardiovascular disease. Therefore, HF-outbred Sprague-Dawley rats, no

genetic factor involved, were selected as a model for studying metabolic syndrome and related cardiovascular risk factors.

2.4.2 F

F, a PPARα agonist, is a well-known pharmacotherapy for dyslipidemic patients. F is an isopropyl ester that is hydrolyzed completely in the intestine. Its plasma half-life is 20 hours. Sixty percent is excreted in the urine as the glucuronide, and about twenty-five percent in feces. The dosage of F is one to three 48 mg tablets (or a single 145 mg tablet). PPARα activation leads to up-regulate or down-regulate multiple target genes involved lipid metabolism. It is usually used to treat hypercholesterolemia, hypertriglyceridemia, and mixed dyslipidemia (Chapman, Redfern, McGovern, & Giral, 2010).

Previous studies showed that the treatment with F improved the metabolic dysfunctions in animal model such as decreased body mass, hypertrophic adipocyte (Fernandes-Santos, Carneiro, de Souza Mendonca, Auguila, & Mandarim-de-Larcerda, 2009), decreased blood TG and LDL-C levels (Ferreira, Parreira, Green, & Botion, 2006), and decreased hepatic TG levels by increasing the β-oxidation (Jeong et al., 2004). Recent studies revealed pleiotropic effects of F on cardiovascular tissue (Balakumar, Rohilla, & Mahadevan, 2011; Marchesi & Schiffrin, 2008). F was able to prevent the development of cardiac dysfunction and hypertrophy by decreasing the cardiac expression of NF-κB, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1). F has been shown to have direct anti-inflammatory and anti-atherosclerotic effects by decreased NF-κB and increased eNOS expression (Watts & Staels, 2004). In the current study, the treatment of F was considered as a positive control to standardize the experiments. However, long-term F treatment was able to induce vascular inflammation and dysfunction (Blanco-Rivero et al., 2007). Therefore, F treatment was not used as a positive control for intermediate-term study.

2.5 Rice bran, MS and cardiovascular diseases

Rice is the staple source of energy for various countries in the world including Thailand and the appropriate consumption of rice in order to gain the most benefit to health is concerned. Rice is a member of the grass family (*Gramineae*) and belongs to the genus *Oryza* under tribe *Oryzeae*. The genus *Oryza* includes 20 wild species and 2 cultivated species (cultigens). Of the two cultivated species, African rice (*Oryza glaberrima* Steud.) is confined to West Africa, whereas common or Asian rice (*Oryza sativa* Linnaeus or *Oryza sativa* Linn.) is now commercially grown in 112 countries, covering all continents.

Rice bran is a by-product of rice milling process in the generation of white rice from brown rice, which derives from the outer layer of whole-grain rice. Rice bran is an important source of phytochemicals and nutrients with known carbohydrates (34.1-52.3%), lipids (15-22%), protein (10-16%), dietary fiber (7-11.4%), vitamins, minerals and bioactive components including phenolic compound, γ-oryzanol (a mixture of ferulic acid esters of triterpene alcohols and sterols), vitamin E isoforms or tocols (α-, γ-, δ-tocopherols and tocotrienols), and unsaturated FA (Chen & Bergman, 2005; Cicero & Derosa, 2005). Rice bran also contains other bioactive compounds such as ferulic acid, phytosterols, cellulose, hemicelluloses, albumins, globulins, prolamins, glutelins, arginine, lysine, proline, valine, glutamic acid, aspartic acid, tryptophan, histidine, and cysteine (Fabain & Ju, 2011; Henderson et al., 2012).

In previous studies, a rice bran enzymatic extract (RBEE) intake has been reported to decrease inflammation and oxidative stress in association with a reduction in vascular dysfunction in animals with MS (Justo et al., 2013). RBE and rice bran intakes have also been reported to have cardiovascular protective effects including anti-atherosclerotic and blood pressure-lowering effects (Ardiansyah et al., 2006; Justo et al., 2012; Perez-Ternero et al., 2015). Moreover, treatment with RBEE intake led to decreased hyperlipidemia, hyperglycemia, hyperinsulinemia, glucose intolerance, and adipocyte size in animals with MS (Candiracci, Justo, Castaño, Rodriguez-Rodriguez, & Herrera, 2014; Justo et al., 2015). Consistently, treatment with RBE and rice bran markedly prevented the impaired lipid and glucose metabolism in patients with diabetes mellitus (Cheng et al., 2010; Qureshi et al.,

2002). These data strongly suggest that RBE treatment could be useful for preventing metabolic and cardiovascular diseases. Rice bran also has the nutritional and pharmaceutical potential as an anti-cancer activity and hypoallergenicity (Fabain & Ju, 2011).

The most studied phytochemicals in whole grain are phenolic compounds. Phenolics are compound with one or more aromatic rings and one or more hydroxyl group (Figure 2.13) such as phenols, phenolic acids and flavonoids. Phenolic compounds have antioxidant properties and can protect against degenerative diseases (i.e., heart disease and cancer) in which ROS (i.e., superoxide anion, hydroxyl radicals, and peroxy radicals) are involved (Kishimoto, Tani, & Kondo, 2013; Liu, 2007).

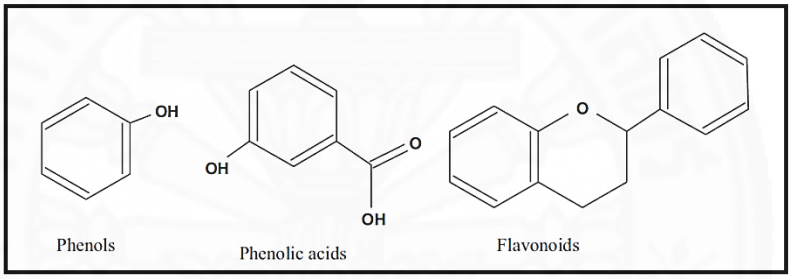

Figure 2.13 Chemical structure of common phenolic compounds. From "Whole grain phytochemicals and health," R. H. Liu, 2007, *Journal of Cereal Science, 46*, p. 208.

γ-Oryzanol is one of major phytochemical components in rice bran. It has been suggested to show blood cholesterol-lowering, anti-inflammatory and antioxidants effects. γ-Oryzanol is a mixture of at least 4 main components including phytosteryl ferulates, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, β-sitosteryl ferulate, and campesteryl ferulate (Figure 2.14). In vivo studies have been reported that treatment with γ-oryzanol inhibits the NF-κB activation and inflammatory responses in the endothelial cells (Sakai e al., 2012). In animal model also demonstrated that the treatment with γ-oryzanol was able to improve hypoadiponectinemia and inhibit obesity and liver fat accumulation (Nagasaka, Yamsaki, Uchida, Ohara, & Ushio, 2011; Wang et al., 2015).

Vitamin E as anti-inflammatory, antioxidant, anticancer (anti-proliferative), hypocholesterolemic, and neuroprotective agents in animal and humans have been confirmed by several investigators. α-Tocopherol is one of 8 compound of

vitamin E that compose of a chromanol ring and a phylyl tail (Figure 2.15). Consumption of α-Tocopherol has been shown to be associated with beneficial effects on metabolic and atherosclerotic diseases (Kim, Kim, Ham, & Choue; 2013; Peluzio et al., 2001)

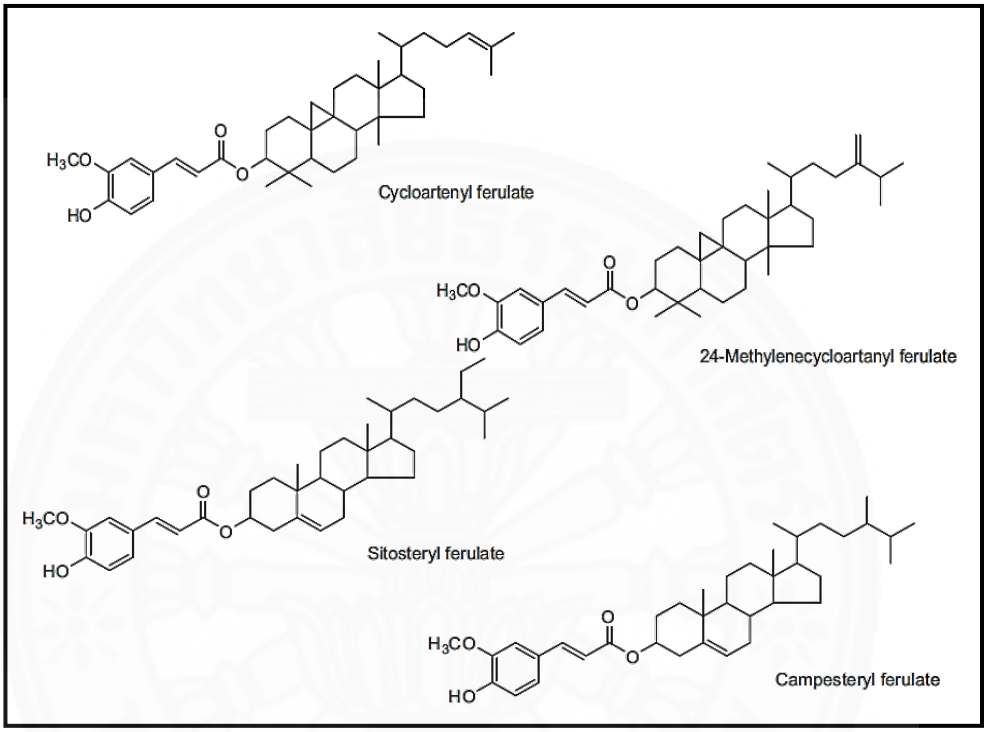

Figure 2.14 Chemical structure of the four main components of γ-oryzanol. From "γ-Oryzanol recovers mouse hypoadiponectinemia induced by animal fat ingestion," R. Nagasaka, T. Yamsaki, A. Uchida, K. Ohara, & H. Ushio, 2011, *Phytomedicine, 18*, p. 670.

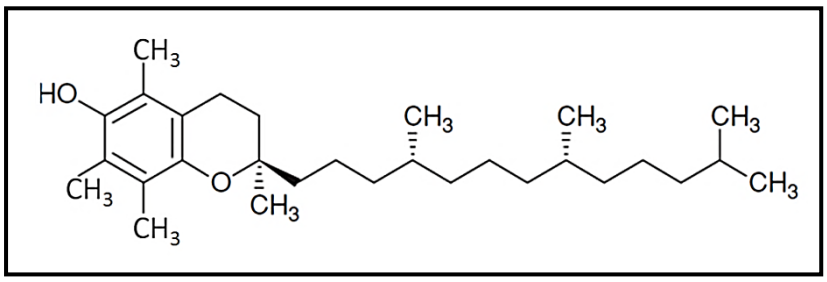

Figure 2.15 Chemical structure of tocopherols. Adapted from "Health benefits of whole grain phytochemicals," N. Okarter, & R. H. Liu, 2010, *Critical Reviews in Food Science and Nutrition, 50*, p. 203.

Various international rice cultivars demonstrate nutritional and phytochemical diversity that may translate into differential improvement mechanisms of MS and cardiovascular disease. The studies on the nutraceutical compositions and properties of rice bran across diverse varieties are still needed. Thai rice, KDML 105 or white Jasmine rice, is the most abandon commercial crop in Thailand and the world market. Previous studies have shown that the treatment with RBE from the KDML 105 rice variety significantly reduced insulin resistance, as well as abdominal and hepatic fat deposition in rats fed an HF for 4 weeks (Chotip Chakhonpunya et al., 2011; Noppamat Kandee et al., 2009). Although those studies suggested KDML 105 RBE could reduce the cardiovascular risk factors, cardiovascular protection has not been yet elucidated. Thus, this study aimed at verifying the effects of RBE on the heart and vasculature of HF-fed rats so as to provide a model for prevention of metabolic and cardiovascular diseases.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials

Instruments

1. Electrical balance (Mettler-Toledo, Thailand)
   (Precisa Instrument, Switzerland)
2. Autopipettes (Rainin Instrument LLC, USA)
3. Hotplate and stirrer (Sterillin, France)
4. Hot air oven (ULM 600) (Memmert, Germany)
5. Autoclaver (Hirayama, Japan)
6. Centrifuge machine (Beckman coulter, USA)
   Centrifuge machine (Thermo Electron Corporation, USA)
   (Sorvall RC6 -PLUS)
   Refrigerated centrifuge machine (Hettich Zentrifugen, Germany)
7. Microcentrifuge machine (Denville Scientific, USA)
   (Bertec Enterprise, Taiwan)
8. Spectrophotometer (Shimadzu, Japan)
9. PowerWave XS Microplate reader (BioTek, USA)
10. Digital Dry Bath Incubator (Boekel Scientific, USA)
11. Incubator shaker (JP Selecta, Spain)
12. Orbital Shaker OS-20 (Biosan, Latvia)
13. Freeze dryer (Lyophilization Systems Inc., USA)
14. ACCU-CHEK performa and strips (Roche Diagnostics, Switzerland)
15. Sonicator (Elma, Germany)
16. Homoginizer (Eberbach corporation, USA)
17. Olympus CX31 microsco equipped to (Oympus, Japan)
    a Olympus DP20 microscope camera
18. Eclipse Ci-L microscope equipped to (Nikon, Japan)
    a DS-Fi2 microscope camera

19. Water bath (Memmert, Germany)
20. High perfomance liquid chromatography (HPLC) (TSP, USA)
    -Pump: ConstaMetric 4100
    -Detector: SpectroMonitor 4100 UV (λ=292 nm)
    -Column: C18 4.6 × 250 mm (5 µm), Phenomenex with guard column
23. NanoDrop 2000 spectrophotometer (Thermo Scientific, USA)
24. MyCycler thermal cycler (Bio-Rad, USA)
25. StepOnePlus Real-Time PCR System (Applied Biosystems, USA)
26. MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems, USA)
    and MicroAmp Optical Adhesive Film
27. Gel electrophoresis system (Bio-Rad, USA)
    (Mini-PROTEIN Tetra Cell)
28. Power supply (Bio-Rad, USA)
    (PowerPac 200)
29. Wet/Tank blotting system (Bio-Rad, USA)
    (Mini Trans-Blot Electrophoretic Transfer Cell)
30. Nitrocellulose membrane, 0.2 µM (Bio-Rad, USA)
31. Odyssey Fc Imaging System (LI-COR® Bioscience, USA)

Chemicals

1. Amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich, USA)
2. Alpha-amylase heat stable from *Bacillus licheniformis* (Sigma-Aldrich, USA)
3. Sodium chloride (NaCl) (Amresco, USA)
4. Phosphate buffered saline (PBS) tablets (Amresco, USA)
5. Fenofibrate (Abbot laboratories , Thailand)
6. D-glucose ($C_6H_{12}O_6$) (Amersham Biosciences, USA)
7. Folin-Ciocalteu pherol reagent (Fluka, Switzerland)
8. Sodium carbonate (Na_2CO_3) (Alpha, India)
9. Gallic acid (Sarstedt, Germany)
10. γ-oryzanol (Wako, Japan)
11. α-tocopherol (Sigma-Aldrich, USA)
12. Ethanol absolute (Merck, Germany)

13. Chloroform, HPLC grade and analytical reagent (RCI labscan, Thailand)
14. Methanol, HPLC grade (RCI labscan, Thailand)
15. Methanol, analytical reagent (Quality control chemical, New Zealand)
16. Acetonitrile, HPLC grade (RCI labscan, Thailand)
17. Butylate hydroxytoluene (BHT) (Merck, Germany)
18. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Fluka, USA)
19. Pentobarbital sodium (nembutal) (Jagsonpal, India)
20. Formaline (40%) (International Resin, China)
21. Sodium dihydrogen phosphate monohydrate (Merck, Germany) ($NaH_2PO_4{}^*H_2O$)
22. Di-Sodium hydrogen phosphate (Quality control chemical, New Zealand) anhydrous (Na_2HPO_4)
23. Fluitest Cholesterol reagent (Analyticon Biotechnologies AG, Germany)
24. Fluitest TG reagent (Analyticon Biotechnologies AG, Germany)
25. Fluitest HDL-C precipitation reagent (Analyticon Biotechnologies AG, Germany)
26. Potassium hydroxide (KOH) (Merck, Germany)
27. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, USA)
28. Glycerol standards (Sigma-Aldrich, USA)
29. Free glycerol reagent (Sigma-Aldrich, USA)
30. RNA*later* RNA stabilization reagent (Qiagen, Germany)
31. TRIzol reagent (Invitrogen, USA)
32. 2-propanol (Sigma-Aldrich, USA)
33. Diethylpyrocarbonate (DEPC)-treated water (Ambion, USA)
34. High Capacity cDNA Reverse Transcription Kits (Applied Biosystems,USA)
35. TaqMan Gene Expression Master Mix (Applied Biosystems, USA)

36. TaqMan Gene Expression Assay (Applied Biosystems, USA)
    - PPARα (Assay ID Rn00566193_m1)
    - FAT/CD36 (Assay ID Rn02115479_g1)
    - GLUT-4 (Assay ID Rn01752377_m1)
    - NF-κB p65 (Assay ID Rn01399583_m1)
    - eNOS (Assay ID Rn02132634_s1)
    - β-actin (Assay ID Rn00667869_m1)
    - GAPDH (Assay ID Rn99999916_s1)
37. Cell lysis buffer (Cell Signaling Technology, USA)
38. Protease Inhibitor Cocktail (Cell Signaling Technology, USA)
39. Phenylmethylsulfonyl fluoride (PMSF) (Cell Signaling Technology, USA)
40. Quick Start Bradford Protein Assay kit 2 (Bio-Rad, USA)
41. Sodium dodecyl sulfate (Amresco, USA)
42. 30% acrylamide/bis solution, 29:1 (Bio-Rad, USA)
43. Ammonium sulfate (Bio-Rad, USA)
44. N, N, N′, N′-tetra-methyl-ethylenediamine (TEMED) (Bio-Rad, USA)
45. 2-mercaptoethanol (Bio-Rad, USA)
46. Tris (Amresco, USA)
47. Glycine (Amresco, USA)
48. Glycerol (Merck, Germany)
49. Bromophenol blue (Amresco, USA)
50. ColorPlus Prestained Protein Ladder, Broad Range (NEB,USA)
51. Cromassie brilliant blue (Research Organics, USA)
52. Ponceau S staining solution (Sigma-Aldrich, USA)
53. Primary antibody
    -PPARα antibody (BioVision, USA)
    -FAT/CD 36 antibody (Santa Cruz Biotechnology, USA)
    -NF-κB p65 antibody (Cell Signaling Technology, USA)
    -GLUT-4 antibody (Cell Signaling Technology, USA)
    -eNOS antibody (Santa Cruz Biotechnology, USA)
    -β-actin antibody (Cell Signaling Technology, USA)

54. Secondary antibody (Cell Signaling Technology, USA)
    -Anti-rabbit immunoglobulin (H+L)
    (Dylight 680 conjugate)
    -Anti-mouse immunoglobulin (H+L)
    (Dylight 680 conjugate)
55. Tween 20 (Prolabo, UK)
56. Odyssey Blocking Buffer (LI-COR Bioscience, USA)

3.2 Preparation of RBE

3.2.1 Rice bran samples and its stabilization

Rice bran of KDML 105 rice variety was purchased from the local mill in Surin province, Thailand. KDML 105 rice was grown in the organic farm approved by the Organic Agriculture Certification of the Department of Agricultural Extension (Bangkok, Thailand). These rice grains were harvested and milled to separate husk from brown rice at the rice mill. The brown rice was then polished to obtain bran. Rice bran was stabilized in hot air oven at 130–140°C for 3 min (lipase inactivation).

3.2.2 Preparation of digestive enzymes solution

A 100 mg of α-amylase was dispersed in 65 mL distilled water (DW) and stirred on magnetic stirrer plate until it was dissolved. Then, an aliquot of 50 µL of α-amyloglucosidase solution was added.

3.2.3 Hot water extraction digestion of stabilized rice bran

The procedure of preparation was described in details by previous studies (Qureshi et al., 2002). About 2,000 g of stabilized rice bran was boiled in 8,000 mL of DW for 1 h at 70 °C. After cooling to room temperature, the suspension was centrifuged at 8,000 rpm (12,000 × g) for 10 min at 25 °C. . The supernatant was then mixed with digestive enzymes solution. The mixture was shaken and incubated for 1 h at 37 °C. The mixture was then centrifuged at 8,000 rpm for 10 min at 25 °C. The supernatant was transferred to a stainless steel tray and then freeze-dried into powdered extract by using a freeze dryer. The lyophilized powder was stored at −40 °C until further studies (Figure 3.1).

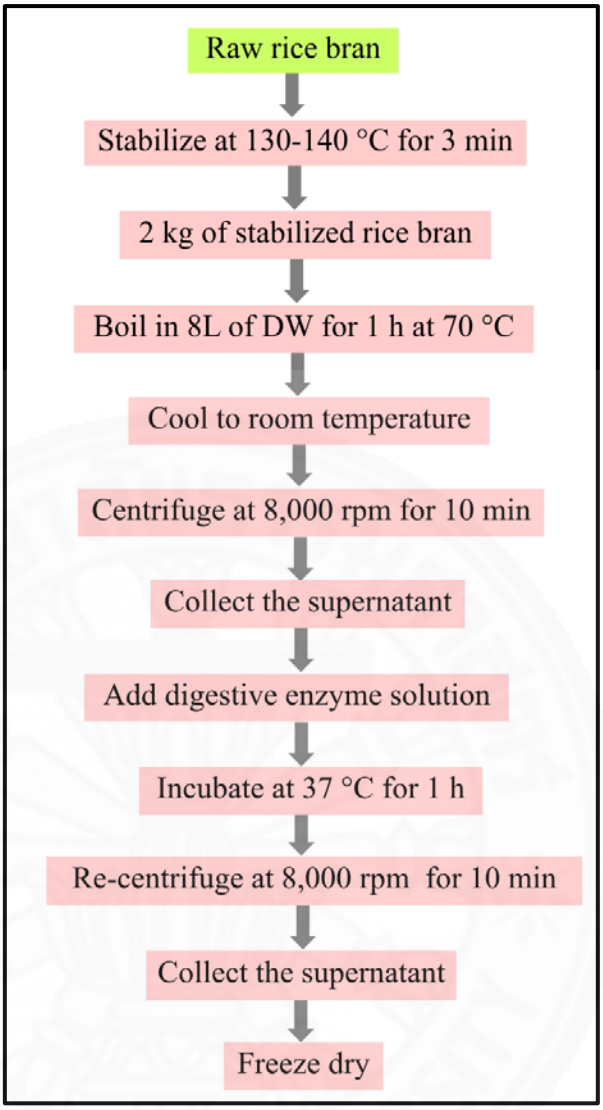

Figure 3.1 A flowchart of rice bran water extraction.

3.3 Determination of phytochemicals and antioxidant property of RBE

3.3.1 Determination of total phenolic compounds

The total phenolic compounds of RBE were determined using the procedure of previous studied with some modification (Singleton, & Rossi, 1965; Sunan Butsat & Sirithon Siriamornpun, 2010). Briefly, 70 µL of RBE solution were pipetted into a test tube. A 525 µL of diluted Folin-Ciocalteu reagent (1:5 with DW) was added to each of the test tubes. This mixture was shaken for a min. 525 µL of 7.5 % Na_2CO_3 was added and shaken. The color of mixture was developed for 30 min at

room temperature. The absorbance of the mixture was measured at 760 nm against the reagent blank.

The total phenolic compound in samples was quantified by the calibration curve of reference standard. Using gallic acid as a reference standard, total phenolic compounds are expressed as mg of gallic acid equivalents (GAE)/g dry weight of RBE.

3.3.2 Determination of γ-oryzanol and α-tocopherol contents

The contents of γ-oryzanol and α-tocopherol in RBE were determined using HPLC equipped with a UV-V is detector according to the method described by other studied (Rohrer & Siebenmorgen, 2004; Sunan Butsat & Sirithon Siriamornpun, 2010). About 200 mg of RBE were dissolved in 2 mL chloroform and filtered through a 0.45 μm pore size syringe-driven filter before injection. A 10 μL sample was injected into the HPLC column via a fixed loop coupled to an autosampler. Chromatography was performed using a C18 column (4.6 × 250 mm, 5 μm) with a guard column. The elution was isocratic with 25% methanol as the mobile phase A and 75% acetonitrile as the mobile phase B at a flow rate of 1.2 mL/min with 40 min total running time.

γ-Oryzanol and α-tocopherol were measured using spectrophotometric detector at wave length of 325 nm and 292 nm, respectively. Quantification of γ-oryzanol and α-tocopherols in the samples were indentified by comparing their relative retention times and calibration curves of peak area with the external standards (authentic standards). The concentration ranges of calibration standards were 50, 100, 200 and 1,000 μg/mL for both active constituents. The concentration of γ-oryzanol and α-tocopherols in the samples were considered by the coefficient of determinations (R^2) of the standard curves ranged from 0.999 to 1. Data was analyzed using PC 1000 (TSP) software.

3.3.3 Determination of antioxidant activity

Antioxidant activity of RBE was determined by scavenging effect on DPPH generated by the chemical method according to a protocol by Yu et al., 2002. RBE was diluted in 70% ethanol, to a final concentration of 10, 50, 100, 250, 500 and 1,000 μg/mL. An aliquot of 500 μL of sample solution was mixed with 500 μL of 6×10^{-5} M DPPH in absolute ethanol. The absorbance of each sample was measured at

517 nm by a microplate reader after the reaction mixture is allowed to stand for 30 min at room temperature in dark. Compare DPPH radical scavenging capacity of each sample with BHT. BHT was used as reference standard tested in the same system. All tests were conducted in triplicate assay. The results were expressed as the inhibition percentage determined from the difference in absorbance (A) of DPPH between the control and sample. The result is reported as half maximal effective concentration (EC50). EC50 value was calculated by Prism GraphPad software (GraphPad Software, Inc., USA). A percentage inhibition activity was calculated by using the formula below.

$$\text{Inhibition (\%)} = \frac{(\text{A control} - \text{A sample}) \times 100}{\text{A control}}$$

3.4 Animal study

3.4.1 Animals and ethical approval

All experimental procedures involving animals were pre-approved by the Animal Ethics Committee of the Faculty of Medicine, Thammasat University, Pathum Thani, Thailand (Permission Number AE 002/2013, Appendix A). Male outbred Sprague-Dawley rats (6–8 weeks old and body weight, 180–220 g) were purchased from the National Laboratory Animal Center, Nakhon Pathom, Thailand. All animals were maintained under controlled temperature (24 ± 1 ºC) with 60% humidity and a 12-h light and 12-h dark cycle (lights on at 6 A.M.).

3.4.2 Experimental design and animal grouping

After a week of acclimatization, rats were assigned to the following 4 groups:

(1) Control (C) group: rats received standard chow (n = 16)

(2) HF group: rats received HF (n = 16)

(3) HF + RBE group: rats received HF and co-fed with RBE at the dose of 2,205 mg/kg rat weight/day (n = 16)

(4) HF + F group: rats received HF and co-fed with F at the dose of 5 mg/kg rat weight/day for 4^{th} day to 16^{th} day. Then, it was increased to 15 mg/kg rat weight/day until the end of 4^{th} week (n = 8).

After acclimatization, animals in group 4 were fed with a HF alone for the first three days. After 4 weeks, half of animals (n = 8/group) in group 1 to 3 and all animals in group 4 were sacrificed, and then collected the target samples. The treatment was continued to the rest of the rats (n = 8/group) in group 1 to 3 until the end of treatment period of 16 weeks. In the present study, 4 and 16 weeks of treatment were determined as a short- and intermediate-term, respectively (Wilson, Tran, Salazar, Young & Taegtmeyer, 2007). The experimental design and groups of rats are shown in Figure 3.2.

3.4.3 Animals diets and feeding

Animals in all groups were fed ad libitum with water and experimental diets throughout study period. The standard chow (Number 082G, C.P., Thailand) consisted of 13%, 55% and 31% of energy derived from fat, carbohydrate and protein, respectively (energy density of 3.04 kcal/g). The HF was modified from the diet-induced obesity in which 65% of total energy was derived from fat (Claret et al. 2004). It consisted of 65%, 24%, and 11% of total energy derived from fat, carbohydrate, and protein, respectively (energy density of 5.12 kcal/g). The HF pellets were prepared in the kitchen of the Nutrition Unit, Thammasat University Hospital, Thammasat University, Thailand. The HF was stored at 4 °C. Fresh diet was replaced every 24 h for the complete experimental period. The ingredients of HF are shown in Table 3.1.

RBE was dissolved in DW in a dose of 2,205 mg/kg rat weight/day. Consistent with previous reports (Chotip Chakhonpunya et al., 2011; Noppamat Kandee et al., 2009), our preliminary study showed that the treatment with RBE at a dose of 2,205 mg/kg/day was highly effective in improving the impaired lipid and glucose metabolism in HF-fed rats. Thus, this dose was chosen for the present study. F was dissolved in DW and administered orally in a dose of 5 mg/kg body weight per day for 4^{th} day to 16^{th} day. Then, it was co-fed at a dose of 15 mg/kg body weight per day until the end of co-feeding period of 4 weeks. The dosage and feeding was modified according to therapeutic dose for dyslipidimic patients. The feeding processes were done by oral gavage. The same volume of DW was gavaged to the C and HF groups. All experimental procedures were done at the same time (9-10 A.M.).

3.4.4 Measurement of animal body weight and dietary intake

Body weight and dietary intake (food and energy intakes) of the rats were recorded daily throughout the study period.

3.4.4.1 Calculation of food intake

The food intake per rat in each day was calculated as follows:
Food intake (g/day) = Initial food weight (g) – Leftover food weight (g)

3.4.4.2 Calculation of energy intake

The energy intake per each rat per day was calculated as:
Energy intake of control diet (kcal/day) = Consumed food weight (g) × 3.04 (kcal/g)
Energy intake of HF (kcal/day) = Consumed food weight (g) × 5.12 (kcal/g)

3.4.4.3 Calculation of body weight gain

The initial body weight and final body weight of each rat was represented by body weight on first day and last day before termination, respectively. The body weight gain per each rat was calculated as follows:

Body weight gain (g) = Final body weight (g) – Initial body weight (g)

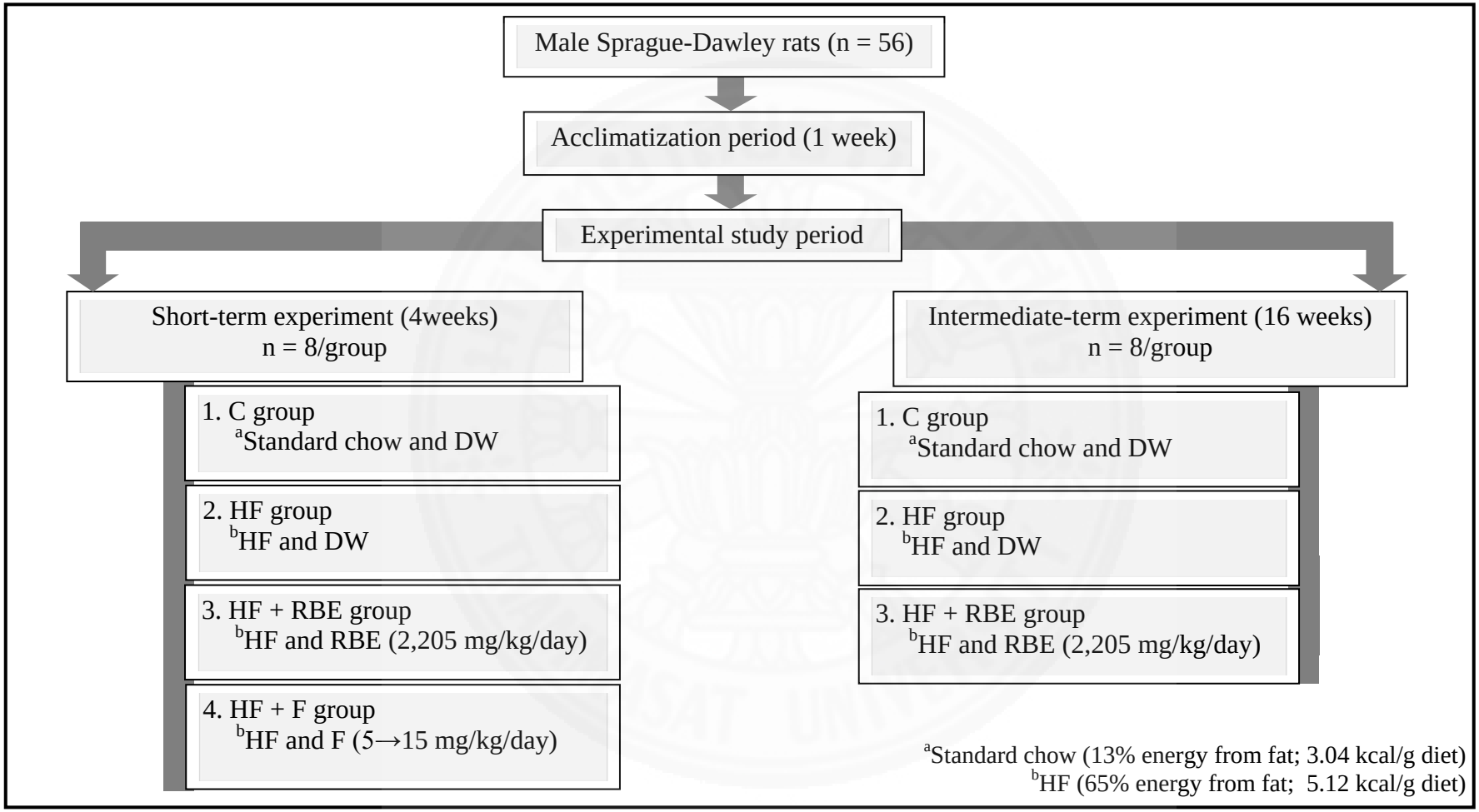

Figure 3.2The experimental design of animal study.

Table 3.1

Compositions of HF as were estimated by Nutri Survey Program from Nutrition Divisions, Department of Health, Ministry of Public Health

Ingredients	Amount (g)	Carbohydrate (g)	Protein (g)	Fat (g)	Energy (kcal)
Sugar	58	57.7	-	-	223.5
Standard chow	100	41.8	24	4.5	304
Wheat flour	150	114.5	15.5	1.5	546.5
Pork belly	100	2.8	13.9	33.5	368.1
Pork liver	100	2.4	19.9	4	126
Egg yolk (hen)	200	4	28.6	60.2	672.1
Egg white (hen)	33	-	3.3	0.4	16.8
Margarine	200	-	0.8	172.6	1,558.8
Total	941	223.2	106	276.7	3,816
% kcal	-	23.39	11.11	65.26	-

Note. 941 g of composition mixture gave a result of 745 g biscuit.

3.4.5 Collection of serum and tissue samples

At the end of treatment period, the animals were sacrificed by intraperitoneal injection of high dose (150 mg/kg) of pentobarbital sodium after an approximately 16-h fasting overnight period (Eu, Lim, Ton, & bin Abdul Kadir, 2010). After opening the thorax, blood was drawn from the apex of cardiac ventricle (cardiac puncture). Blood samples were collected into a glass container and centrifuged at 1,500 × *g* for 10 min at room temperature. An aliquot part of serum was transferred to a new microcentrifuge tubes and stored −20 °C prior to use for determination of biochemical parameters.

The heart and aorta were rapidly excised and promptly placed in a beaker containing cold (4 °C) PBS, gently cleaned of adhesive and contaminating tissue and weighted. Cardiac ventricles and aortic tissues were divided into four and three portions, respectively. The first portion was stored at −20 °C in RNA*later* reagent until the measurement of messenger ribonucleic acid (mRNA) levels. The second portion was rapidly frozen in liquid nitrogen until the measurement of protein levels. The third portion was fixed in 10% formalin for histological analysis. The fourth portion of heart (300 mg) was digested immediately with 500 µL ethanolic potassium hydroxide (KOH) overnight for measurement of TG contents (Figure 3.3).

Besides, other tissue or organ samples of interest, namely the abdominal fat tissue, epididymal fat pads, liver, pancreas and kidney were promptly harvested, rinsed with cold PBS, weighed and then stored in 10% formalin. In addition to the histological study, 300 mg of liver tissue were digested with 500 µL ethanolic KOH for measurement of TG contents (Figure 3.2).

The absolute organ weight of each rat was normalized by the final body weight and reported as a relative value of organ weight per 100 g final body weight. Relative organ weight (% of body weight or g/100 g body weight) was calculated as:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)} \times 100}{\text{Final body weight (g)}}$$

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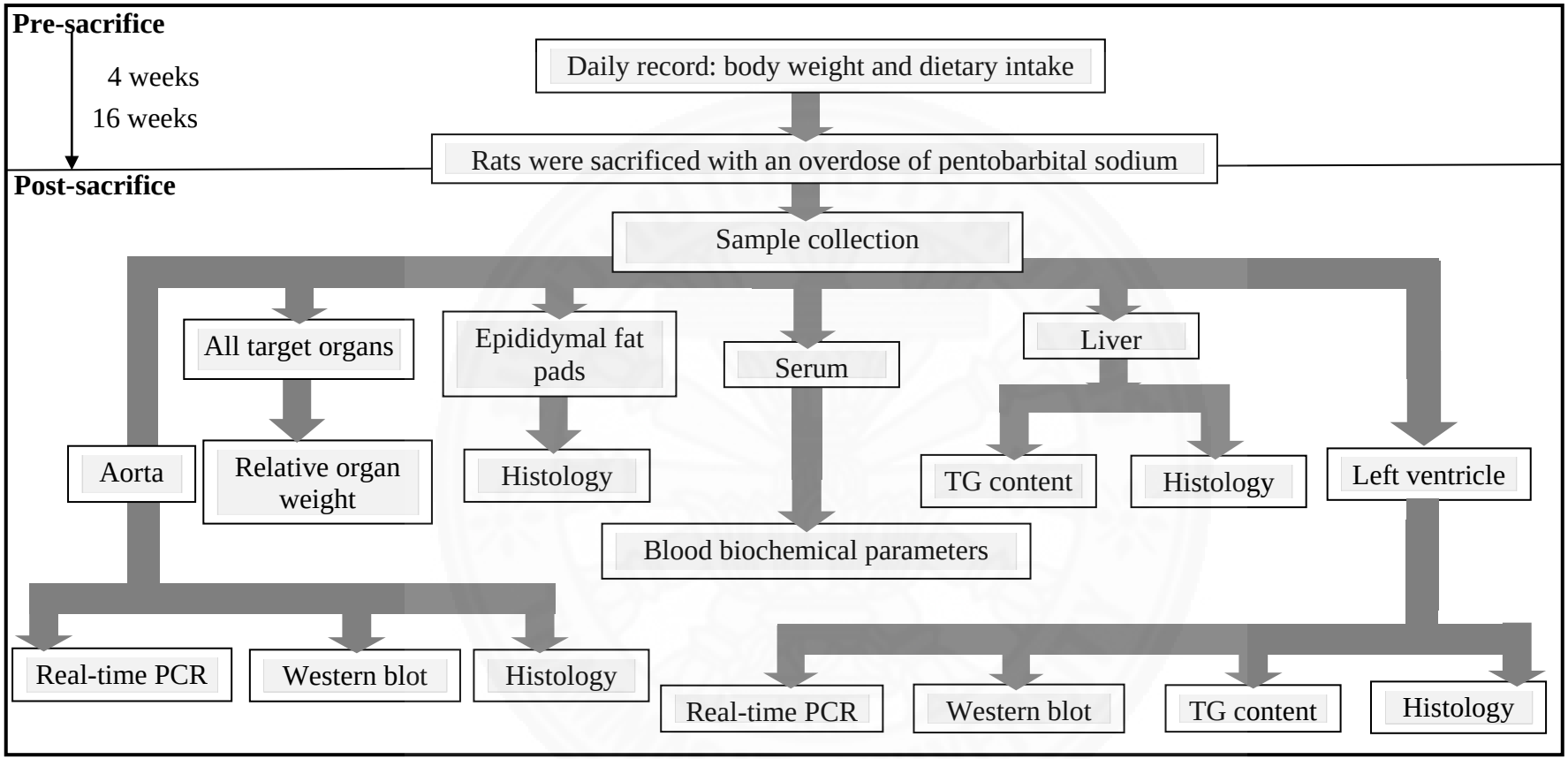

Figure 3.3 The collection of animal data and samples for each experiment.

3.5 Hematoxylin and Eosin (H and E) staining of tissue sections

Samples of epididymal adipose tissue, liver, left ventricle and thoracic aorta were obtained from rats. Tissues were fixed in 10% formalin, embedded in paraffin, cut into 5 µm sections, and fixed on slides. Tissue sections were subsequently stained with hematoxylin and eosin (H and E). The processes of H and E staining is shown in Figure 3.4. Images were detected under a light microscope coupled to a digital microscope camera. The average area of 100 adipocytes and 50 cardiomyocytes was measured for each rat (n = 3/group) by AxioVision software (Carl Zeiss, Germany). The average area of the aortic wall was also measured for each rat (n = 3/group) by this software and calculated as follows: Area of the aortic wall = (Cross-sectional area of the whole aorta) – (Cross-sectional area of the lumen).

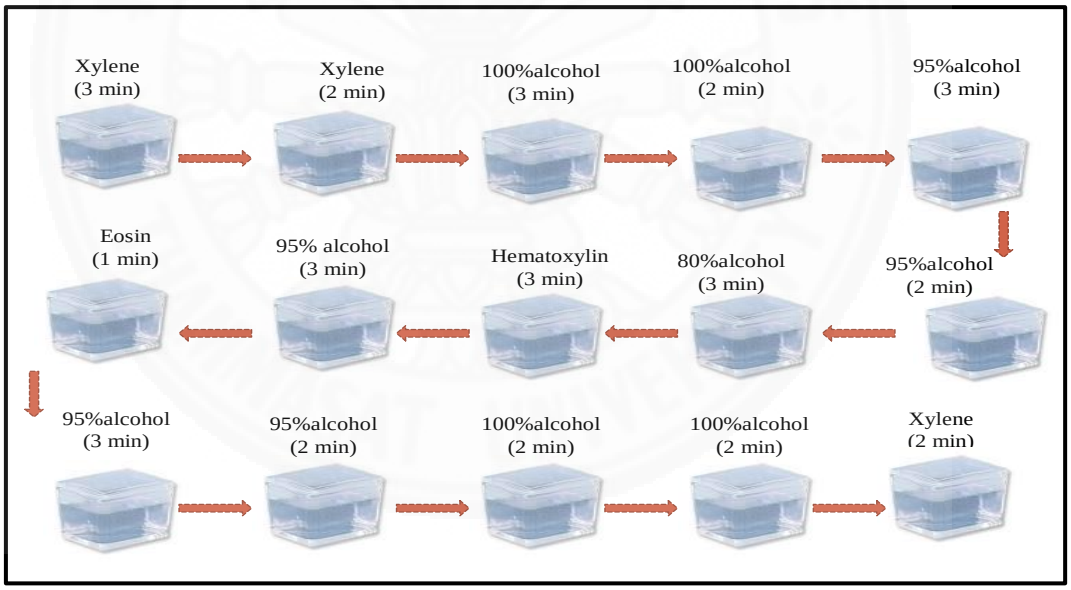

Figure 3.4 A flowchart for H and E staining.

3.6 Blood biochemical measurement

The methods and instruments for the determination of blood biochemical parameters are shown in Table 3.2.

Table 3.2

Summary of methods and instruments for determination of blood biochemical parameters

Blood biochemical parameters	Methods	Instruments
Glucose	Enzymatic oxidation of glucose	Glucosemeter
Lipid profile	Enzymatic-colorimetric method	Spectophotometer
Liver function test	Kinetic UV method	Automate (Modular P8000)
CK-MB	Microparticle enzyme immunoassay method	Automate (Axsym)
Total LDH	Multi-point kinetic methods	Automate (Integra 400)

3.6.1 Determination of serum lipid profile

3.6.1.1 Determination of total-C

The levels of cholesterol in serum were determined using the enzymatic colorimetric method described by Richmond et al. in 1992. Cholesterol is determined enzymatically using cholesterol esterase, cholesterol oxidase and peroxidase. The color intensity of quinieimine dye is directly proportional to cholesterol concentration and can be determined photometrically. The reactions are as follows:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{FA}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinieimine dye} + 4H_2O$$

A 10 µL of serum samples and cholesterol calibrator (200 mg/dL or 5.17 mmol/L) was added to a 1000 µL of cholesterol reagent (R1 reagent), mixed and subsequently incubated for 10 min at room temperature. Cholesterol reagent consisted of pipes buffer (90 mmol/L), phenol (26 mmol/L), cholesterol esterase (300 U/L), cholesterol oxidase (200 U/L), peroxidase (1,250 U/L) and 4-aminoantipyrine (0.4 mmol/L). Within 60 min read absorbance (A) of cholesterol calibrator and serum samples against reagent blank. The concentration of cholesterol was quantitated by spectrophotometer at the wavelength of 546 nm. The concentration of total-C is expressed as mg/dL. Cholesterol concentration was calculated according to the following equation:

$$\text{Cholesterol concentration} = \frac{\Delta A \text{ sample} \times \text{Calibrator concentration}}{\Delta A \text{ calibrator}}$$

3.6.1.2 Determination of HDL-C

The levels of HDL-C in serum were determined using the enzymatic colorimetric method. The chylomicrons, VLDL, and LDL-C are precipitated by phosphotungstic acid and magnesium chloride in precipitating reagent or HDL reagent. After centrifugation the supernatant contains the HDL-fraction, their cholesterol content is determined enzymatically. A 200 µL of serum samples was added to a 500 µL of working precipitating reagent (dilute 4 parts of precipitating reagent with 1 part of DW), mixed and subsequently incubated for 10 min at room temperature. The mixture was then centrifuged at 4,000 × *g* for 10 min at room temperature. After centrifugation, the mixture was separated the HDL-containing supernatant from the precipitated lipoprotein. A 100 µL of HDL supernatant was

mixed with 1,000 µL of cholesterol reagent. The mixture was incubated for 10 min at room temperature. Then, the mixture was detected at 546 nm in the assay using the spectrophotometer. The concentration of HDL-C is expressed as mg/dL.Cholesterol concentration in HDL was calculated according to the following equation:

$$\text{Cholesterol in HDL concentration} = \frac{\Delta A \text{ sample} \times \text{Calibrator concentration} \times 3.5}{\Delta A \text{ calibrator}}$$

3.6.1.3 Determination of TG

The levels of TG in serum were determined using the enzymatic colorimetric method described by Wahlefeld et al. in 1974. The TG is hydrolyzed by LPL to generate glycerol and FA. Glycerol is phosphorylated by glycerol kinase to produce the glycerol-3-phosphate (G-3-P) and adenosine diphosphate (ADP). The G-3-P is converted by G-3-P peroxidase to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). The H_2O_2 is then reacted with 4-aminophenazone and 4-chlorophenol under the catalytic reaction of oxidase to give a red quinoneimine dye (Trinder endpoint reaction). This red dye stuff can be measured spectrophotometrically at 546 nm. The reactions are as follows:

$$TG + 3H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + FA$$

$$\text{Glycerol} + ATP \xrightarrow{\text{Glycerol kinase}} \text{G-3-P} + ADP$$

$$\text{G-3-P} + O_2 \xrightarrow{\text{G-3-P oxidase}} DAP + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{4-(ρ-benzoguinone-monoimino)-phenazone} + 2H_2O + HCL$$

A 10 µL of serum samples and TG calibrator (200 mg/dL or 2.28 mmol/L) was added to a 1000 µL of TG reagent (R1 reagent), mixed and subsequently incubated for 10 min at room temperature. TG reagent consisted of

pipes buffer (50 mmol/L), ρ-chlorophenole (2 mmol/L), lipoprotein lipase (150,000 U/L), glycerol kinase (800 U/L), G-3-P oxidase (4,000 U/L), peroxidase (440 U/L), 4-aminoantipyrine (0.7 mmol/L), ATP (0.30 mmol/L, magnesium chloride (40 mmol/L), Na-cholat (0.20 mmol/L) and potassium-hexacyanoferrat (II) (1µmol/L). Then read absorbance of serum samples and TG calibrator against reagent blank within 60 min after start. The concentration of TG was quantitated by spectrophotometer at the wavelength of 546 nm. The concentration of TG is expressed as mg/dL. TG concentration was calculated according to the following equation:

$$\text{TG concentration} = \frac{\Delta A \text{ sample} \times \text{Calibrator concentration}}{\Delta A \text{ calibrator}}$$

3.6.1.4 Determination of LDL-C

The concentration of LDL-C was expressed as mg/dL. The serum low-density lipoprotein-cholesterol (LDL-C) level was determined using the Friedewald equation (Friedewald, Levy & Fredrickson, 1972), represented as follows:

$$\text{LDL-C} = (\text{Total-C}) - (\text{HDL-C}) - (\text{Triglyceride}/5)$$

3.6.1.5 Determination of atherogenic index and coronary risk index

Atherogenic index (Abbott, Wilson, Kannel, & Castelli, 1988) and coronary risk index (Alladi & Shanmugasundaram, 1989) were calculated according to the following equations (Haglund's method):

$$\text{Atherogenic index} = \frac{\text{LDL-C (mg/dL)}}{\text{HDL-C (mg/dL)}}$$

$$\text{Coronary risk index} = \frac{\text{Total-C (mg/dL)}}{\text{HDL-C (mg/dL)}}$$

3.6.2 Determination of fasting blood glucose (FBG) and oral glucose tolerance test (OGTT)

The levels of glucose in blood were determined with a glucometer. Rats were fasted overnight (16 h), weighed and then clipped the tip tail for glycemic determinations. Blood samples were collected immediately prior to a glucose solution administration (at 0 min) for measurement of fasting glucose concentrations and at 30, 60, 90, 120 and 150 min after receiving of oral glucose loading (2 g/kg body weight) for the assay of OGTT. The glucose dehydrogenase enzyme in test strip, in the presence of the coenzyme, converts glucose in the blood sample to gluconolactone. A series of half-hourly blood glucose of each rats are analyzed and glucose concentrations are plotted against the time of blood withdrawals. Area under the curve of blood glucose levels (AUC-G) was calculated using trapezoidal rule. This method was used for determination of glucose concentrations and glucose tolerance as previously described with minor modification (Wolever, Jenkins, Jenkins, & Josse, 1991).

3.6.3 Determination of liver function test and cardiac markers

Serum alanine transaminase (ALT), aspatate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were measured by automate instrument. Serum liver function test, CK-MB and LDH were determined by kinetic UV method, microparticle enzyme immunoassay and multi-point kinetic method, respectively.

3.7 Determination of hepatic and cardiac TG contents

Liver and left ventricular TG levels were quantified according to the method reported by Salmon and Flatt (1985) with some modifications**.** A total of 300 mg of tissue were added to 500 µL of ethanolic KOH (2 parts of 90% ethanol and 1 part of 30% KOH) and incubated overnight at 55 °C (no oil layer visible). The mixture was adjusted to a final volume of 1 mL with 50% ethanol and centrifuged at 14,000 rpm (22,780 × *g*) for 5 min. Eight hundred microliters of supernatant were added to 400 µL of 50% ethanol and vortex-mixed. Then 200 µL of mixture was transferred to a new microcentrifuge tube containing 215 µL of 1M $MgCl_2$, and tubes

were left on ice for 10 min followed by centrifugation for 5 min at 14,000 rpm. Then 10 µL of supernatant, glycerol standard (2.5 mg/mL) or DW (blank) was mixed with 800 µL of free glycerol reagent and incubated at 37 °C for 5 min. The absorbance was assayed spectrophotometrically at 540 nm.

Glycerol contents in the supernatant were evaluated using enzyme reactions. Glycerol is phosphorylated by ATP to glycerol-1-phosphate (G-1-P) and ADP in the reaction catalyzed by glycerol kinase. G-1-P was then oxidized by glycerol phosphate oxidase to DAP and H_2O_2. Peroxidase catalyzed the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine (ESPA) to produce a quinoneimine dye that showed an absorbance maximum at 540 nm. The TG levels were normalized to the weight of the tissue and expressed as mg/g tissue. The reaction steps were shown as scheme below. The increase in absorbance at 540 nm was directly proportional to the free glycerol concentration of the sample.

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{G-1-P} + \text{ADP}$$

$$\text{G-l-P} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{ESPA} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + H_2O$$

The content of glycerol was calculated using the formula:

$$\text{Glycerol content} = \frac{\Delta A \text{ sample} \times \text{Standard concentration}}{\Delta A \text{ calibrator}}$$

3.8 Determination of target mRNA expression

The differential displays of cardiac mRNA for PPARα, FAT/CD 36, NF-κB and GLUT-4 and aortic mRNA for eNOS and NF-κB were measured by reverse transcriptase-polymerase chain reaction (RT-PCR)/quantitative PCR combined technique.

3.8.1 Total RNA isolation and purification

Total RNA was extracted from rat left ventricles and thoracic aortas using guanidinium thiocyanate-phenol-chloroform method according to the manufacturer's recommendations. Left ventricles and thoracic aortas were washed with cold PBS prior to use for isolation of total RNA. The tissue samples were grinded by sterile pestle and mortar on liquid nitrogen. A 1 mL of TRIzol reagent (TRIzol reagent consisted of phenol, guanidinium thiocyanate and other components) was added to grinded tissue. The samples were homogenized and subsequently incubated at room temperature for 5 min. The homogenates were mixed with a 200 μL of chloroform, shaken vigorously for 15 s and then incubated at room temperature for 3 min. Then, the mixture was centrifuged at 12,000 × *g* for 15 min at 4 °C. This centrifuged mixture were separated into three phase. The colorless upper aqueous phase was collected and placed to a 500 μL of 2-propanol (isopropanol). The mixture was incubated at room temperature for 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. The supernatant was removed from the tube. The RNA pellet was washed with a 1 mL of 75% ethanol, mixed and then centrifuged at 7,500 × *g* for 5 min at 4 °C. The RNA pellet was dried at room temperature for 10-15 min. Dried RNA was dissolved with DEPC-treated water and incubated at 55 °C for 10 min. Total RNA concentrations were determined using the NanoDrop 2000 spectrophotometer by determining the absorbance values at 260 nm and 280 nm (A260/A280 ratio) where a ratio of 1.8-2.0 was considered pure. RNA solutions were stored at −80 °C until required for reverse transcription (RT). A flowchart summarizing the basic steps of RNA extraction is presented in Figure 3.5.

3.8.2 Complementary deoxyribonucleaic acid (cDNA) synthesis

Total RNA was reverse transcribed to synthesize single-stranded cDNA using the High Capacity cDNA RT Kits. A 10 μL of total RNA (20 ng/μL) was converted to cDNA with reverse transcriptase in a 10 μL volume of RT reaction mixture containing RT buffer RT buffer, RT random primers, deoxyribonucleotide triphosphates (dNTPs) mix, RNase inhibitor and DEPC-treated water. The reaction components for cDNA synthesis are shown in Table 3.3. The MyCycler thermal cycler conditions were as follows: an initial set up cycle at 25 °C for 10 min and 37 °C for 120 min, followed by cycles at 85 °C for 5 min, and 4 °C for infinity (Table 3.4). Each cDNA solution was stored at −80 °C.

3.8.3 Quantitative real-time PCR

cDNA was quantitatively amplified by StepOne Real-Time PCR System using the TaqMan Gene Expression Master Mix [TaqMan Gene Expression Master Mix contains: AmpliTaq Gold DNA Polymerase, Ultra Pure, Uracil-DNA glycosylase (UDG), dNTPs with deoxyuridine triphosphate (dUTP), ROX Passive Reference and Optimized buffer components] and TaqMan Gene Expression Assay. Each reaction contained 4 μL of cDNA solution (40 ng), 10 μL of TaqMan Gene Expression Master Mix, 1.5 μL of TaqMan Gene Expression Assay and 4.5 of μL DEPC-treated water in a final volume of 20 μL (Table 3.5). This mixture was transferred into each well of a 96-well plate reaction. Plate was loaded into StepOne Real-Time PCR System. All the samples were done in duplicates. Cycling conditions were as follows: an initial set up cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. The cycling program used is presented in Table 3.6.

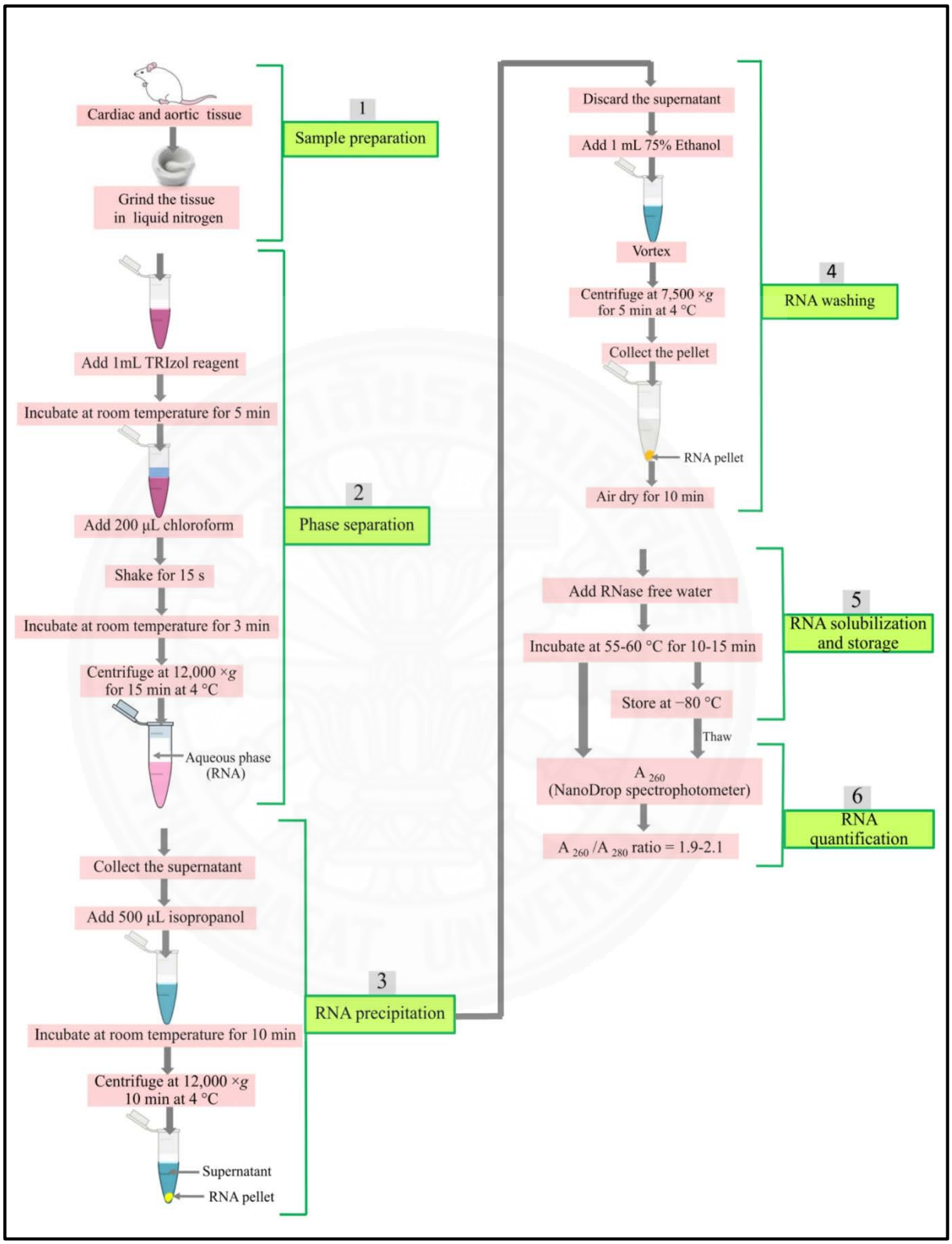

Figure 3.5 A flowchart of RNA isolation and purification.

Table 3.3

Reaction components for cDNA synthesis

Reaction components	Volume/reaction (μL)
	Kit with RNase inhibitor
2× RT master mix:	
1) DEPC-treated water	3.2
2) 10× RT buffer	2
3) 10× RT random pimers	2
4) 25× dNTP Mix (100mM)	0.8
5) RNase Inhibitor	1
6) MultiScribe Reverse Transcriptase	1
Total per reaction	10
cDNA RT reaction:	
1) 2× RT master mix	10
2) RNA sample	10
Total per reaction	20

Table 3.4

Incubating protocol for cDNA synthesis

	Step 1 (primer extension)	Step 2 (cDNA synthesis)	Step 3 (reaction termination)	Step 4 (hold)
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Table 3.5

Reaction components for real-time PCR

Reaction components	Volume/reaction
1) TaqMan Gene Expression Master Mix	10 µL
2) TaqMan Gene Expression Assays (target genes or reference gene)	1.5 µL
3) DEPC-treated water	4.5 µL
4) cDNA	4 µL
Reaction volume/final volume	20 µL
Total amount of cDNA	40 ng

Table 3.6

Cycling protocol for real-time PCR

Step	UDG incubation	AmpliTaq Gold, UP enzyme activation	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/extend
Temperature (°C)	50	95	95	60
Time	2 min	10 min	15 s	1 min

Relative mRNA levels were measured using the system above via TaqMan analysis that employed gene-specific primers and probes [FAM dye-labeled minor groove binder (MGB) probe or TaqMan MGB probe]. TaqMan MGB probes composed of reporter dye (FAM dye) and nonfluorescent quencher (NFQ)-linked MGB at 5´and 3´ end, respectively. The quencher dye has a suppressible activity to fluorescence in the reporter. During PCR, the reporter dye was separated from the quencher dye by 5´ exonuclease activity of the DNA polymerase. The releasing of the reporter dye results in elevated fluorescence. This elevation was detected for

determination of real-time PCR products (Appendix B). The real-time PCR results were quantified base on the number of cycles required for amplification-produced fluorescence to reach a specific threshold of detection (the threshold cycle or C_T value). The relative mRNA expression levels of each gene were analyzed by the comparative $2^{-\Delta\Delta CT}$ method. The expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used for normalization. The following steps were used to calculate relative quantitation (RQ) value:

Step 1: calculate the ΔC_T

$$\Delta C_{T(\text{test/calibrator sample})} = C_{T(\text{target gene, test/calibrator sample})} - C_{T(\text{reference gene, test/calibrator sample})}$$

Step 2: calculate the $\Delta\Delta C_T$

$$\Delta\Delta C_{T(\text{test/calibrator sample})} = \Delta C_{T(\text{test/calibrator sample})} - \Delta C_{T(\text{calibrator sample})}$$

Step 3: calculate the RQ value

$$RQ = 2^{-\Delta\Delta CT}$$

3.9 Determination of target protein expression

The differential displays of cardiac proteins for PPARα, FAT/CD 36, GLUT-4 and NF-κB and aortic proteins for eNOS and NF-κB were measured by Western blot analysis (immunoblot analysis).

3.9.1 Total protein isolation

Rat left ventriculars and thoracic aortic segments were washed in cold PBS. The tissue samples were subsequently grinded in 1 mL ice-cold cell lysis buffer containing 1 mM PMSF and proteinase inhibitor (Appendix C) by pestle and mortar on liquid nitrogen. The grinded tissue samples were then homogenized by homogenizer in a cold condition (4 °C). Homogenates were incubated on ice for 5 min, followed by sonication. Homogenates were centrifuged at 14,000 × *g* for 10 min at 4 °C. A portion of cleared supernatant was used for the determination of total protein concentrations using Bradford protein assay kit. Briefly, a 20 µL of unknown sample solution and bovine serum albumin (BSA) standard with a known concentration (125, 250, 500, 750 and 1,000 µg/mL) were mixed with a 1 mL of dye

reagent (dye reagent composed of Coomassie Brilliant Blue-G-250 dye and other components). This mixture was incubated at room temperature for at least 5 min and detected at 595 nm in the assay using a spectrophotometer. The protein concentrations of unknown sample were determined by using the standard curve of BSA standard. Protein solutions were stored at −80 °C until required for western blot analysis. A flowchart of protein extraction protocol is shown in Figure 3.6.

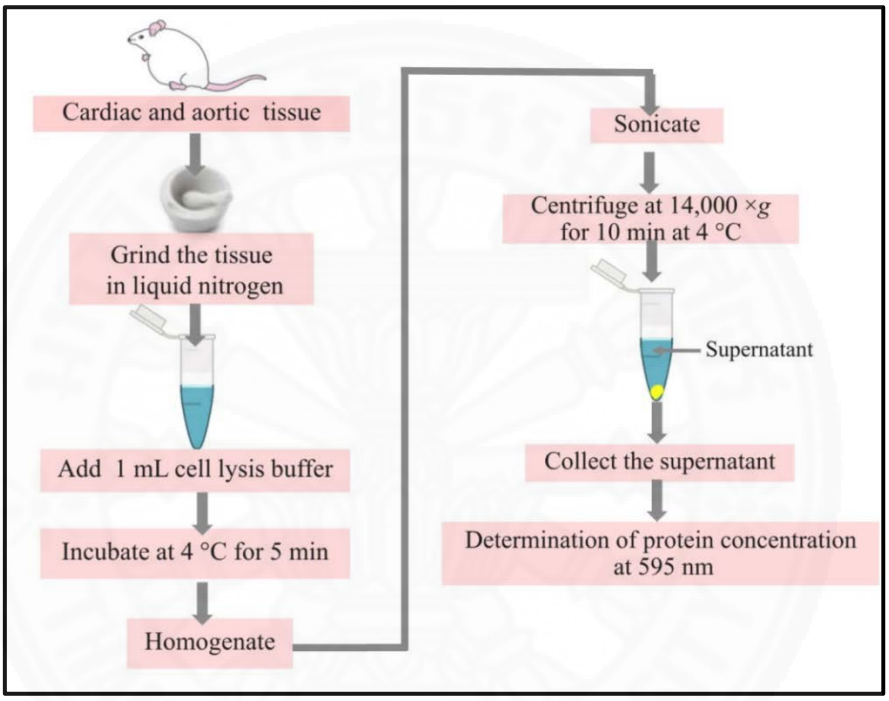

Figure 3.6 A flowchart of protein isolation.

3.9.2 Western blot analysis

Three parts of protein samples were mixed with one part of 4X loading buffer (Appendix D). After that the mixture was denatured in boiling water for 10 min and then rapidly placed in a cold condition. In an electrophoretic separation, equal amounts of protein samples were then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was run at 100 volts for 120 min using running buffer and gel electrophoresis apparatus (Appendix E and F). ColorPlus Pretianed Protein Ladder, Broad Range (10-230 kDa), was used as standard molecular weight (MW) of protein ladder for the comparison to the apparent protein ladder of test samples. pack and transferred The separated proteins-containing gel was

placed in electrophoretic blotting apparatus along with ice pack and electroblotted to nitrocellulose membrane at 250 milliampere for 180 min using Towbin transfer buffer (Appendix G). Following electrophoretic transfer, nitrocellulose membranes were blocked by a mixture of Odyssey blocking buffer and Tris-buffered saline (TBS) (1:1) at room temperature for 1 h. Electroblotted membranes were incubated and shaken with specific primary antibody against the target proteins including anti-PPARα antibody (1:800), anti-FAT/CD36 antibody (1:700), anti-GLUT-4 antibody (1:500), anti-p65 NF-κB antibody (1:500), anti-eNOS antibody (1:500) and anti-β-actin (1:1,000) overnight at 4 °C. The membranes were washed thrice with TBS containing 0.1% Tween-20 (TBST) (Appendix H), incubated with secondary antibody (Dylight 680 conjugated) (1:10,000) for 1 h in the dark at room temperature. After washing, protein bands were visualized by near-infrared (NIR) fluorescence detection. The densities of protein bands were determined using the Odyssey Fc Imaging System. β-actin was used as a reference protein, and its levels were used to normalize the expression levels of target proteins. The relative protein expression value (fold of control) was calculated according to the following steps:

Step 1: calculate the normalized value

$$\text{Normalized value}_{(\text{test/calibrator sample})} = \frac{\text{Density of target protein band}_{(\text{test/calibrator sample})}}{\text{Density of reference protein band}_{(\text{test/calibrator sample})}}$$

Step 1: calculate the relative protein expression value

$$\text{Relative protein expression value}_{(\text{test/calibrator sample})} = \frac{\text{Normalized value}_{(\text{test/calibrator sample})}}{\text{Normalized value}_{(\text{calibrator sample})}}$$

3.10 Statistical analysis

The results were statistical analyzed using Statistics Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc., USA) and reported as means ± standard error of the means (S.E.M.). Untreated HF group was served as a reference group for comparison with the other groups. The unpaired 2-tailed Student's *t* test was used to assess significance difference between two groups. For multiple group comparisons, data were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference's (LSD) post hoc test. Statistically, the significant difference was considered as $p < 0.05$.

CHAPTER 4
RESULTS

4.1 Characterization of RBE

The yield of crude extract was 18.33 ± 0.24%. The contents of total phenolic compounds and γ-oryzanols were 4.59 ± 0.25 mg GAE/g of RBE and 4.59 ± 0.14 μg/g of RBE, respectively (Table 4.1A and Figure 4.1). The EC50 of the extract in scavenging DPPH radical was 416.82 ± 11.48 μg/mL as compared to that of BHT with 12.71 ± 1.31 μg/mL (Table 4.1B).

Table 4.1

Phytochemical compositions and antioxidant capacity of RBE

	Concentrations or EC50
A. Phytochemical composition	
Total phenolic content	4.59 ± 0.25 mg GAE/g of RBE
γ-Oryzanol	4.59 ± 0.14 μg/g of RBE
α-Tocopherol	N.D.
B. DPPH radical-scavenging activity	
RBE	416.82 ± 11.48 μg/mL
BHT	12.71 ± 1.31 μg/mL

Abbreviation: N.D., non-detectable.

Values are expressed as mean ± S.E.M. (n = 3).

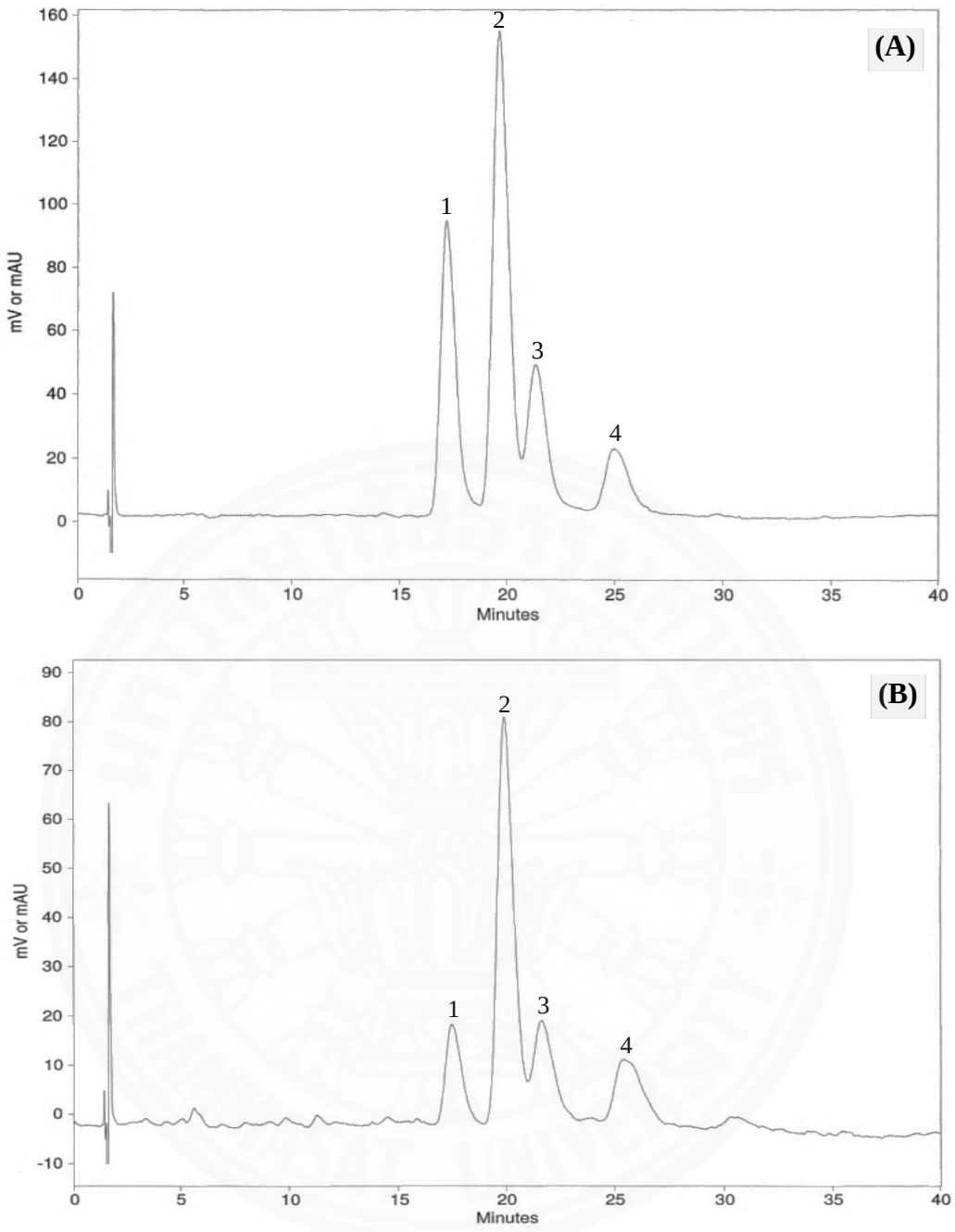

Figure 4.1 Typical chromatograms of standard γ-oryzanol (A) and RBE (B). Peaks: (1) cycloartenyl ferulate, (2) 24-methylene cycloartanyl ferulate, (3) campesteryl ferulate and (4) β-sitosteryl ferulate.

4.2 Effects of RBE on daily dietary intakes and body weights

4.2.1 Short-term experiment

Data of daily dietary intakes and body weights in all short-term groups are presented in Table 4.2A and 4.2B, respectively. The control animals consumed significantly more daily food intakes than the HF-fed animals. On the other hand, the energy intakes were significantly higher in all HF-fed groups than in the standard chow-fed group. Daily food and energy intakes did no change between untreated HF and treated HF groups. At the beginning of the experimental period, there were no differences between experimental groups for the mean body weights of rats. After 4 weeks, body weight and body weight gain of rats were significantly increased in HF group compared with the C group. In contrast, the final body weight and body weight gain of groups those receiving RBE and F were significantly lower than the group that was fed HF alone.

4.2.2 Intermediate-term experiment

Data of daily dietary intakes and body weights in all intermediate-term groups are shown in Table 4.3A and 4.3B, respectively. The C group consumed significantly more daily food intake than the HF group. However, the energy intakes were significantly higher in both HF and HF + RBE groups than in the C group. Food and energy intakes were not significantly different between the HF and HF + RBE groups. The initial body weight in rats before treatment did not significantly differ among the experimental groups. After 16 weeks, final body weights and body weight gain were all significantly higher in the HF group than those in the C group, but the levels were not significantly different between the HF and RBE-administered groups.

4.3 Effects of RBE on relative organ weights

4.3.1 Short-term experiment

Relative organ weights of rats of the short-term groups are shown in Table 4.2C. After 4 weeks of treatment period, the relative weights of liver and abdominal fat and epididymal fat tissues were all significantly increased in the HF group as compared with the C group, whereas relative weights of pancreas and kidney of HF group was significantly decreased. An oral administration of RBE caused a significant decrease in these levels when compared to rats fed an HF alone. The same trends were observed for F treatment. However, an administration of F showed the increased liver weight when compared with the HF group. In this experiment, there were no significant differences in the relative heart weights among the groups.

4.3.2 Intermediate-term experiment

Compared to the C group, the relative weights of liver and abdominal fat and epididymal fat tissues were significantly increased, whereas the relative weight of kidney was reduced in the HF group (Table 4.3C). However, the relative weights of heart and pancreas were no differences between the C and HF groups. RBE treatment did not change these parameters in the HF + RBE group.

4.4 Effects of RBE on histology and histopathology of the epididymal fat pads, liver, left ventricle and thoracic aorta

4.4.1 Short-term experiment

According to H and E staining, the epididymal adipocyte size in the HF-fed group was markedly larger than in the C group (Table 4.2D and Figure 4.2). Conversely, the adipocyte size was smaller in the HF + RBE and HF + F groups than in the HF group.

Histological examination of the liver showed that intracytoplasmic vacuoles (Figure 4.3, black arrows) were observed in the hepatocytes of the HF group compared with the C group; however, intracytoplasmic vacuoles were decreased in HF + RBE group (blue arrows). No alterations were found in the livers of the rats fed with the F. The intracytoplasmic fats or lipid-laden cells were assumed as clear vacuoles in the hepatocytes.

H and E-stained left ventricles and thoracic aortas from all animal groups exhibited normal structure with no evidences of pathological lesions (Figure 4.4 and 4.5, respectively). In parallel, there were no significant differences in the cross-sectional area of cardiomyocytes and aortic wall between three groups (Table 4.2D). However, RBE-fed rats displayed a trend towards decreased area of left ventricular cardiomyocytes compared to the HF-fed rats.

4.4.2 Intermediate-term experiment

H and E-stained epididymal fat pads, the adipocytes size was greater in rats fed an HF alone than in standard chow-fed rats (Table 4.3D and Figure 4.2). This change was attenuated by RBE consumption.

Examination of H and E-stained slides from all HF-fed rats showed an increase in hepatic vacuoles compared to normal architecture of livers in control diet-fed rats (Figure 4.3, black and blue arrows).

Histological analysis of the left ventricles showed that cardiomyocytes cross-sectional area was significantly larger in the HF group than in the C group (Table 4.3D and Figure 4.4). There was a downward trend in cardiomyocytes cross-sectional area in the group fed an RBE compared to the HF group.

In the C and HF + RBE groups (all rats, n = 3), H and E-stained sections of aortas showed that the tunica intima was smooth (blue arrows), and all aortic layers remained intact (yellow arrows); in the HF group (1/3, n = 3), the tunica intima was rough (black arrow) and the elastic laminas were partly disturbed (red arrow) (Figure 4.6). However, there was no significant difference in the area of the aortic wall among the experimental groups (Table 4.3D).

4.5 Effects of RBE on hepatic and cardiac TG contents

4.5.1 Short-term experiment

After 4 weeks, HF promoted the elevation of hepatic TG levels in rats as compared to the normal rats (Table 4.2E). On the other hand, hepatic TG levels were found to be significantly decreased in both RBE- and F-treated groups when compared with the HF group. Left ventricular TG contents did not change significantly between experimental groups (Table 4.2E).

4.5.2 Intermediate-term experiment

Compared with the controls, HF-fed rats had significantly had significantly increased hepatic TG contents (Table 4.3E). Hepatic TG contents did not alter significantly between HF and HF + RBE groups. No differences were observed among the animal groups with respect to left ventricular TG contents after the experimental period (Table 4.3E).

Table 4.2

Daily dietary intakes, body weights, relative organ weights, cell areas, aortic wall areas and tissue lipid contents of the short-term experimental groups

Parameters	Short-term groups			
	C	HF	HF + RBE	HF + F
A. Dietary intake				
Food intake (g/day)	23.22 ± 0.08*	19.43 ± 0.33	19.05 ± 0.50	19.67 ± 0.16
Energy intake (kcal/day)	70.59 ± 0.24*	99.51 ± 1.73	97.57 ± 2.58	100.75 ± 0.80
B. BW				
Initial BW (g)	243.87 ± 2.09	243.59 ± 1.85	243.99 ± 1.59	243.54 ± 2.26
Final BW (g)	366.17 ± 0.94*	412.31 ± 2.81	368.56 ± 7.39*	392.54 ± 5.13*
BW gain (g)	123.45 ± 1.36*	169.53 ± 2.27	123.03 ± 5.47*	148.38 ± 4.63*
C. Relative organ weight				
Heart (% BW)	0.38 ± 0.00	0.38 ± 0.01	0.39 ± 0.01	0.39 ± 0.00
Liver (% BW)	3.27 ± 0.08*	3.59 ± 0.06	3.34 ± 0.09*	5.25 ± 0.08*
Pancreas (% BW)	0.56 ± 0.01*	0.44 ± 0.01	0.52 ± 0.02*	0.55 ± 0.02*
Kidney (% BW)	0.84 ± 0.01*	0.78 ± 0.01	0.81 ± 0.01*	0.82 ± 0.01*
Abdominal fat (% BW)	1.29 ± 0.07*	2.17 ± 0.08	1.48 ± 0.08*	1.39 ± 0.14*
Epididymal fat (%BW)	1.48 ± 0.04*	2.05 ± 0.05	1.69 ± 0.07*	1.48 ± 0.10*
D. Area of cells or tissue				
Adipocyte size (μm^2)	3216 ± 94*	4998 ± 7	3485 ± 59*	3332 ± 39*
Cardiomyocyte size (μm^2)	173.33 ± 25.11	194.94 ± 11.66	157.85 ± 15.83	180.56 ± 6.00
Aortic wall area (mm^2)	0.29 ± 0.04	0.28 ± 0.02	0.28 ± 0.01	0.29 ± 0.02
E. Tissue lipid contents				
Liver (mg/g)	12.56 ± 0.92*	32.59 ± 2.69	20.05 ± 1.87*	13.01 ± 1.87*
Left ventricle (mg/g)	4.31 ± 0.27	4.39 ± 0.25	4.55 ± 0.13	4.15 ± 0.15

Abbreviation: BW, body weight. Values are expressed as means ± S.E.M. (n = 8).

HF group was used as a reference group. *$p < 0.05$ versus HF group.

Table 4.3

Daily dietary intakes, body weights, relative organ weights, cell areas, aortic wall areas and tissue lipid contents of the intermediate-term experimental groups

Parameters	Intermediate-term groups		
	C	HF	HF + RBE
A. Dietary intake			
Food intake (g/day)	25.58 ± 0.51*	20.50 ± 0.41	19.57 ± 0.24
Energy intake (kcal/day)	78.25 ± 1.66*	105.00 ± 2.12	100.2 ± 1.22
B. BW			
Initial BW (g)	256.08 ± 2.27	256.52 ± 2.06	256.42 ± 2.09
Final BW (g)	522.65 ± 5.21*	544.48 ± 7.38	551.94 ± 7.42
BW gain (g)	265.61 ± 4.61*	289.01 ± 8.98	292.89 ± 6.16
C. Relative organ weight			
Heart (% BW)	0.29 ± 0.00	0.29 ± 0.00	0.29 ± 0.00
Liver (% BW)	2.91 ± 0.03*	3.17 ± 0.04	3.09 ± 0.04
Pancreas (% BW)	0.37 ± 0.01	0.37 ± 0.02	0.40 ± 0.01
Kidney (% BW)	0.70 ± 0.01*	0.63 ± 0.01	0.62 ± 0.01
Abdominal fat (% BW)	2.42 ± 0.19*	3.58 ± 0.17	3.78 ± 0.08
Epididymal fat (% BW)	1.98 ± 0.13*	2.50 ± 0.09	2.55 ± 0.10
D. Area of cells or tissue			
Adipocyte size (μm^2)	3486 ± 135*	5759 ± 48	5353 ± 37*
Cardiomyocyte size (μm^2)	175.68 ± 4.19*	229.14 ± 10.70	199.07 ± 9.61
Aortic wall area (mm^2)	0.39 ± 0.09	0.45 ± 0.02	0.41 ± 0.00
E. Tissue lipid contents			
Liver (mg/g)	17.39 ± 1.30*	44.38 ± 3.43	48.95 ± 4.24
Left ventricle (mg/g)	4.76 ± 0.40	4.50 ± 0.34	5.11 ± 0.39

Abbreviation: BW, body weight. Values are expressed as means ± S.E.M. (n = 8). HF group was used as a reference group. *$p < 0.05$ versus HF group.

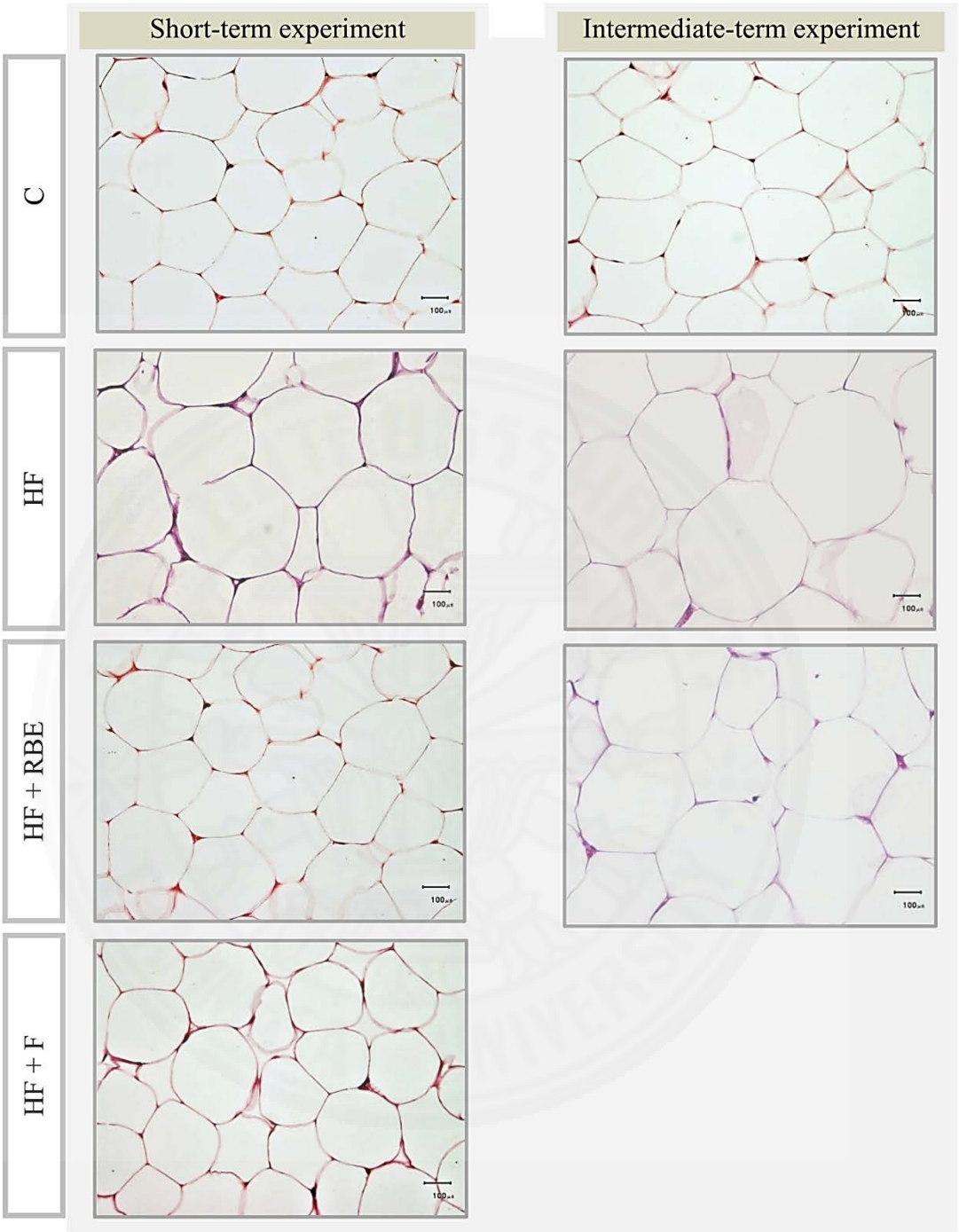

Figure 4.2 Representative microscopic photographs of epididymal fat pads stained with H and E of the short- and intermediate-term experimental groups (magnification ×400; scale bar = 100 μm).

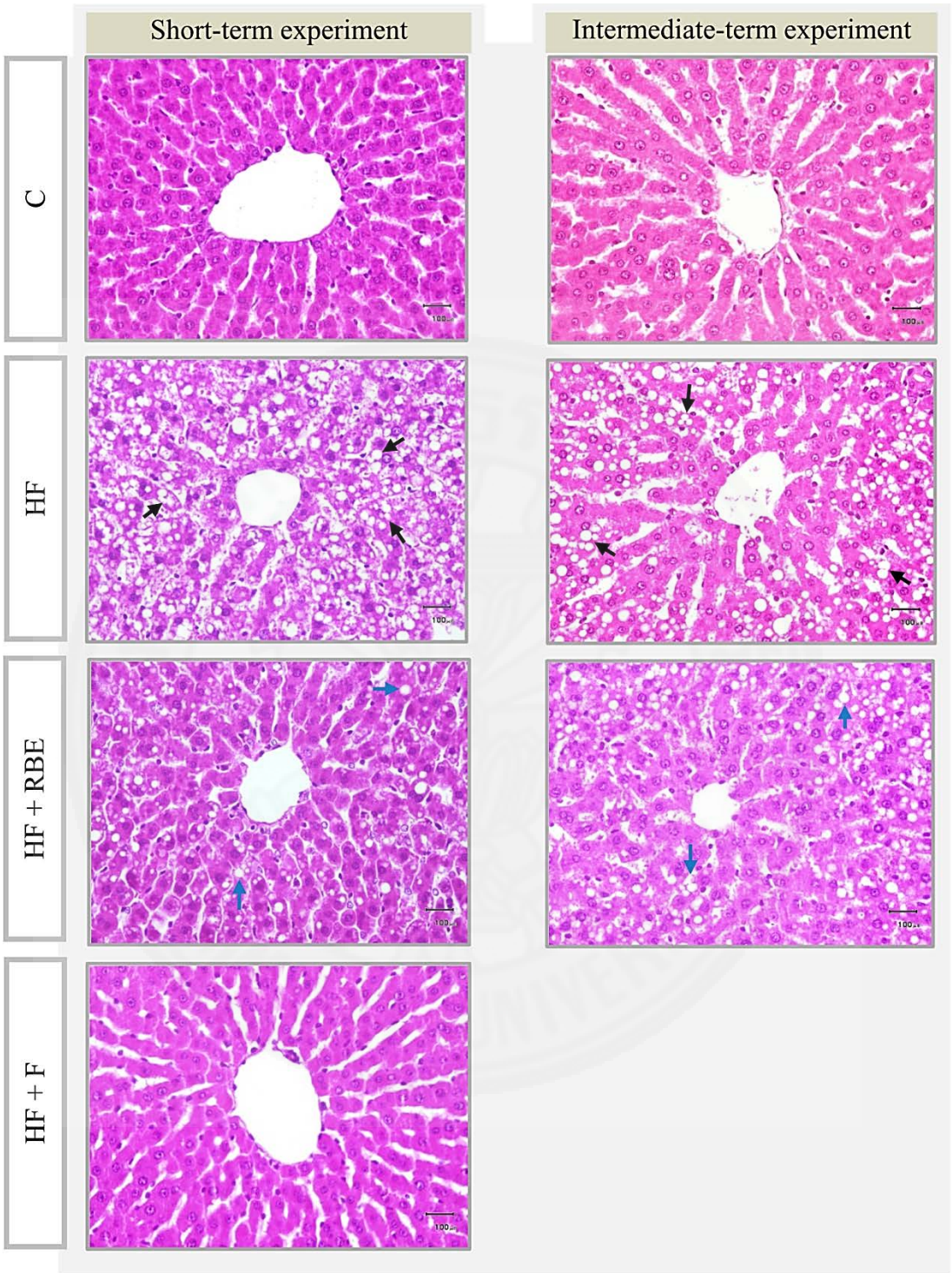

Figure 4.3 Representative microscopic photographs of livers stained with H and E of the short- and intermediate-term experimental groups (magnification ×400; scale bar = 100 μm).

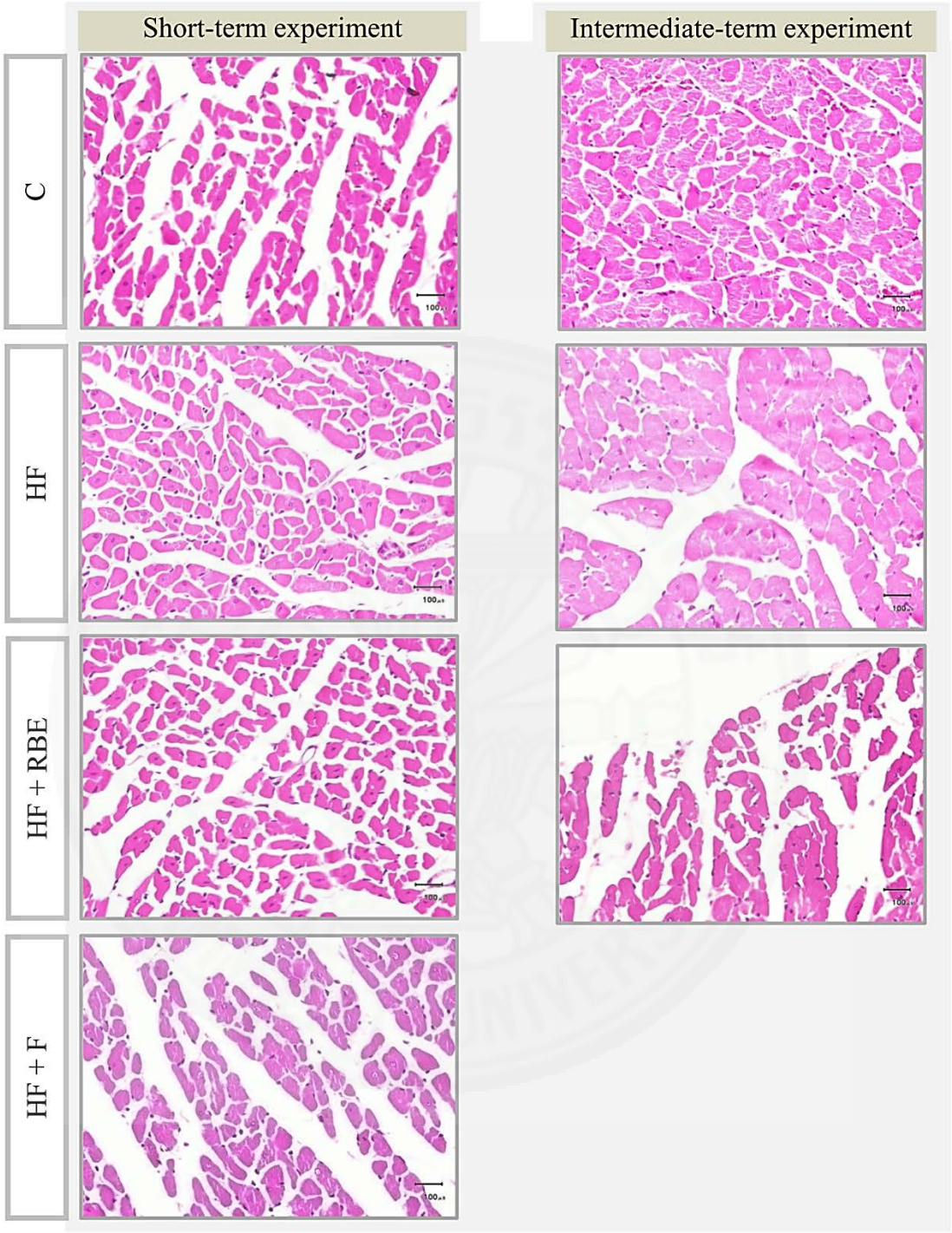

Figure 4.4 Representative microscopic photographs of left ventricles stained with H and E of the short- and intermediate-term experimental groups (magnification ×400; scale bar = 100 μm).

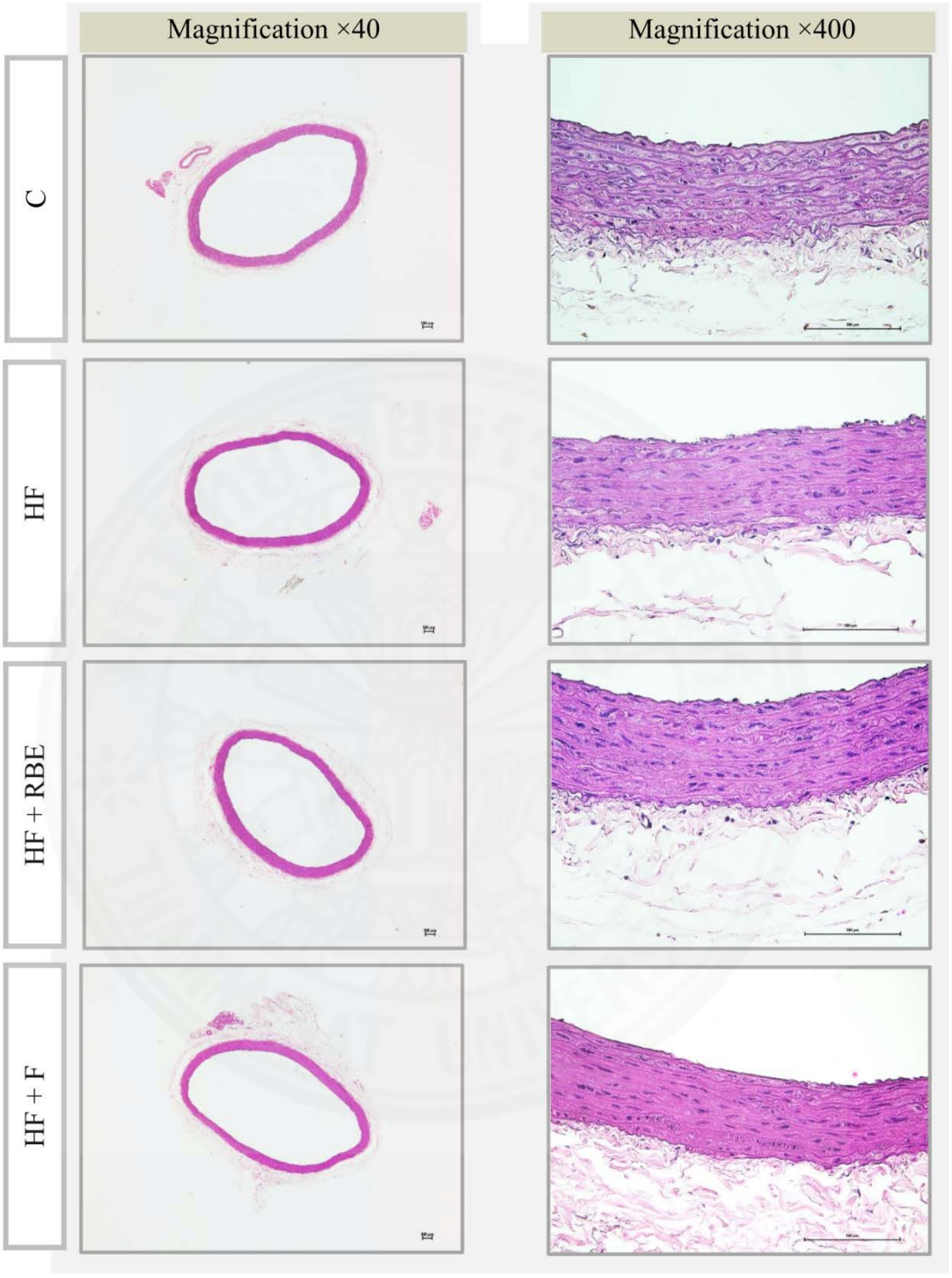

Figure 4.5 Representative microscopic photographs of aortas stained with H and E of the short-term experimental groups (left panel, magnification ×40; right panel, magnification ×400; scale bar = 100 μm).

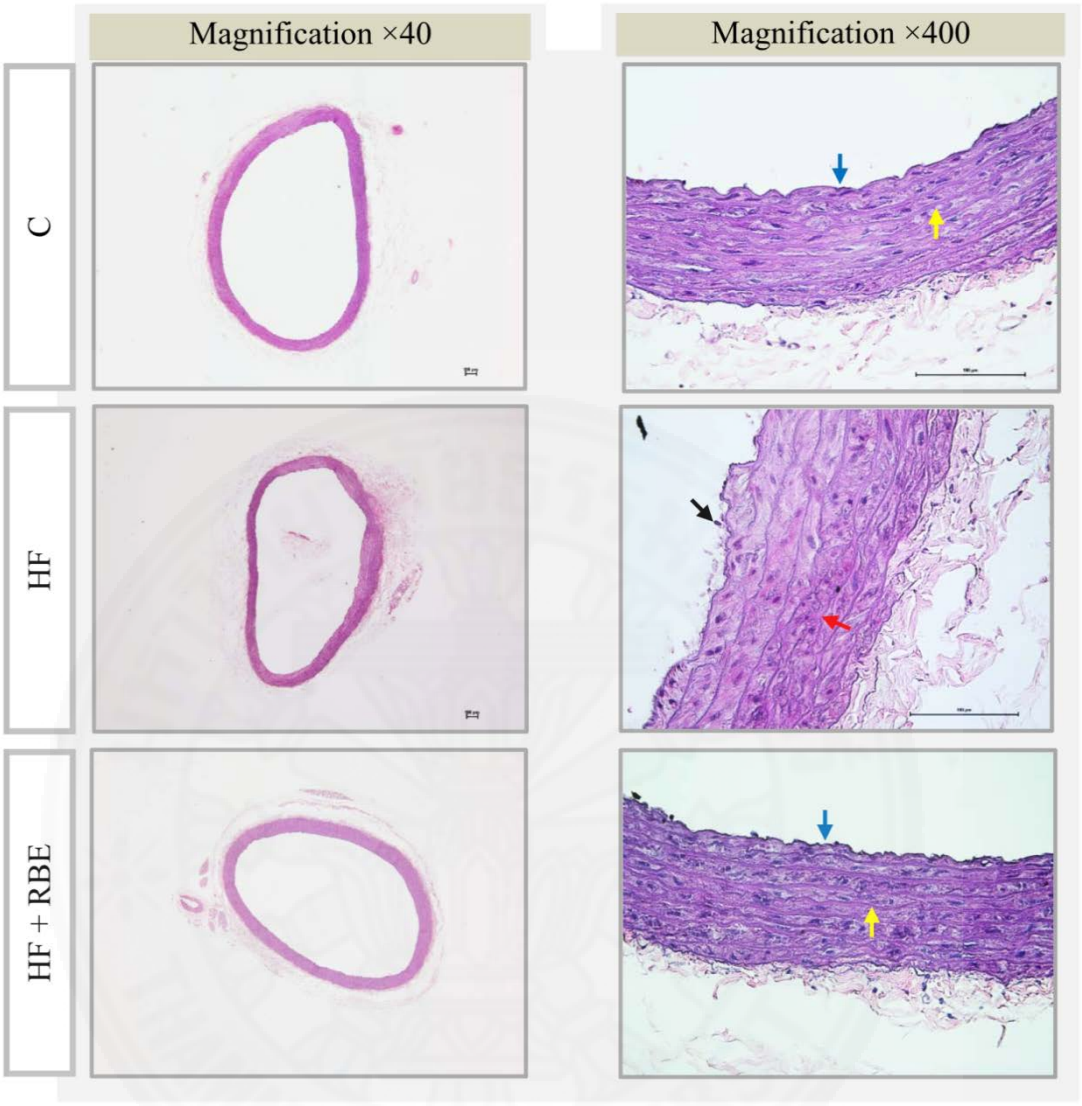

Figure 4.6 Representative microscopic photographs of aortas stained with H and E of the short-term experimental groups (left panel, magnification ×40; right panel, magnification ×400; scale bar = 100 μm).

4.6 Effects of RBE on serum lipid profile

4.6.1 Short-term experiment

Serum lipid profile is shown in Table 4.4A. Compared with rats fed a normal diet, the HF-fed rats had elevated mean serum values for LDL-C, atherogenic index and coronary risk index as well as decreased mean HDL-C level. Total-C and TG levels in serum also showed a tendency to increase in the HF group, but the differences were not statistically significant. In contrast, RBE-treated rats had significantly lower levels of serum total-C and exhibited a trend towards decreased LDL-C levels (19%) compared to HF-fed rats. The serum total-C and TG levels of F-treated rats were significantly lower than those of the HF-fed rats. However, other parameters of serum lipid profile, including HDL-C, atherogenic index and coronary risk index, were not significantly improved in all treatment groups.

4.6.2 Intermediate-term experiment

The increased levels of total-C, LDL-C, atherogenic index and coronary risk index and decreased levels of HDL-C were observed in the HF group (Table 4.5A). Although serum TG levels were not significantly increased in the HF group compared to the C group; but their levels were decreased in the HF + RBE group. In addition, RBE treatment tended to reduce the atherogenic index (18%) when compared with the HF group. However, there were no significant differences in total-C, LDL-C, HDL-C and coronary index between HF and HF + RBE groups.

4.7 Effects of RBE on glucose homeostatic parameters

4.7.1 Short-term experiment

The parameters of glucose homeostasis are shown in Table 4.4B. In the short-term study, FBG and AUC-G were higher than the C group when rats were fed with HF. Treatment of HF-fed rats with RBE significantly reduced FBG when compared with the HF-fed rats. However, there was no significant change between AUC-G in rats fed HF and RBE. F co-treatment significantly increased FBG as compared to HF group. F co-treatment also showed a tendency to decrease in AUC-G when compared to the HF group.

4.7.2 Intermediate-term experiment

As shown in Table 4.5B, there was no significant difference in FBG among the groups. However, AUC-G was significantly higher in HF-fed animals, but the high AUC-G was attributed to 14% decreases in RBE-treated animals.

4.8 Effects of RBE on liver functions test and cardiac markers

4.8.1 Short-term experiment

The serum AST and ALT levels in rats did not significantly differ among the experimental groups (Table 4.4C). However, serum ALT levels were higher than the HF group when rats were co-fed with F. Serum CK-MB and LDH levels were not statistically significant in all groups (Table 4.4D).

4.8.2 Intermediate-term experiment

Data relevant to liver functions test and cardiac markers are presented in Table 4.5C and 4.5D, respectively. Intermediate-term feeding of HF demonstrated the elevated serum AST and ALT levels as compared to the control animals. Intermediate-term treatment with RBE resulted in significant decreases in the AST and ALT levels compared to HF alone-fed rats. All experimental groups did not show a significant difference of serum cardiac markers.

Table 4.4

Blood biochemical parameters of the short-term experimental groups

Parameters	Short-term groups			
	C	HF	HF + RBE	HF + F
A. Lipid profile				
Total-C (mg/dL)	88.00 ± 2.32	95.39 ± 3.66	85.00 ± 2.81*	89.34 ± 2.13*
TG (mg/dL)	36.20 ± 3.13	41.70 ± 2.63	37.94 ± 2.37	35.66 ± 2.57*
LDL-C (mg/dL)	25.95 ± 2.62*	38.52 ± 3.62	31.25 ± 1.86	39.67 ± 2.39
HDL-C (mg/dL)	53.72 ± 1.07*	48.75 ± 1.63	45.18 ± 2.59	44.11 ± 0.53
Atherogenic index	0.48 ± 0.06*	0.78 ± 0.07	0.74 ± 0.09	0.92 ± 0.06
Coronary risk index	1.61 ± 0.06*	1.95 ± 0.07	1.92 ± 0.10	2.10 ± 0.06
B. Glucose homeostasis				
FBG (mg/dL)	104.88 ± 1.41*	115.38 ± 0.92	105.62 ± 1.49*	129.63 ± 1.51*
AUC-G (mg/dL.min)	2764 ± 174*	3285 ± 121	3198 ± 308	2921 ± 176
C. Liver function test				
AST (U/L)	137.75 ± 8.92	129.63 ± 7.55	118.88 ± 9.14	121.13 ± 4.97
ALT (U/L)	36.63 ± 1.85	34.63 ± 1.32	34.13 ± 1.71	41.00 ± 1.27*
D. Cardiac markers				
CK-MB (U/L)	< 0.1	< 0.1	< 0.1	< 0.1
LDH (mU/L)	2.63 ± 0.31	2.21 ± 2.22	2.17 ± 2.32	2.37 ± 1.85

Values are expressed as means ± S.E.M. (n = 8).

HF group was used as a reference group. * $p < 0.05$ versus HF group.

Table 4.5

Blood biochemical parameters of the intermediate-term experimental groups

Parameters	Intermediate-term groups		
	C	HF	HF + RBE
A. Lipid profile			
Total-C (mg/dL)	93.13 ± 2.97*	114.38 ± 4.83	117.13 ± 3.39
TG (mg/dL)	59.86 ± 2.53	55.27 ± 1.43	45.89 ± 2.69*
LDL-C (mg/dL)	20.31 ± 3.13*	55.71 ± 4.26	49.15 ± 2.33
HDL-C (mg/dL)	60.34 ± 2.27*	52.96 ± 1.71	55.47 ± 2.22
Atherogenic index	0.35 ± 0.06*	1.02 ± 0.10	0.89 ± 0.04
Coronary risk index	1.55 ± 0.07*	2.24 ± 0.11	2.09 ± 0.05
B. Glucose homeostasis			
FBG (mg/dL)	102.75 ± 1.81	104.00 ± 1.70	108.00 ± 1.97
AUC-G (mg/dL.min)	2903 ± 168*	4697 ± 430	4043 ± 379
C. Liver function test			
AST (U/L)	190.63 ± 5.74*	234.63 ± 9.33	172.85 ± 11.80*
ALT (U/L)	39.25 ± 1.85*	46.50 ± 2.39	36.50 ± 2.75*
D. Cardiac markers			
CK-MB (U/L)	< 0.1	< 0.1	< 0.1
LDH (mU/L)	4.76 ± 0.32	5.45 ± 4.27	4.89 ± 3.78

Values are expressed as means ± S.E.M. (n = 8).

HF group was used as a reference group. * $p < 0.05$ versus HF group.

4.9 Effects of RBE on left ventricular gene expression

4.9.1 PPARα gene expression

4.9.1.1 Short-term experiment

Figure 4.7A shows the effect of RBE on cardiac PPARα expression of the short-term experimental groups. PPARα mRNA and protein levels showed a tendency to decrease in the HF group compared with the C group, but the differences were not statistically significant. Compared with the HF group, HF + RBE group showed a significant increase in PPARα mRNA expression. Likewise, there was a trend toward an increase in PPARα protein expression in rats fed an RBE, but the difference was not significant. Treatment with F in the HF + F group significantly increased both PPARα mRNA and protein expressions as compared with the untreated HF group.

4.9.1.2 Intermediate-term experiment

As shown in Figure 4.7B, the expression levels of PPARα were lower in the HF group than in the C group. HF + RBE group tended to have higher PPARα mRNA and protein expression levels compared to the HF groups, but these levels were not significantly different between the HF and HF + RBE groups.

4.9.2 FAT/CD36 gene expression

4.9.2.1 Short-term experiment

The relative levels of FAT/CD36 transcript and protein were significantly up-regulated in the left ventricles of HF group when compared with the C group; however, the rats in both HF + RBE and HF + F groups exhibited a significant decrease in the FAT/CD36 expression levels (Figure 4.8A).

4.9.2.2 Intermediate-term experiment

As illustrated in Figure 4.8B, the levels of FAT/CD36 mRNA were significantly increased in the hearts from HF-fed rats as compared to the normal rats. Consistently, there was a trend toward an increase in FAT/CD36 protein levels in rats fed an HF alone, but the differences were not significant. However, these increases were reduced to basal levels by RBE treatment.

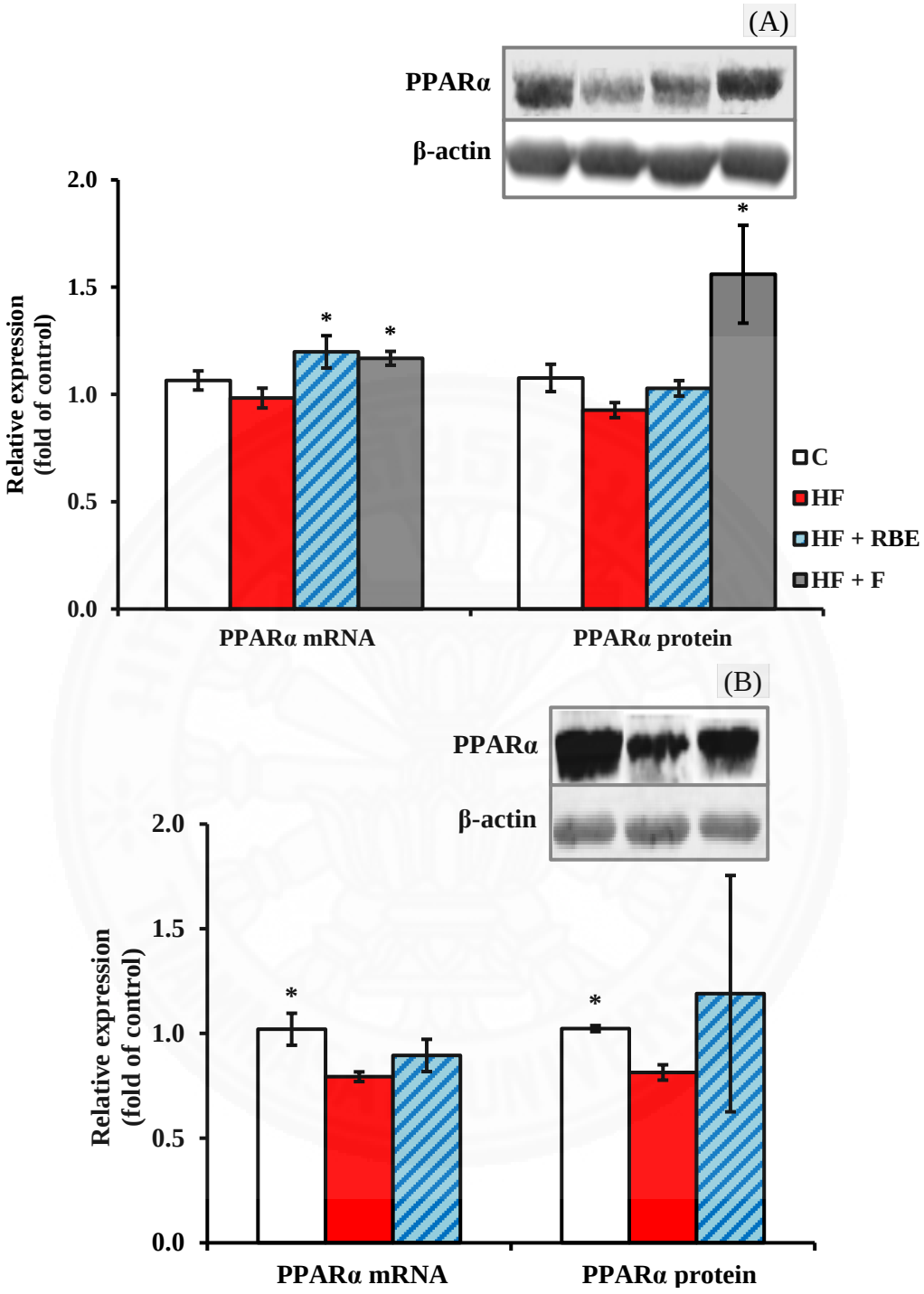

Figure 4.7 Left ventricular PPARα mRNA and protein expression levels of the (A) short- and (B) intermediate-term experimental groups. Values are expressed as means ± S.E.M. (n = 6 for mRNA expression and protein expression in the short-term experiment and n = 3 for protein expression in the intermediate-term experiment). HF group was used as a reference group. *$p < 0.05$ versus HF group.

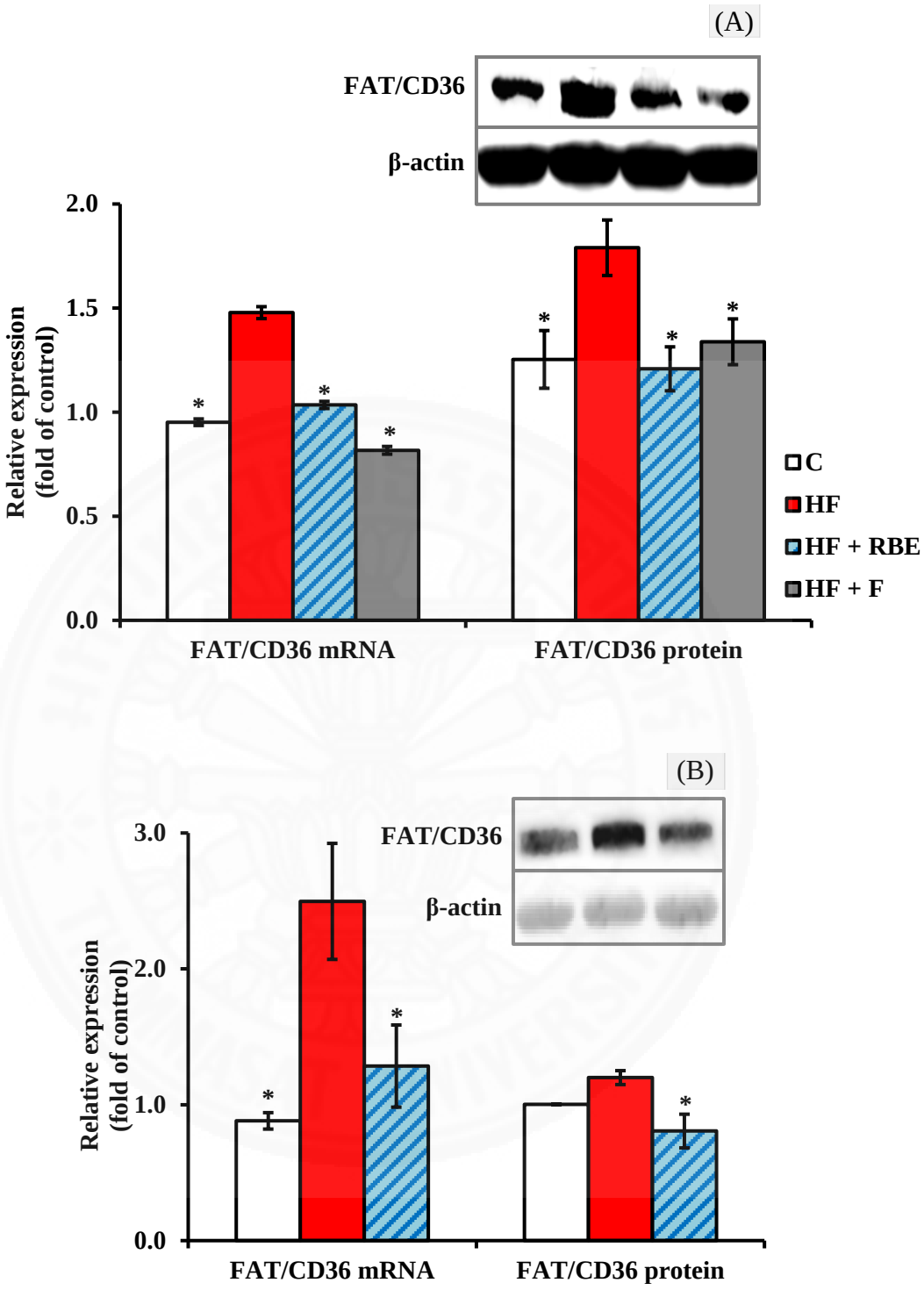

Figure 4.8 Left ventricular FAT/CD36 mRNA and protein expression levels of the (A) short- and (B) intermediate-term experimental groups. Values are expressed as means ± S.E.M. (n = 6 for mRNA expression and protein expression in the short-term experiment and n = 3 for protein expression in the intermediate-term experiment). HF group was used as a reference group. *$p < 0.05$ versus HF group.

4.9.3 GLUT-4 gene expression

4.9.3.1 Short-term term experiment

The effect of RBE on cardiac GLUT-4 gene expression for 4 weeks is presented in Figure 4.9A. Rats fed an HF alone showed a significant reduction in GLUT-4 mRNA and protein expression levels as compared to the control rats. Among the experimental groups, only the HF + RBE group showed a significant increase in the GLUT-4 protein levels. However, RBE treatment had no effect on the transcript levels of GLUT-4 in the HF + RBE group.

4.9.3.2 Intermediate-term experiment

No significant changes were found in the relative mRNA levels of GLUT-4 among the animal groups (Figure 4.9B). However, GLUT-4 protein levels of the HF-fed group were significantly lower than those of the C group. RBE-treated rats had significantly higher levels of GLUT-4 protein compared to the HF-fed rats.

4.9.4 NF-κB p65 gene expression

4.9.4.1 Short-term experiment

Although there were no significant differences in the NF-κB p65 expression levels of the hearts between the C and HF groups, administration of RBE showed the decreased expression levels of NF-κB p65 gene when compared to the HF group (Figure 4.10A). Lower protein levels of NF-κB p65 was also found in the HF + F group than those of the HF group.

4.9.4.2 Intermediate-term experiment

In the HF hearts, the mRNA expression levels of NF-κB p65 were significantly up-regulated compared with the C hearts, whereas its protein expression levels were similar between the HF and C groups (Figure 4.10B). After treatment with RBE, the mRNA levels of NF-κB p65 were lower in HF + RBE group than in the HF group.

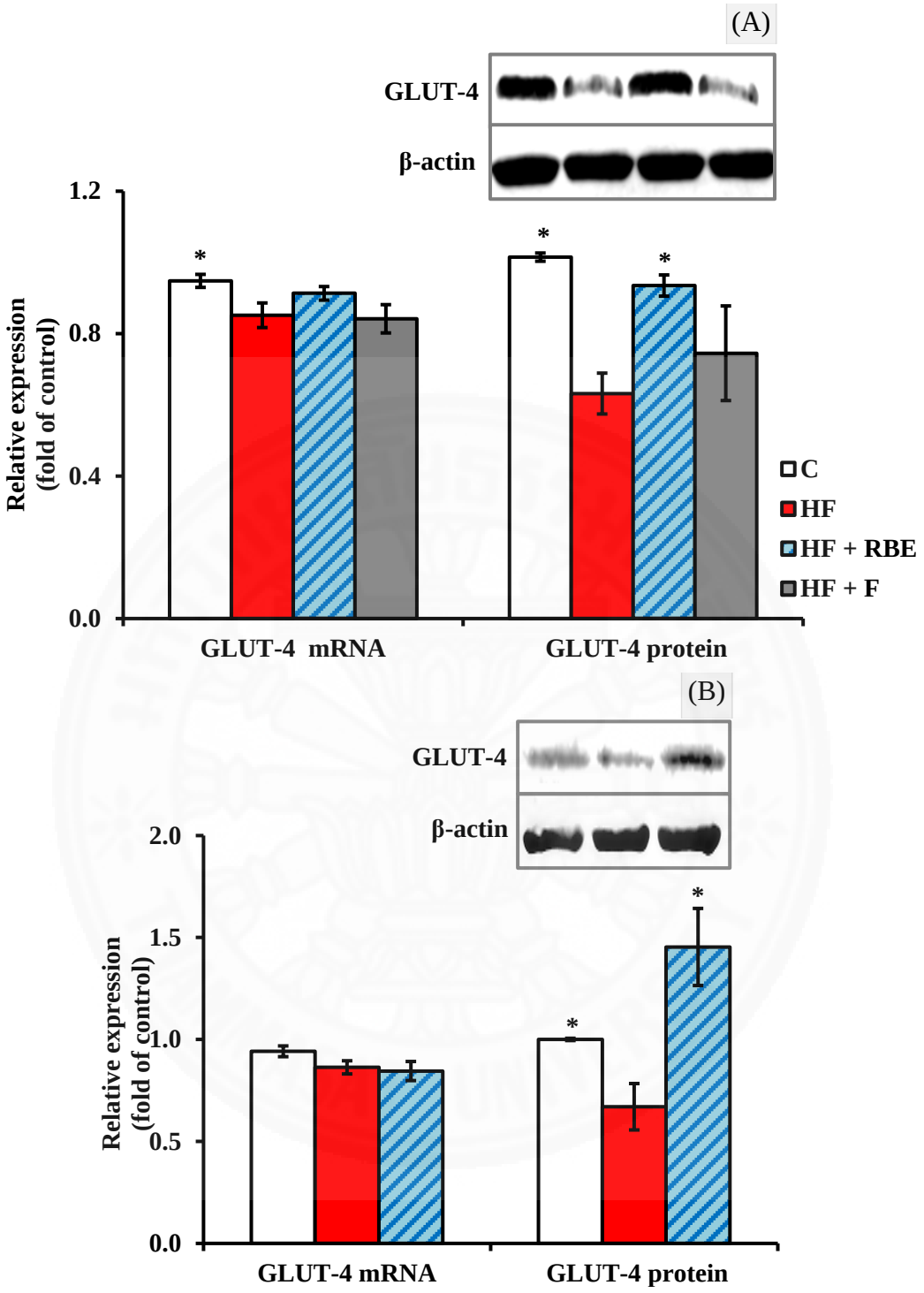

Figure 4.9 Left ventricular GLUT-4 mRNA and protein expression levels of the (A) short- and (B) intermediate-term experimental groups. Values are expressed as means ± S.E.M. (n = 6 for mRNA expression and protein expression in the short-term experiment and n = 3 for protein expression in the intermediate-term experiment). HF group was used as a reference group. *$p < 0.05$ versus HF group.

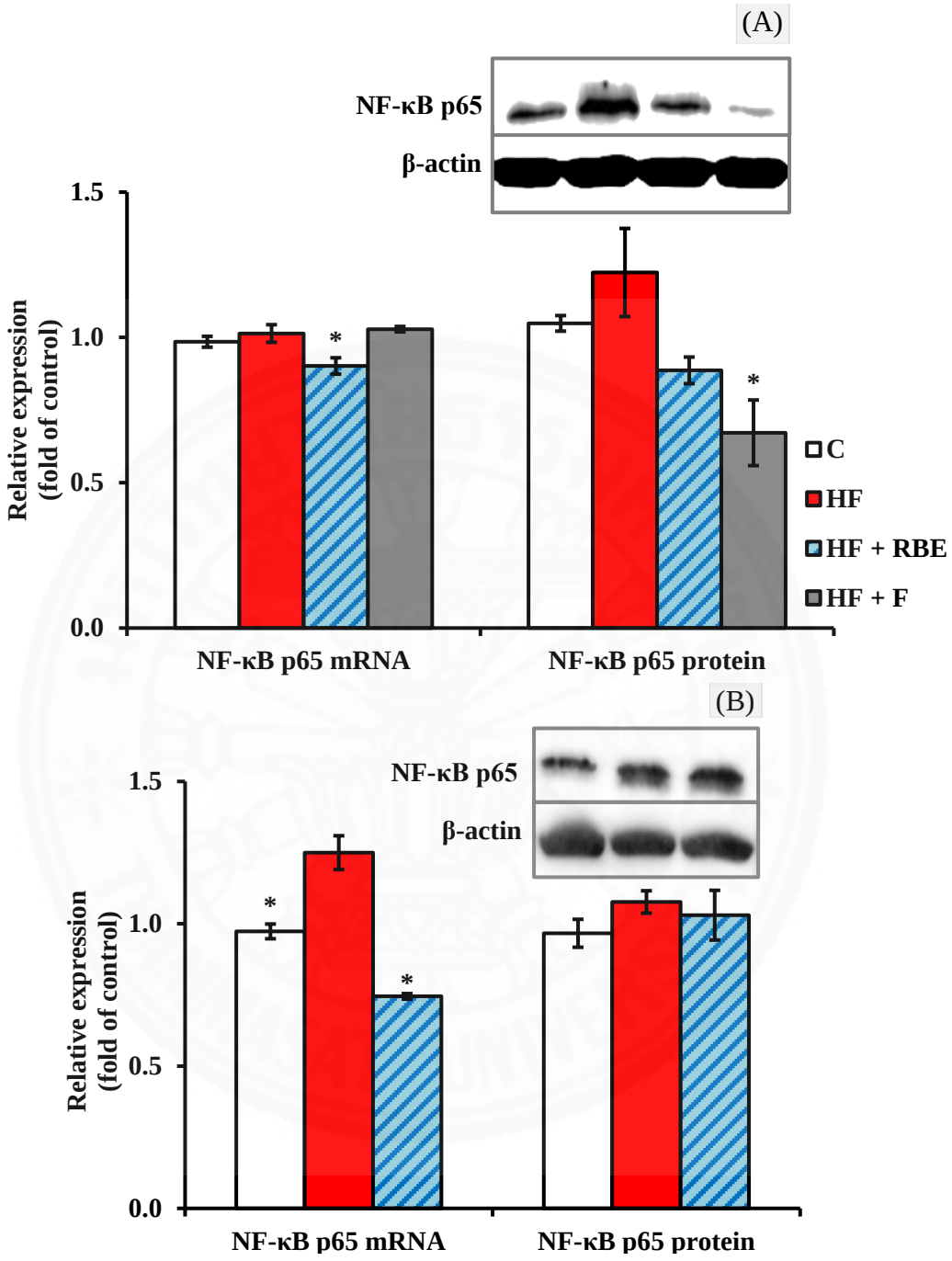

Figure 4.10 Left ventricular NF-κB p65 mRNA and protein expression levels of the (A) short- and (B) intermediate-term experimental groups. Values are expressed as means ± S.E.M. (n = 6 for mRNA expression and protein expression in the short-term experiment and n = 3 for protein expression in the intermediate-term experiment). HF group was used as a reference group. *$p < 0.05$ versus HF group.

4.10 Effects of RBE on aortic gene expression

4.10.1 eNOS gene expression

4.10.1.1 Short-term experiment

The results are presented in Figure 4.11A. Both real-time PCR western blotting demonstrated that eNOS mRNA and protein levels in the hearts were all significantly decreased in the HF group compared with the C group. In contrast, the eNOS expression levels in both RBE- and F-treated groups significantly increased compared with those in HF group.

4.10.1.2 Intermediate-term experiment

As presented in Figure 4.11B. HF-fed group had significantly lower expression levels of eNOS gene compared to the control diet-fed group. However, the expression levels of eNOS were not significantly up-regulated in the HF + RBE group.

4.10.2 NF-κB p65 gene expression

4.10.2.1 Short-term experiment

The effect of RBE on aortic NF-κB mRNA and protein expression is shown in Figure 4.12A. In comparison with the C group, NF-κB mRNA and protein expression levels were elevated in the HF group. Administration of RBE resulted in significant decreases in the expression levels of this gene compared to HF alone-fed rats. The levels of NF-κB p65 mRNA were also significantly decreased in aortic tissue of F-fed rats as compared to the HF group.

4.10.2.2 Intermediate-term experiment

The relative mRNA and protein levels of NF-κB p65 were significantly increased in the aortas of the HF-fed rats when compared with the normal diet-fed rats (Figure 4.12B). After 16 weeks of treatment with RBE, NF-κB p65 expression levels were significantly decreased in HF + RBE group compared with the HF group.

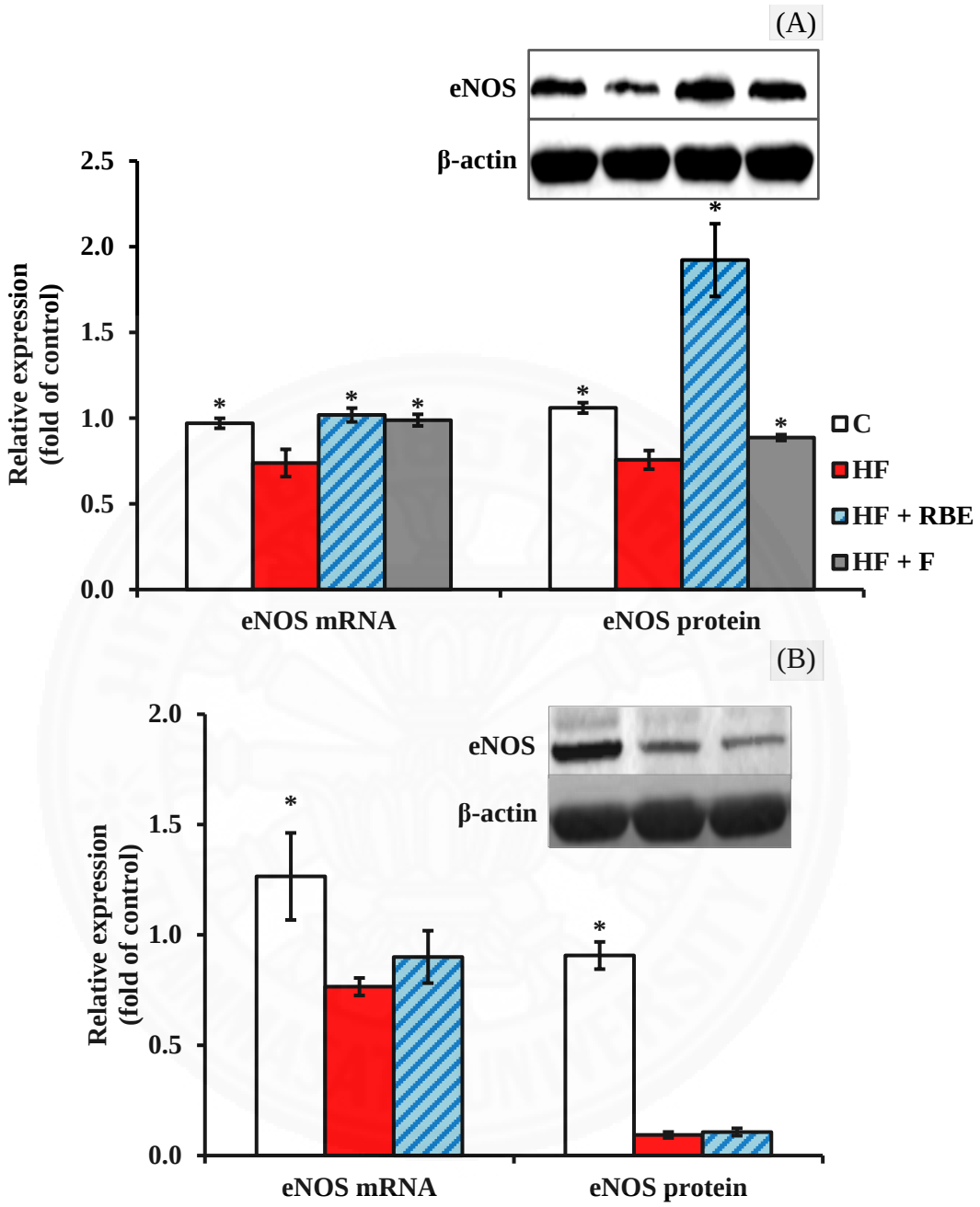

Figure 4.11 Aortic eNOS mRNA and protein expression levels of the (A) short- and (B) intermediate-term experimental groups. Values are expressed as means ± S.E.M. (n = 6 for mRNA expression and protein expression in the short-term experiment and n = 3 for protein expression in the intermediate-term experiment). HF group was used as a reference group. $^{*}p < 0.05$ versus HF group.

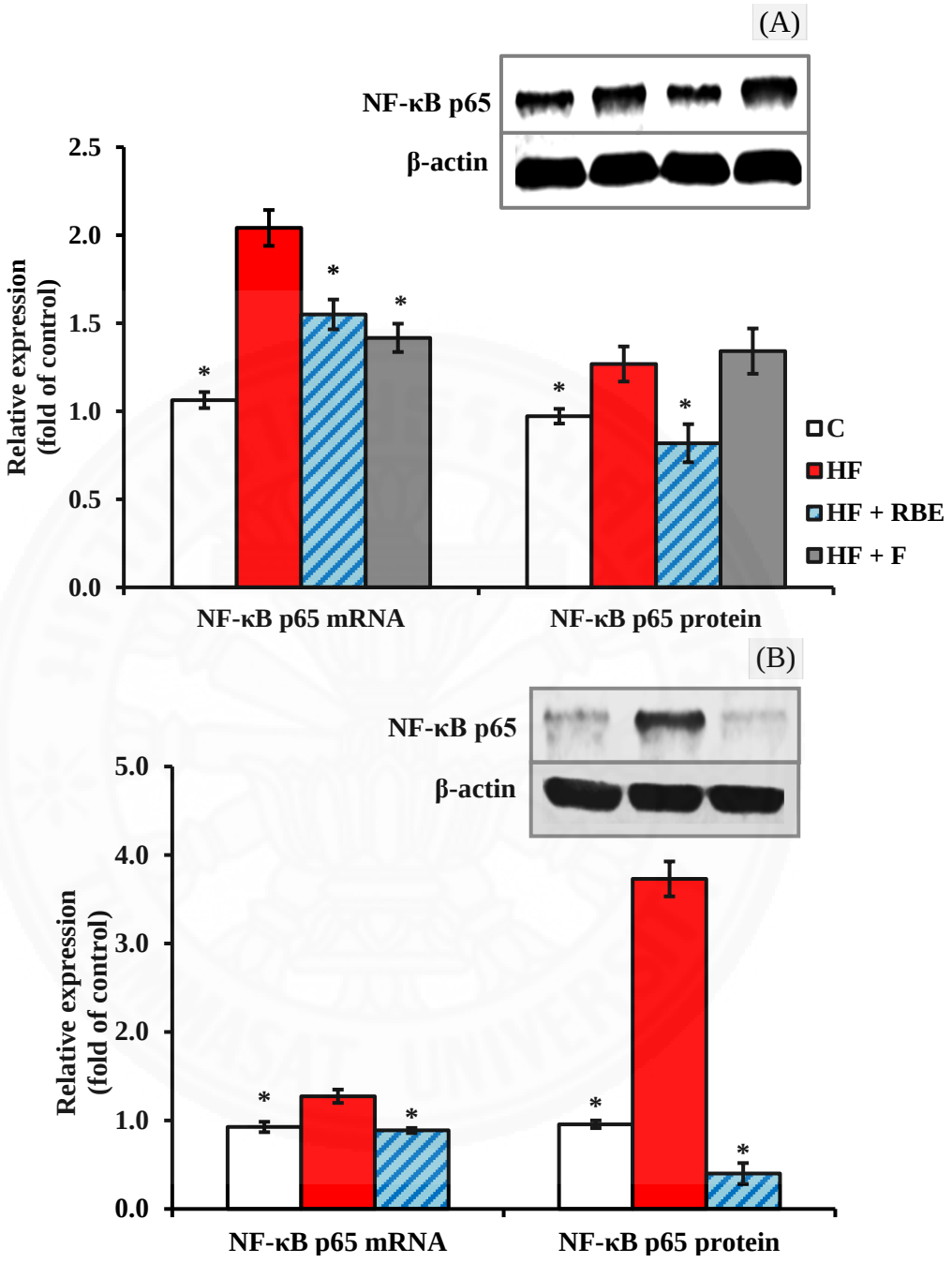

Figure 4.12 Aortic NF-κB p65 mRNA and protein expression levels of the (A) short- and (B) intermediate-term experimental groups. Values are expressed as means ± S.E.M. (n = 6 for mRNA expression and protein expression in the short-term experiment and n = 3 for protein expression in the intermediate-term experiment). HF group was used as a reference group. $^{*}p < 0.05$ versus HF group.

CHAPTER 5
DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

The present study investigated the effects of RBE from KDML 105 rice on five main factors associated with the development of MS and cardiovascular disease: (1) abdominal obesity, (2) liver fat accumulation, (3) dyslipidemia, (4) hyperglycemia and glucose intolerance and (5) cardiovascular lipotoxicity. This study was also designed to characterize the phenolic compounds, γ-oryzanol and α-tocopherol composition of RBE and to evaluate its antioxidant activity.

Although the pathogenesis of the cardiovascular disease-related MS is not completely understood, high dietary fat intake is an important cause of cardiovascular risk factors in both human and experimental animals (Lottenberg et al., 2012; Panchal & Brown, 2011). In the current study, rats fed an HF for 4 and 16 weeks developed metabolic disturbances, which were characterized by obesity, liver fat overaccumulation, dyslipidemia, hyperglycemia and glucose intolerance, compared with rats fed a control diet. It is well-established that these metabolic disturbances are involved in the development of cardiovascular disease (Anstee et al., 2013; Bays et al., 2013; Bergman et al., 2006; Hamaguchi et al., 2007; Madonna & De Caterina, 2011; Rader, 2007; Targher et al., 2013). The prevention of these metabolic disturbances is necessary for the reduction of cardiovascular disease. In the current study, cardiovascular abnormalities were also developed after rats were fed an HF. Thus, these HF-fed rats could be used as a suitable model for studying the effects of RBE on metabolic and cardiovascular diseases, as described below.

5.1 Protective effects of RBE on HF-induced obesity in rats

Obesity, especially abdominal obesity, is a well-established risk factor for MS and cardiovascular disease (Bergman et al., 2006; Rader, 2007). Obesity is characterized by excess adipose tissue mass resulting from hypertrophy and/or hyperplasia of adipocytes (Lionetti et al., 2009). Furthermore, human study has revealed that omental adipocyte size correlated positively with the degree of insulin

resistance and liver fat accumulation (O'Connell et al., 2010). Consistent with previous studies, obesity, increased abdominal fat mass and hypertrophic adipocyte were observed in rats fed an HF alone when compared with rats fed a standard chow (Eu et al., 2010; Kim et al., 2000; Wu et al., 2010). In contrast to the HF group, the short-term administration of RBE caused a significant decrease in the body weight, intra-abdominal fat deposition and adipocyte size independently of food intake. The mechanism of anti-obesity effect may, therefore, be mediated via the inhibition of adipocyte hypertrophy, thereby decreasing adipose tissue mass. In addition, RBE may also inhibit HF-induced obesity by reducing ectopic fat accumulation in the liver. Similar results were obtained in rats fed an F (Jeong et al., 2004). The current results are in agreement with previous reports indicating that RBE treatment caused a significant decrease in abdominal fat weight and adipocyte size in HF-fed rats (Chotip Chakhonpunya et al., 2011; Noppamat Kandee et al., 2009). Previous studies have also reported the anti-obesity effects of RBEE and rice bran in animals with MS (Candiracci et al., 2014; Justo et al., 2015; Kang, Kim, Rico, & Lee, 2012). Consistent with a short-term study, intermediate-term administration of RBE significantly inhibited HF-induced adipocyte hypertrophy in rats. However, adipocyte size, abdominal fat weight and body weight of RBE-administered rats were still higher compared with those of the C group. These data suggest that the short-term administration of RBE is more effective than the intermediate-term administration of RBE in regulating the elevation in body weight and abdominal fat mass of animals under diet-induced obesity condition. Additional studies are necessary to fully investigate its anti-obesity effects.

5.2 Protective effects of RBE on HF-induced liver fat overaccumulation in rats

An excessive TG accumulation within lipid droplets in the liver cells is the hallmark of NAFLD (Zámbó et al., 2013). NAFLD is strongly associated with obesity and high-energy diet consumption that may progress to non-alcoholic steatohepatitis (Li et al., 2009). Hepatocellular damage is often occurs in parallel with the hepatic TG overaccumulation and dyslipidemia (Li et al., 2009; Mopuri, Ganjayi, Banavathy, Parim, & Meriga; 2015). Recently, NAFLD is considered to be a

significant contributing factor for the pathogenesis of cardiovascular disease (Anstee et al., 2013; Hamaguchi et al., 2007; Targher et al., 2013). In the present study, lipid droplets were observed due to TG accumulation, confirming that HF consumption induces hepatic fat overaccumulation. Moreover, intermediate-term HF feeding caused a marked increase in serum AST and ALT levels, the major biomarkers of hepatic injury, suggesting the role of HF feeding in developing steatohepatitis in experimental rats. In comparison with the HF group, the short-term RBE-treated group had significantly decreased liver weight and lipid accumulation, whereas the intermediate-term RBE-treated group did not have any significant changes. However, intermediate-term RBE treatment was found to reduce the increase in serum AST and ALT levels. These findings indicate that the RBE treatment may protect against HF-induced NAFLD by decreasing liver lipid accumulation and damage. The current findings are supported by a previous study that showed the decreased TG and total-C accumulation in the liver of RBE-treated rats (Wang, Li, Sun, & Yu, 2014). These results are also in agreement with a previous study demonstrating that the consumption of RBE decreased TG deposition in the livers of HF-fed rats by inhibiting the activity of acetyl CoA carboxylase (ACC) (Chotip Chakhonpunya et al., 2011). Moreover, the treatment with protein hydrolysates, one of the important bioactive compounds in KDML105 rice bran, was also found to suppress the expression of hepatic lipogenic proteins, sterol response element-binding protein-1 and fatty acid synthase (Kampeebhorn Boonloh et al., 2015). Similar data were also reported with HF + F group (Jeong et al., 2004). Compared with the HF group, only the HF + F group showed a significant increase in the liver weight and serum ALT levels, suggesting a role of F in promoting hepatotoxicity in this model of MS. These data are concordant with those of Jeong et al. (2004) and Kobayashi et al. (2009).

5.3 Protective effects of RBE HF-induced dyslipidemia in rats

Dyslipidemia, a major component of MS, is modifiable risk factors of cardiovascular disease (Bays et al., 2013). It is generally accepted that dyslipidemia, including the increased circulating FFA levels, are involved in the development of cardiovascular disease (Boden, 2008; Breitling, Rothenbacher, Grandi, März, &

Brenner, 2011). In the present study, short-term treatment with RBE decreased total-C and TG levels in serum without affecting the serum levels of LDL-C and HDL-C, which are similar to the previous observations (Wang et al., 2014). Intermediate-term treatment with RBE also decreased TG levels in serum. The same trends were observed for F treatment (Jeong et al., 2004). Unlike present results, however, previous studies showed RBE treatment significantly increased the serum HDL-C levles, but decreased the serum LDL-C levels in animal models of MS (Justo et al., 2013; Wang et al., 2014). These differences may be due to the types of rice bran, extraction methods, and experimental designs. In addition to the abnormal lipoprotein metabolism, elevated levels of FFA were observed in the serum of rats fed an HF alone (short-term control 14.76 ± 0.38 mg/dL; short-term HF 17.65 ± 0.14 mg/dL; intermediate-term control 9.59 ± 0.29 mg/dL; intermediate-term HF 13.48 ± 1.06 mg/dL). Both RBE treatments markedly decreased serum FFA levels (short-term HF + RBE 14.29 ± 0.50 mg/dL; intermediate-term HF + RBE 11.11 ± 0.94 mg/dL). These results are in agreement with those of Cheng et al. (2010) showing that the treatment with rice bran could ameliorate blood FFA concentrations in diabetic patients. Future researches will be required to explore the specific mechanisms underlying the effects of this RBE against the dyslipidemia.

5.4 Protective effects of RBE on HF-induced hyperglycemia and glucose intolerance in rats

Abnormalities of glucose metabolism, such as impaired fasting glucose, impaired glucose tolerance and insulin resistance, are the basic characteristics of obesity, and diabetes mellitus and are associated with cardiovascular disease (Bock et al., 2006; Singla, Bardoloi, & Parkash, 2010; Weyer et al., 1999). Hyperglycemia can initiate changes in cardiovascular structure and function such as vascular dysfunction, myocardial hypertrophy and cardiovascular oxidative stress (Balteau et al., 2014; Madonna & De Caterina, 2011; Xu et al., 2014). An important mechanism in the development of impaired glucose metabolism in animals is the reduction of the pancreatic cell mass, which may lead to the loss of insulin sensitivity, hyperglycemia and impaired glucose tolerance (Couturier et al., 2010; Sone & Kagawa, 2005). In

animals fed a short-term HF, the levels of blood glucose as well as AUC-G, were higher than in the C group. Furthermore, their pancreas weight was markedly decreased compared with the controls. Although there was no significant difference in FBG between the intermediate-term groups, HF-alone group showed an impaired OGTT when compared to the C group. Short-term RBE treatment suppressed these HF-induced increases in the FBG. The AUC-G was slightly decreased in both RBE-treated groups. Consistent with a decrease in FBG and AUC-G, the pancreatic mass was normalized after the short-term treatment with RBE, suggesting that RBE might prevent the impaired glucose homeostasis via the improvement of pancreatic mass. To support this notion, previous reports have showed that KDML 105 RBE and rice bran protein treatments could prevent high-calorie diet-induced impaired glucose homeostasis in animals (Kampeebhorn Boonloh et al., 2015; Noppamat Kandee et al., 2009). Additionally, treatment of HF-fed mice with rice bran and phytic acid resulted in a marked decrease in blood glucose levels by stimulating glucose utilization but inhibiting gluconeogenesis (Kim, Rico, Lee, & Kang, 2010). Additional observations are needed to investigate the RBE-mediated mechanisms that improve glucose homeostasis. Rats fed only with F showed significantly increased levels of FBG compared to the HF group. In line with this result, Liu et al. (2011) found that F induced hyperglycemia and β-cell dysfunction in animals after 12 weeks of the treatment.

5.5 Protective effects of RBE on HF-induced cardiovascular abnormalities in rats

5.5.1 Cardiolipotoxicity

Cardiolipotoxicity is one of the important pathophysiological features associated with MS (Geetha et al., 2014; Marfella et al., 2009; Zhou et al., 2000). Unfortunately, the cardiac TG accumulation and markers were found to be unaffected even after the rats were fed with HF for 4 and 16 weeks. These results are concordant with those of Jeckel et al. (2011). However, alterations in the expression of the PPARα, FAT/CD36 and GLUT-4 were observed in the hearts of rats fed an HF alone. Present results suggest that the abnormalities of metabolic gene expression, rather than the abnormalities of cardiac lipid metabolism and injury, are probably the early

pathological events in rats fed with short- and intermediate-term HF. It is widely accepted that the pathogenesis of cardiolipotoxicity is initially found to be associated with altered expression of metabolic genes (Drosatos & Sschulze, 2013; Wende et al., 2012). To clarify the molecular mechanisms underlying the cardioprotective effects of RBE, the expression of genes involved in energy metabolism were evaluated.

PPARα is one of the important lipid sensors that have been suggested to play a key role in the cardiac lipid metabolism (Finck & Kelly, 2002; Lopaschuk et al., 2010). PPARα can be activated by FA and lipid metabolites. The activation of this nuclear receptor can stimulate FA catabolic pathways, such as mitochondrial FA uptake and FA β-oxidation. PPARα also plays a major role in the regulation of cardiac inflammation (Smeets, Planavila, van der Vusse, & van Bilsen, 2007). Some *in vivo* and *in vitro* studies of MS have revealed that the down-regulation of PPARα and its target genes encoding FA oxidation enzymes resulted in impaired FA oxidation in the heart (Aroor et al., 2012; Geetha et al., 2014; Haffar, Bérubé-Simard, & Bousette, 2015; Yan et al., 2009; Zhou et al., 2000). Furthermore, treatment with PPARα agonist could reduce myocardial TG contents in mice fed an HF (Barbieri et al., 2012). The present results provided evidence that the rats fed the HF alone failed to induce PPARα expression during the metabolic disturbances in the hearts. The down-regulations of this gene may contribute to lose lipid-sensing pathways and accordingly cause metabolic derangement in the heart. With a slight increase in PPARα proteins, RBE treatment markedly increased the levels of PPARα mRNA expression in the heart. Up-regulated expression of PPARα was clearly detected in the positive control group. The present findings suggest that the up-regulation of PPARα expression by RBE treatment may be an early molecular compensation that helps to enhance the oxidation of the increased accumulation of lipids in the heart. In line with these findings, a study by Ronis, Badeaux, Chen and Badger (2010) has revealed that the rice protein consumption effectively increased the hepatic PPARα expression in rats fed an HF-high cholesterol diet.

The increases in circulating FFA and TG have been reported in both animal and human studies in the setting of the MS (Sumiyoshi, Sakanaka, & Kimura, 2008; Zhang et al., 2014). This hyperlipidemia may also predispose to increased myocardial lipid delivery**.** In this view, FAT/CD36 is a major sarcolemmal FA

transporter that facilitates the entry of long-chain FA into cardiac myocytes. However, the elevated FAT/CD36 gene expression and FA uptake are associated with cardiac contractile dysfunction and cardiomyopathy (Ouwens et al., 2007; Yang, Sambandam, et al., 2007). In the models of MS, FAT/CD36 gene has been shown to be up-regulated in mouse and rat hearts (Bonen et al., 2009; García-Rú et al., 2012; Geetha et al., 2014) and its inhibition in rat cardiomyocyte was associated with a reduction in TG accumulation and contractile dysfunction (Angin et al., 2012). The present study showed that the treatment of HF-fed rats with RBE prevented the increased serum levels of FFA and TG as well as cardiac FAT/CD36 gene expression, thus suggesting that an RBE intake might attenuate the delivery of FA to the heart with a concomitant decrease in FA uptake.

In addition to the abnormal fat metabolism, the defects in glucose uptake and oxidation have been also observed in the hearts from patients and animal models with MS (Cook et al. 2010; Ko et al., 2009). The glucose uptake process, which is the rate-limiting step in cardiomyocyte glucose metabolism, is mediated by the members of GLUT protein family, especially the GLUT4 (Manchester, Kong, Nerbonne, Lowry, & Lawrence, 1994). Decreased expression of GLUT4 was found in cardiac tissue from both the diabetic rats and patients (Bonen et al., 2009; Cook et al., 2010). GLUT-4 down-regulation in the cardiac tissue could occur in the presence of hyperlipidemia, type 2 diabetes mellitus, insulin resistance and cardiac lipid overaccumulation (Armoni, Harel, Bar-Yoseph, Milo, & Karnieli, 2005; Cook et al., 2010; Glatz, Bonen, & Luiken, 2007). Moreover, transgenic mice with overexpression of GLUT4 could improve insulin sensitivity and hypertriglyceridemia in the setting of an HF intake (Atkinson, Griesel, King, Josey, & Olson, 2013). In line with this notion, compared with FA, glucose has also been reported to be an effective substrate for preventing cardiac metabolic maladaptation and dysfunction under ischemic and diabetic conditions (Hafstad, Khalid, How, Larsen, & Aasum, 2007; Ardehali et al., 2012). The results showed that GLUT-4 was down-regulated in the left ventricles of rats fed an HF alone. The insufficient expression of GLUT4 gene may, therefore, cause dysregulation of glucose metabolism in the heart. However, both the short- and intermediate-term RBE consumptions markedly prevented the down-regulation of GLUT4 protein in the left ventricles of HF-fed rats. Thus, we speculate that the RBE

consumption may improve the impairment of cardiac glucose uptake and metabolism by enhancing the up-regulation of GLUT4 gene.

5.5.2 Cardiac hypertrophy

Cardiac hypertrophy is considered to be an important factor for heart failure, arrhythmia and sudden death (Bernardo, Weeks, Pretorius, & McMullen, 2010). NF-κB is a well-known transcription factor for inflammation and oxidative stress, a principal contributor to the development of cardiac disease (Lorenzo et al., 2011; Mariappan et al., 2010). In addition to its role in inflammation, previous study has suggested that activation of NF-κB signaling pathway is essential for developing cardiomyocyte hypertrophy (Gupta et al., 2005). Results from this study suggest that the treatment with NF-κB inhibitor can suppress cardiac hypertrophy induced by hypertension in spontaneously hypertensive rats. Gupta and colleagues (2008) have also reported that knocking down of the NF-κB p65 gene in myocytes and mice exhibited a significant reduction of myocyte growth and cardiac mass, respectively. Their results suggest that the activation of NF-κB p65 signaling pathway may be one of the pathological mechanisms of cardiac hypertrophy. In the intermediate-term study, the HF hearts revealed the increased cardiomyocyte size, which is a sign of cardiac hypertrophy in comparison with the normal rat hearts. Similar results were also reported with short-term HF group. These findings are coincident with previous data (Wang, Li, Zhao, Peng, & Zuo, 2015). In parallel with increased cardiomyocyte size, NF-κB p65 gene was up-regulated in the left ventricles of HF-fed rats. Moreover, other mechanisms may also play an important role in initiating the myocardial hypertrophy. They include the down-regulation of PPARα and GLUT-4 and up-regulation of FAT/CD36 (Domenighetti et al., 2010; Smeets et al., 2008; Sung et al., 2011). Interestingly, the treatment with fenofibrate, a PPARα agonist, has been reported to reduce the myocardial fibrosis and diastolic dysfunction through the inactivation of NF-κB pathway in an animal with hypertensive heart disease (Ogata et al., 2004). In the present study, RBE-fed rats tended to have lower cardiomyocyte size compared to the HF-fed rats. Consistent with an improvement in PPARα, GLUT-4 and FAT/CD36 expressions, the administration of RBE was also able to reduce NF-κB p65 expression in the heart. NF-κB expression was also attenuated by F treatment. Thus, the anti-hypertrophic effect of RBE may be mediated, at least in part, by the

improvement of the NF-κB p65, PPARα, GLUT-4 and FAT/CD36 gene expressions. Metabolic stimuli involved in pathogenesis of cardiac disease, such as high levels of TG, FFA and glucose, have been shown to stimulate the NF-κB signaling pathway (Nizamutdinova et al., 2013; Sun et al., 2012). Therefore, the decreased expression of NF-κB in the heart by RBE treatment may be associated with the reduced levels of TG, FFA and glucose. However, further experiments are required to explore the details of these mechanisms underlying the effects of RBE against left ventricular hypertrophy.

5.5.3 The initial pathogenesis of vascular disease

To provide the molecular mechanisms for the vasoprotective effects of RBE, the present study analyzed the expression of vascular genes that are involved in NO production (eNOS) and inflammation (NF-κB). Although the pathogenesis of vascular disease in MS is complex, the decreased NO synthesis and enhanced inflammatory responses are involved in the early stages of vascular disease (Kim et al., 2008). eNOS is a major enzyme that generates NO in the blood vessels as well as is an anti-inflammatory, anti-hypertensive, and anti-atherosclerotic molecules (Zhao et al., 2015). By contrast, NF-κB is considered as a pro-inflammatory transcription factor involved in the initiation of atherosclerosis (de Winther et al., 2005). In blood vessels, activation of NF-κB pathway in response to various atherogenic stimuli results in the production of pro-inflammatory molecules, such as TNF-α and iNOS. The results showed that eNOS was down-regulated while NF-κB p65 was up-regulated in the aorta of rats fed an HF. These findings are consistent with Wilson et al.'s study (2000) reporting that the down-regulation of eNOS expression and up-regulation of NF-κB p65 expression were observed in the initial stage of vascular diseases.

Interestingly, short-term RBE treatment significantly increased eNOS mRNA and protein levels when compared with HF-fed rats, which is consistent with previous findings that the obese Zucker rats treated with the RBEE showed higher eNOS protein levels in blood vessels (Justo et al., 2013). However, intermediate-term RBE treatment had no effect on eNOS expression. Moreover, all RBE treatments significantly inhibited HF-induced vascular NF-κB p65 expression in rats. The results are supported by previous studies reporting that supplementation with

the RBEE decreased the levels of pro-inflammatory markers (e.g., TNF-α and iNOS) in the vasculature and serum of obese Zucker rats (Justo et al., 2012; Justo et al., 2013). Alterations in the expression of eNOS and NF-κB p65 in the vascular cells and tissues have been shown to be associated with hypercholesterolemic and hyperglycemic conditions (Srinivasan et al., 2004; Williams et al., 2012; Wilson et al., 2000). Zhang et al. (2013) have found that treatment with palmitate decreased eNOS expression in endothelial cells. In addition, expression of NF-κB p65 has been reported to elevate in the endothelium of FA-infused rats (Li, Li, Bao, Zhang, & Yu, 2011). In the current study, the modulatory effects of RBE on the expression of these genes could be related to its hypocholesterolemic, hypoglycemic and FFA-lowering effects. Compared with the HF-alone group, positive control group could significantly restore the expression of both eNOS and NF-κB genes in the vasculature. These results have been confirmed by Walker et al. (2012) and Wang et al. (2014). In accordance with the molecular changes of the aortic tissues from the HF-fed rats in the intermediate-term study, histological analysis revealed the early signs of atherosclerotic lesions that were characterized by abnormal aorta wall morphology. These morphological changes were prevented by RBE treatment. These results are in agreement with previous studies demonstrating that the supplementation with rice bran and RBEE decreased atherosclerotic plaques in animal models of vascular diseases (Perez-Ternero et al., 2015; Wilson, Idreis, Taylor, & Nicolosi, 2002). Unfortunately, there was no evidence of atherosclerotic lesions in rats fed an HF for 4 weeks.

5.6 Bioactive compounds in RBE

Many studies have suggested that the consumption of phenolic compounds, γ-oryzanols and α-tocopherols is associated with a decreased risk for MS and cardiovascular diseases (Kim et al., 2013; Kishimoto et al., 2013; Peluzio et al., 2001; Wang et al., 2015; Wilson et al., 2007). However, present study showed that RBE from the rice variety contained low levels of these bioactive components and showed low DPPH radical-scavenging capacity. Previous studies have shown many biological activities of rice bran protein and phytic acid, such as hypolipidemic,

hypoglycemic and cardiovascular protective effects (Burris et al., 2010; Kampeebhorn Boonloh et al., 2015; Kang et al., 2012; Kim et al., 2010; Orachorn Boonla et al., 2015; Schutkowski, Hirche, Geissler, Radtke, & Stangl, 2010). Interestingly, the report by Orachorn Boonla et al. (2015) has indicated that treatment with peptides derived from the KDML 105 rice bran significantly enhanced the endothelial function of two kidney-one clip hypertensive rats by elevation of eNOS protein levels in the aorta and NO production. In addition, treatment with KDML 105 rice bran protein significantly attenuated hyperlipidemia, insulin resistance and inflammation in rats fed a high-energy diet (Kampeebhorn Boonloh et al., 2015). These studies led us to hypothesize that protein and/or phytic acid may be bioactive compounds in our RBE for preventing the metabolic and cardiovascular abnormalities. However, the bioactive compounds in RBE are still unknown. Further studies are needed to evaluate the bioactive constituents in RBE.

5.7 Conclusions and recommendations

The present results indicate that the RBE treatment can prevent the derangement of cardiac energy metabolism in the setting of diet-induced metabolic disturbances, partly by decreasing the cardiac FA delivery as well as by improving the expression of genes involved in the lipid sensor (PPARα), lipid uptake (CD36) and glucose uptake (GLUT4). RBE treatment also exhibited preventive effects against HF-induced cardiac NF-κB expression, which may contribute to the improvement of cardiac hypertrophy. Furthermore, RBE treatment showed vasoprotective effects through the regulation of eNOS and NF-κB p65 gene expressions in the vasculature of HF-induced metabolic disturbances. Although RBE treatment did not reduce the overall risk factors for vascular disease, it had a tendency to decrease some of the risk factors associated with cardiovascular diseases. The preventive effects of RBE are likely due to its hypolipidemic and hypoglycemic effects. However, short-term treatment with RBE is more effective than intermediate-term treatment with RBE in inhibiting metabolic dysfunctions. The protective effects and possible molecular mechanisms of KDML 105 RBE are summarized in Figure 5.1 and 5.2. Taken together, RBE could be a potential food supplement for the prevention of

cardiovascular diseases in the setting of the MS. Evaluation of the bioactive compounds in RBE will be further studied to correlate their effects on the prevention of metabolic and cardiovascular disease.

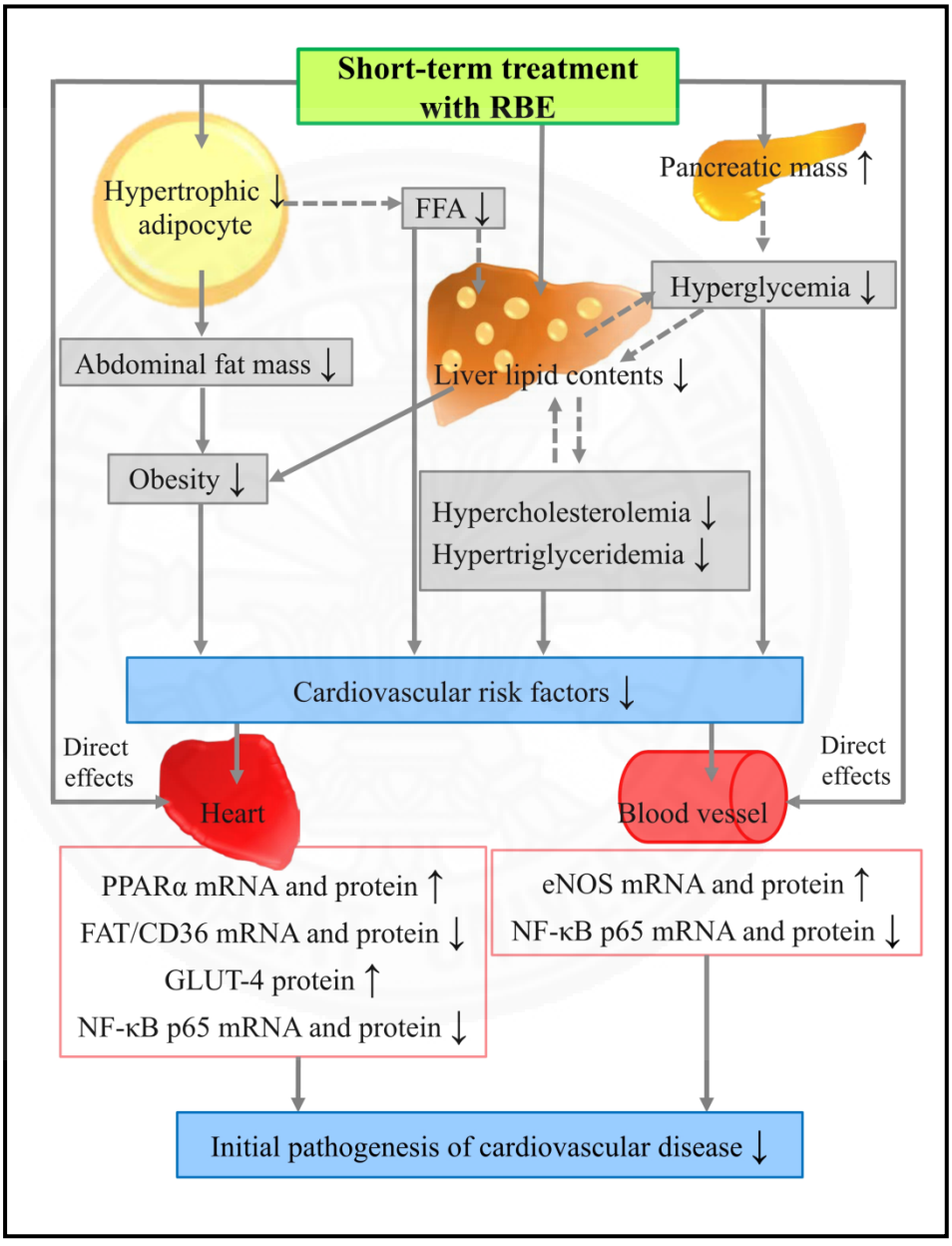

Figure 5.1 Conclusion of the proposed mechanisms responsible for the short-term effects of KDML 105 RBE on metabolic and cardiovascular abnormalities.
↑, increased; ↓, decreased.

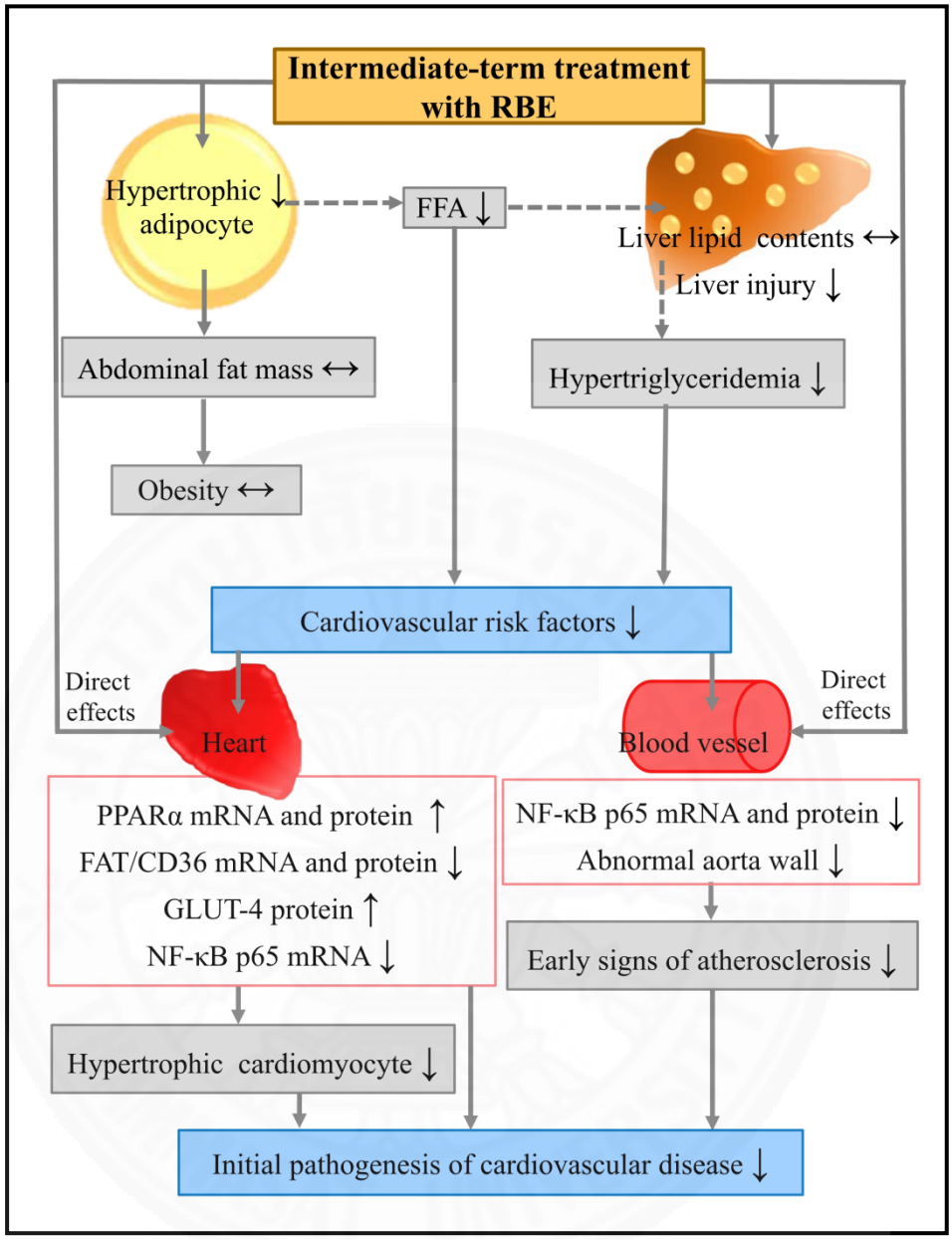

Figure 5.2 Conclusion of the proposed mechanisms responsible for the intermediate-term effects of KDML 105 RBE on metabolic and cardiovascular abnormalities.
↑, increased; ↓, decreased; ↔, no effect.

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APPENDICES

APPENDIX A
THE ANIMAL ETHIC APPROVAL

เลขที่ AE 002/2013

ใบรับรองให้ดำเนินการวิจัยในสัตว์ทดลอง

คณะกรรมการจรรยาบรรณการใช้สัตว์เพื่องานทางวิทยาศาสตร์

คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

ชื่อข้อเสนอการวิจัย

(ภาษาไทย) ผลของสารสกัดรำข้าวต่อภาวะไขมันเป็นพิษต่อระบบหัวใจหลอดเลือดในหนูที่ได้รับอาหารไขมันสูง

(ภาษาอังกฤษ) The effect of rice-bran extract on cardiovascular lipotoxicity in rats fed a high-fat diet

ชื่อ-สกุล ผู้เสนอโครงการวิจัย นายณรงค์ศักดิ์ มั่นคง

หน่วยงานสังกัด (คณะ/กอง) นักศึกษาบัณฑิตศึกษา คณะแพทยศาสตร์

(มหาวิทยาลัย/กรม) มหาวิทยาลัยธรรมศาสตร์

(กระทรวง)

คณะกรรมการจรรยาบรรณการใช้สัตว์เพื่องานทางวิทยาศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์ มีมติให้โครงการดังกล่าวสามารถดำเนินการวิจัยได้ โดยยึดหลักจรรยาบรรณการใช้สัตว์อย่างเคร่งครัด หากได้ตรวจสอบพบว่าผู้ดำเนินการวิจัยในโครงการนี้ได้ดำเนินการวิจัยผิดหลักจรรยาบรรณการใช้สัตว์ คณะกรรมการจรรยาบรรณการใช้สัตว์เพื่องานทางวิทยาศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์มีสิทธิเพิกถอนใบรับรองนี้

ลงนาม..... ลงนาม.....

(รองศาสตราจารย์ ดร. นายสัตวแพทย์สิวบูรณ์ สิริรัฐวงศ์) (รองศาสตราจารย์ นายแพทย์จักรชัย จึงธีรพานิช)

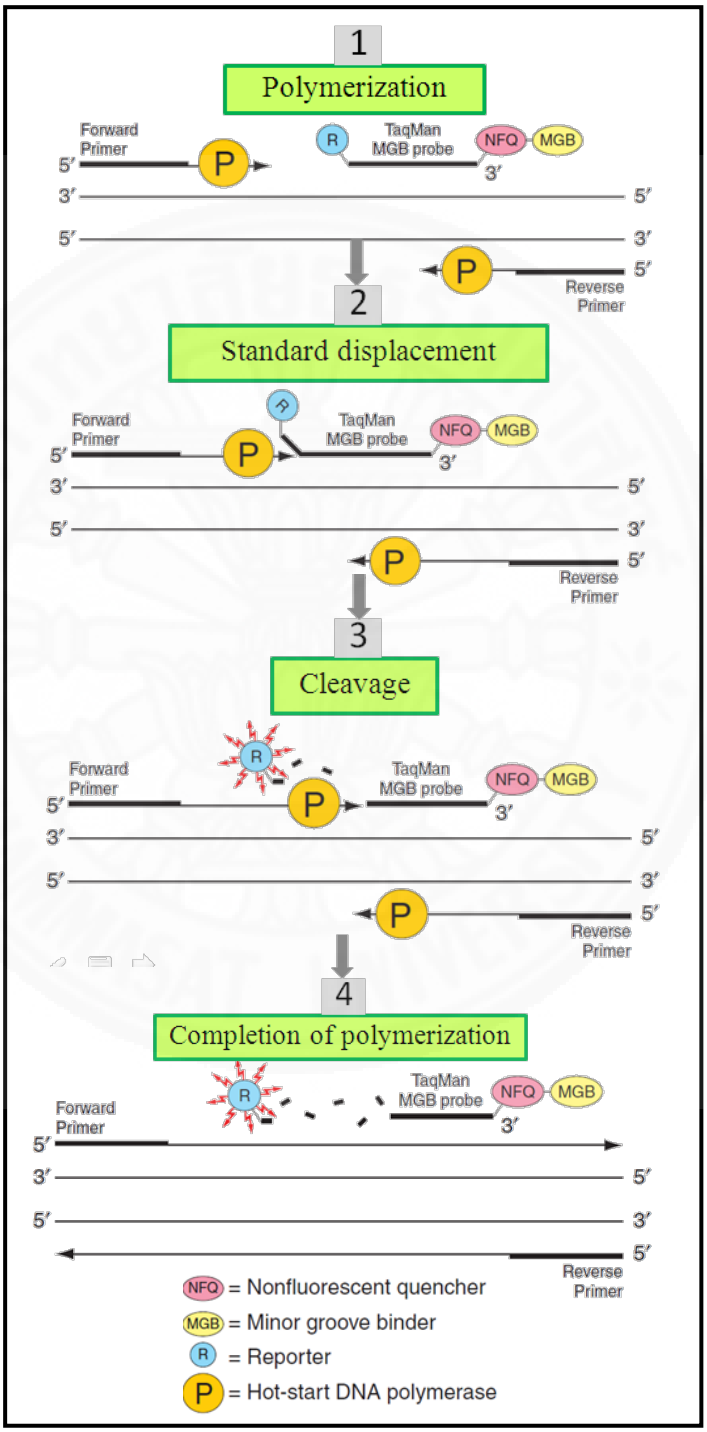
ประธานคณะกรรมการจริยธรรม รองคณบดีฝ่ายวิจัย

การใช้สัตว์เพื่องานทางวิทยาศาสตร์

วัน/เดือน/ปี ๒๕ มีนาคม ๒๕๕๖ วัน/เดือน/ปี ๒๕ มีนาคม ๒๕๕๖

APPENDIX B

THE PRINCIPLE OF TaqMan REAL-TIME PCR



Retrieved form http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldo cuments/cms_039284.pdf

APPENDIX C
WORKING CELL LYSIS BUFFER

Components of 1× cell lysis buffer

- 20 mM Tris-HCl (pH 7.5)
- 150 mM NaCl
- 1 mM Na_2EDTA
- 1 mM EGTA
- 1% Triton
- 2.5 mM sodium pyrophosphate
- 1 mM b-glycerophosphate
- 1 mM Na_3VO_4
- 1 μg/ml leupeptin
- 1mM PMSF
- 1× Proteinase inhibitor

APPENDIX D

PREPARATION FOR LOADING BUFFER (LAEMMLI BUFFER)

4× Loading buffer

- 0.5 M Tris-HCL (pH 6.8)	4.4 mL
- Glycerol	4.4 mL
- 20% SDS	2.2 mL
- 2-Mercaptoethanol	0.5 mL
- 1% Bromophenol blue	0.5 mL

APPENDIX E
PREPARATION FOR SDS-PAGE AND RUNNING BUFFER

Reagent and buffer

1. 10% SDS
   - SDS 10 g
   - Adjust to a final volume of 100 mL with deionized distilled water (dd H_2O)

2. 1% Ammonium persulfate (MW 228.19)
   - Ammonium persulfate 0.1 g
   - Adjusted to a final volume of 10 mL with dd H_2O

3. 4× Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)
   - Tris-base (MW 121.1) 6.06 g
   - dd H_2O 80 mL
   - Adjustto pH 6.8 with HCl and adjust to a final volume of 100 mL with dd H_2O

4. 4× Separating gel buffer (1.5 M Tris-HCl, pH 8.8)
   - Tris-base (MW 121.1) 36.33 g
   - dd H_2O 150 mL
   - Adjust to pH 8.8 with HCl and adjust to a final volume of 200 mL with dd H_2O

APPENDIX F
PREPARATION FOR SDS-PAGE AND RUNNING BUFFER (CONTINUED)

Reagent and buffer

5. 4% Stacking gel (final volume 10 mL)

- 30% Acrylamide/bis solution, 29:1	660 µL
- 4× Stacking gel buffer	1.26 mL
- 10% SDS	50 µL
- dd H_2O	3 mL
- 1% Ammonium persulfate	25 µL
- TEMED	5 µL

6. 10% Separating gel (final volume 10 mL)

-30% Acrylamide/bis solution, 29:1	3.3 mL
- 4× Separating gel buffer	2.5 mL
- 10% SDS	100 µL
- dd H_2O	4.05 mL
- 1% Ammonium persulfate	50 µL
- TEMED	5 µL

7. 10× Stocking running buffer (0.25 M Tris-base, 1.92 M glycine, 1% SDS)

- Trise-base	30.3 g
- Glycine (MW 75.07)	144 g
- SDS	10 g
- Adjust to a final volume of 1,000 mL with dd H_2O	

8. 1× Working running buffer

- 10× Stocking running buffer 100 mL
- Adjust to a final volume of 1,000 mL with dd H_2O

APPENDIX G

PREPARATION FOR TOWBIN TRANSFER BUFFER

1. 10× Stocking Towbin's buffer (240 mM Tris-base, 1,920 mM Glycine)
   - Trise-base 29 g
   - Glycine 144 g
   - Adjust to a final volume of 1,000 mL with deionized water

2. 1× Working Towbin's buffer (final volume 1,000 mL)
   - 10× Stocking Towbin's buffer 100 mL
   - Deionized water 700 mL
   - *Methanol 200 mL

   (*methanol was added last to prevent precipitation)

APPENDIX H
PREPARATION FOR TBS AND TBST

1. 10× Stocking TBS (200 mM Tris-base, 1.5 NaCl, pH 7.5)

- Trise-base 24.2 g
- NaCl (MW 58) 80.6 g
- Adjust to pH 7.5 and adjust to a final volume of 1,000 mL with DW

2. 1× Working TBS

- 10× Stocking TBS 100 mL
- Adjust to a final volume of 1,000 mL with DW

3. 0.1 % TBST

- Tween 20 1 mL
- Adjust to a final volume of 1,000 mL with 1× working TBS

BIOGRAPHY

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Publications

Jarinyaporn Naowaboot, Pritsana Piyabhan, Narongsuk Munkong, Wason Parklak, & Patchareewan Pannangpetch. (2016). Ferulic acid improves lipid and glucose homeostasis in high-fat diet-induced obese mice. *Clinical and Experimental Pharmacology and Physiology 2016, 43*(2), 242-250.

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