Proteomic Study of the Effect of Berberine on the Adipose Tissue of db/db Mice and 3T3-L1 Adipocytes

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Declaration

All experimental works reported in this thesis were performed by the author, unless stated otherwise.

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Abstract of thesis entitled:

Proteomic Study of the Effect of Berberine on the Adipose Tissue of db/db Mice and 3T3-L1 Adipocytes

Submitted by Wu Hoi Yan

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Diabetes mellitus (DM) has become an epidemic and is estimated to affect 366 million people worldwide by 2030. In particular, the prevailing type 2 diabetes mellitus is a chronic disease that can hardly be cured. Current treatment includes lifestyle intervention and medications like oral hypoglycemic agents. Common oral hypoglycemic agents have different adverse effects like nausea and vomiting for metformin, hypoglycemia for sulphonylureas and unwanted weight gain and even potential heart or liver failure for thiazolidinedione. Investigation for alternative hypoglycemic agents with fewer side effects is needed. In this project, the effect of berberine, an isoquinoline alkaloid extracted from Tradition Chinese Medicine *Coptis chinensis* or Huanglian, on glucose control in obese, diabetic db/db mice was investigated, with special interest on the proteomic alterations in visceral adipose tissues of berberine-treated db/db mice. Berberine treatment led to loss of body

weight in db/db mice by 25.33%. The average fasting blood glucose level of berberine-treated mice was 266.80mg/dL, compared with 466.83mg/dL of that of control mice. Glucose-lowering ability of db/db mice was also slightly improved after berberine treatment. Berberine could also inhibit triglyceride accumulation in 3T3-L1 adipocytes. However, it inhibited lipolysis in 3T3-L1 adipocytes in short-term (24h) treatment but enhanced lipolysis during prolonged (72h) treatment.

Proteomic study with 2D gel electrophoresis and mass spectrometry revealed increase in glycolysis, lipolysis and beta-oxidation and inhibition of TCA cycle and oxidative phosphorylation in visceral adipose tissue of berberine-treated db/db mice and berberine-treated 3T3-L1 adipocytes. ER stress-related proteins were also up-regulated after treatment. Western blotting confirmed that berberine activated AMPK but inhibited JNK activity in adipocytes, which may be attributed to the anti-diabetic action of berberine.

摘要

糖尿病已成為一種流行病。據估計，到二零三零年，全球將有三億六千六百萬人患上糖尿病。這是一種難以根治，只能輔助治療及加以控制的長期病，其中二型糖尿病最為流行。現時糖尿病的治療主要依靠病人的日常生活管理及服用降血糖藥物。然而市面上之降血糖藥物各有其副作用，如鹽酸二甲雙胍(metformin)可致噁心及嘔吐，磺醯尿素類藥物(sulphonylureas)可致低血糖症，TZD 類藥物(thiazolidinedione)可能導致體重增加甚至引發心臟或肝衰竭。更新、更少副作用的降血糖藥物仍有待研發。在這個研究項目，我研究了鹽酸小檗鹼（又稱黃連素），一種從黃連中提取的異喹啉生物鹼，對癡肥的二型糖尿 db/db 小鼠的血糖控制的影響，並特別注重黃連素對 db/db 小鼠內臟脂肪組織所引起的蛋白質組改變。黃連素對 3T3-L1 脂肪細胞的影響也在研究範圍之內。服用黃連素使 db/db 小鼠的體重減輕了 25.33%，處理組的平均空腹血糖值為 266.80mg/dL，而對照組的數值為 466.83mg/dL。黃連素亦能輕微改善 db/db 小鼠的降血糖能力，減少 3T3-L1 脂肪細胞的三酸甘油脂積聚。在 3T3-L1 脂肪細胞中，短期處理（二十四小時）能抑制脂肪分解，長期處理（三天）則能增加脂肪分解。

結合二維電泳及質譜分析的蛋白質組研究顯示黃連素在 db/db 小鼠的內臟脂肪及 3T3-L1 脂肪細胞中能增加醣酵解、脂肪分解、beta-氧化作用，同時能抑制檸檬酸循環、氧化磷酸化作用。黃連素也增加了脂肪細胞中內質網壓力相關蛋白的表達。免疫印記法證明黃連素能在脂肪細胞中活化 AMPK 分子並抑制 JNK 分子，或能有助於解釋黃連素的降血糖降體重功效。

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List of Abbreviations

µg	Micro gram
2-DE	Two-Dimensional Gel Electrophoresis
2D	Two dimensional
2D-DIGE	Two-dimensional fluorescence difference gel analysis technology
A2MG	Alpha-2-Macroglobulin
ACN	Acetonitrile
ADA	Amercian Diabetes Association
AMP	5' Adenosine Monophosphate
AMPK	5' Adenosine Monophosphate-activated Protein Kinase
ANAX1	Annexin A1
aP2	Adipocyte Protein 2
AR	Aldose Reductase
ATM	Adipose Tissue Macrophage
ATP	Adenosine 5-triphosphate
BACH2	Basic leucine zipper transcription factor 2
BBR	Berberine Hydrochloride
BSA	Bovine serum albumin
C.I.%	Confidence interval %
C/EBP	CCAAT-Enhancer-Binding Protein
CD36	Cluster of Differentiation 36
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1 -propanesulfonate
CLEC16	C-type lectin domain family 16, member A
CO_2	Carbon dioxide
CoA	Coenzyme A
COFRADIC	Combined Fractional Diagonal Chromatography
CTLA4	Cytotoxic T-lymphocyte-associated protein 4 (CTLA4)
Ctrl	Control
DAG	Diacylglycerol
dL	Deciliter
DM	Diabetes Mellitus
DMEM	Dulbecco Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dpi	Dots per inch
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinases
ESI	Electrospray Ionization
FBG	Fasting Blood Glucose
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid

g	Gram
Gal-3	Galectin-3
GDM	Gestational Diabetes Mellitus
GIIS	Glucose-induced Insuiln Secretion
GLUT4	Glucose Transporter Type 4
GRP	Glucose-Responsive Protein
h	Hour
HbA1c	Glycated Haemoglobin
HCl	Hydrogen chloride
HCl	Hydrochloric acid / Hydrochloride
HFD	High-Fat Diet
HIF	Hypoxia-Inducible Factor
HLA	Human leukocyte antigen
hnRNP	Heterogeneous nuclear ribonucleoprotein
HPLC	High performance liquid chromatography
hr	Hour
HSP90	Heat shock 90kDa protein
ICAT	Isotope-Coded Affinity Tag
IEF	Isoelectric focusing
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IKK β	IkB kinase β
IL	Interleukin
IL-2	Interleukin-2
IL2RA	Interleukin 2 receptor, alpha
INS	Insulin
IPG	Immobilized pH Gradient
IRS	Insulin Receptor Substrate
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
kg	Kilogram
L	Liter
LDL	Low-density Lipoprotein
MALDI	Matrix-Assisted Laser Desorption / Ionization
MALDI- ToF	Matrix-assisted laser desorption ionization - time of flight
MAPK1	Mitogen Activated Protein Kinase 1
MCP-1	Monocyte Chemotactic Protein-1
meter	M
min	Minute
mL	Milli liter
mm	Milli meter
mM	Milli molar
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
MudPIT	Multidimensional Protein Identification Technology

MW	Molecular weight
NaCl	Sodium chloride
NAD^+	Nicotinamide Adenine Dinucleotide
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NEFA	Non-esterified Fatty Acid
NFkB	Nuclear Factor Kappa-light-chain-enhancer
nm	Nano meter
OGTT	Oral Glucose Tolerance Test
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen Activator Inhibitor
PBG	Postprandial Blood Glucose
PBS	Phosphate buffer saline
PDI	Protein disulphide isomerase
pI	Isoelectric point
PI3K	Phosphatidylinositol 3-kinase
PMF	Peptide Mass Fingerprint
PPAR	Peroxisome proliferator-activated receptor
PTPN2	Protein tyrosine phosphate, non-receptor type 2
PTPN22	Protein tyrosine phosphate, non-receptor type 22
PVDF	Polyvinylidene Fluoride
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SELDI	Surface-Enhanced Laser Desorption/Ionization
SGA	Small for Gestational Age
SNP	Single Nucleotide Polymorphism
SREBP-1c	Sterol regulatory element binding protein-1c
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAP1	Antigen peptide transporter 1
TBST	Tris buffer saline with Tween-20
TCA	Trichloroacetic acid
TCA cycle	Citric Acid Cycle
TFA	Trifluoroacetic acid
TG	Triglyceride
TLR4	Toll-like Receptor 4
TNF	Tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
UBASH3A	Ubiquitin-associated and SH3 domain-containing protein A
UPR	Unfolded Protein Response
UV	Ultraviolet
V	Voltage
V	Volt
v/v	Volume per volume
VAT	Visceral Adipose Tissue

Vhr	Voltage hour
VLDL	Very low-density lipoprotein
VNTR	Variable Number of Tandem Repeats
w/v	Weight per volume
WAT	White Adipose Tissue
WHO	World Health Organization
β-cell	Beta cells
µL	Micro liter

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1. Literature Review

1.1 Introduction of diabetes mellitus

1.1.1 Definition and prevalence

Diabetes mellitus (DM) is a group of metabolic disorders marked by high levels of blood glucose resulting from defects in insulin secretion, insulin action or both (Alberti et al., 1998). It is associated with reduced life expectancy, significant morbidity, increased risk of cardiovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), and diminished quality of life (World Health Organization, 2006).

Recent estimates indicate there were 171 million people worldwide suffering from diabetes in 2000 and this was projected to increase to 366 million by 2030 (Wild et al., 2004). Prevalence of diagnosed and undiagnosed diabetes in the United States in 2007 was estimated to be 23.6 million people or 7.8% of the population of all ages, of which 5.7 million people were undiagnosed (Centers for Disease Control and Prevention, 2007). The American Diabetes Association (ADA) estimated that the national costs of diabetes in the USA for 2002 was US$132 billion, and the figure would increase to US$192 billion in 2020 (American Diabetes Association, 2003). In Hong Kong, it is estimated that about 10% of the population is suffering from diabetes (Diabetes Hong Kong, http://www.diabetes-hk.org). A recent review

reported that the percentage of undiagnosed diabetes in Hong Kong and Taiwan combined is 52.6% (Wong et al., 2006).

1.1.2 Diagnosis and classification

Criteria for diagnosis of diabetes and other stages of glucose tolerance have been changed considerably over the last 20 years (National Diabetes Data Group, 1979; World Health Organization, 2006). There are a number of important differences between the ADA and the WHO recommendations which may lead to differences in an individual's classification of glucose tolerance. The ADA adopts fasting plasma glucose as the recommended method for diagnosing asymptomatic diabetes by the ADA while the WHO recommends the oral glucose tolerance test (OGTT), which was discouraged by the ADA in clinical practice for its "inconvenience, less reproducibility, greater cost" (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). The fasting plasma glucose (FPG) value used to define impaired fasting glucose (IFG) also differs. The ADA defines IFG with FPG of 5.6-6.9 mmol/l whereas the defining value of FPG recommended by the WHO ranges from 6.1 to 6.9 mmol/l. Table 1.1 shows the criteria for classification of glucose tolerance status recommended by the WHO and the ADA. It should be noted that individuals with IFG and/or impaired glucose tolerance (IGT) are described as having "prediabetes", which "is a not clinical entity but rather a risk factor for future diabetes and adverse outcomes" (World Health Organization, 2006).

		WHO 1999	ADA 2003
Diabetes mellitus (DM)	Fasting plasma glucose*	≥7.0 mmol/l	≥7.0 mmol/l
		or	or
	2-h plasma glucose**	≥11.1 mmol/l	≥11.1 mmol/l
Impaired glucose tolerance (IGT)	Fasting plasma glucose*	<7.0 mmol/l (if measured)	Not required
		and	
	2-h plasma glucose**	7.8 to 11.0 mmol/l	7.8 to 11.0 mmol/l
Impaired fasting glucose (IFG)	Fasting plasma glucose*	**6.1** to 6.9 mmol/l	**5.6** to 6.9mmol/l
		and	
	2-h plasma glucose**	<7.8 mmol/l (Measurement recommended)	Measurement not recommended

Table 1.1 Diagnostic criteria of different glucose tolerance status according to the WHO and the ADA.
*Fasting is defined as no caloric intake for at least 8h. **Value should be obtained during an OGTT performed as described by the WHO, using a glucose load containing the equivalent of 75g anhydrous glucose dissolved in water. Source: Alberti et al., 1998; The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003.

Diabetes mellitus is classified into different etiological types according to the defects, disorders or processes leading to the development of diabetes mellitus. There are four main types of diabetes: type 1 diabetes mellitus, type 2 diabetes mellitus, other specific types and gestational diabetes mellitus.

Type 1 diabetes mellitus (T1DM), previously known as insulin-dependent diabetes mellitus or juvenile-onset diabetes mellitus, develops from autoimmune destruction of pancreatic β-cells, leading to absolute insulin deficiency. This form of diabetes accounts for only 5-10% of all diagnosed cases of diabetes in adults (Centers for Disease Control and Prevention, 2007). The rate of β-cell destruction and the progression to ketoacidosis are variable. T1DM is fatal and patients must undergo

insulin treatment for survival. There is currently no clinically useful preventive measure against the development of T1DM.

Type 2 diabetes mellitus (T2DM), previously referred to as non-insulin-dependent diabetes mellitus or adult-onset diabetes mellitus, results from insulin resistance and relative insulin deficiency. It accounts for about 90-95% of all diagnosed cases of diabetes. Patients of T2DM do not need insulin treatment for survival, at least initially, and ketoacidosis seldom develops spontaneously in T2DM (American Diabetes Association, 2009).

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Prevalence of GDM may range from 1 to 14% of all pregnancies, depending on the population studied and the diagnostic tests employed (American Diabetes Association, 2004). If untreated, GDM increases the risk of macrosomia (baby being too large for gestational age) of infants (HAPO Study Cooperative Research Group, 2008). Neonates are also at an increased risk of hypoglycemia, jaundice, high red blood cell mass (polycythemia) and low blood calcium (hypocalcemia) and magnesium (hypomagnesemia) (Jones, 2001).

1.1.3 Symptoms and complications

Beside hyperglycemia, classic symptoms of diabetes include frequent urination (polyuria) and increased thirst (polydipsia). T1DM patients may also experience

blurred vision, frequent hunger, unusual weight loss and extreme fatigue and irritability (Alberti et al., 1998). All these symptoms may also appear on T2DM patients, though much more slowly and subtle, except significant weight loss. In severe cases, ketoacidosis or a non–ketotic hyperosmolar state may develop and lead to stupor, coma and even death. Often symptoms are not severe, or may be absent, and consequently long-term hyperglycemia may be sufficient to cause pathological and functional changes before diagnosis is made. Long-term DM may lead to progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including erectile dysfunction. People with diabetes are also at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease (Alberti et al., 1998).

1.1.4 Cause and risk factors

T1DM is an autoimmune disorder in which the insulin-producing pancreatic β-cells are subject to the attack of the host immune system. To date, the cause of T1DM is still not fully understood but both genetic and environmental factors should be taken into account. The lifetime risk of T1DM of an individual in a general population is quoted as 0.4% (Mehers et al., 2008) whereas the sibling risk is 6%, 15 times higher than one in a general population (Risch, 1987). In a population study in Finland, the concordance rates of T1DM in monozygotic twins and dizygotic twins were 27% and

3.8% respectively (Hyttinen et al., 2003), indicating the importance of environmental factors in the trigger of T1DM in genetically susceptible individuals. Studies of the environmental factors and genetic-environmental interactions involved in the development of T1DM are ongoing, with specific emphasis on diets, infections, gestational events and even socioeconomic factors (The Environmental Determinants of Diabetes in the Young, http://teddy.epi.usf.edu).

Different strategies have been used in attempt to identify T1DM susceptibility genes. In 1970s, association between T1DM and human leukocyte antigen (HLA) class II on chromosome 6p21.3 (IDDM1) was shown (Thomsen et al., 1975). It is now considered that HLA-mediated susceptibility represents 50% of the genetic susceptibility to T1D (Ounissi-Benkalha et al., 2008). The second locus found to be associated with T1DM, IDDM2, is a variable number of tandem repeat region in the promoter of the INS gene (insulin) (Bell et al., 1984; Bennett et al., 1995). Advancement in technology makes whole genome screens possible in 1990s. Areas of the genome linked to T1D were assigned IDDM numbers and IDDM 1-18 have been allocated to various regions of the genome (Mehers et al., 2008). However, not all loci's association with T1DM was reproducible in larger scale genome wide association studies which operate at a much higher level of statistical power (Concannon et al., 2009; Grant et al., 2009). Table 1.2 summarizes some known loci/genes associated with T1DM.

Locus	Gene	Function	Allele(s)/SNP(s)/VNTRs linked to T1DM
Chromosome 6p21.3 (IDDM1)	Human leukocyte antigen (HLA)	Regulation of immume system	HLA class II DQB1*0302 on the DR4 haplotype and DQB1*0201 on the DR3 haplotype (DR2-DQB1*0602 is protective)
Chromosome 11p15.5 (IDDM2)	Insulin (INS)	Insulin production	Short VNTR class I. (Longer class III are protective)
Chromosome 2q33 (IDDM12)	Cytotoxic T-lymphocyte-associated protein 4 (CTLA4)	Negative regulation of T-cell activation	SNP rs3087243
Chromosome 1p13	Protein tyrosine phosphate, non-receptor type 22 (PTPN22)	Regulation of T-cell activation	SNP C1858T; SNP rs2488457 C-G
Chromosome 10p15.1 (IDDM10)	Interleukin 2 receptor, alpha (IL2RA) / CD25	Binding of interleukin 2 for regulation of T-cell proliferation	SNP ss52580101, C-A; SNP rs11594656, T-A
Chromosome 16p13.2	C-type lectin domain family 16, member A (CLEC16)/ KIAA0350	Cell surface receptor in immune cells	SNP rs12708716, A-G; SNP rs725613, A-C; SNP rs17673553, A-G; SNP rs2903692, A-G
Chromosome 6q15	Basic leucine zipper transcription factor 2 (BACH2)	Acting as transcriptional activator or repressor	SNP rs3757247
Chromosome 21q22.3	Ubiquitin-associated and SH3 domain-containing protein A (UBASH3A)	Regulation of T-cell activation	SNP rs9976767
Chromosome 18p11.3-p11.2	Protein tyrosine phosphate, non-receptor type 2 (PTPN2)	Regulation of cytokine-induced pancreatic β-cells apoptosis	SNP rs1893217; SNP rs478582

Table 1.2 Some candidate loci/genes linked to T1DM.

Abbreviations: SNP-single nucleotide polymorphism; VNTR-variable numbers of tandem repeats

Source from Bottini et al., 2004; Kawasaki et al., 2007; Wellcome Trust Case Control Consortium, 2007; Hakonarson et al., 2007; Grant et al., 2009; Todd et al., 2007.

T2DM arises as a result of insulin resistance of the body. It is a multifactorial disease and the etiology and molecular basis of insulin resistance is still unknown. Common risk factors of T2DM include prior glucose intolerance (IGT and/or IFG), prior gestational diabetes, family history of type 2 diabetes, hyperinsulinemia, history of dyslipidemia, obesity and/or central obesity, sedentary lifestyle, high-fat diet, history of hypertension, low birth weight and so on.

Hu et al. carried out a 16-year study following up the diets, lifestyle and development of T2DM of about 85000 female nurses (Hu et al., 2001). Obesity was shown to be the most important factor. Women with BMI between 30.0 kg/m^2 and 34.9 kg/m^2 or BMI over 35.0 kg/m^2 had a 20-fold or 40-fold risk of becoming diabetic respectively, compared to women whose BMI was below 23.0 kg/m^2. Weekly exercise for at least 7 h per week reduced the risk of T2DM by 39% compared to those who exercised less than 0.5 h per week. Smoking of >14 cigarettes per day increased diabetic risk by 39% whereas alcohol intake >10g/day lowered the risk by 41%. Diet high in cereal fiber and polyunsaturated fat and low in saturated and trans fat and glycemic load could also reduce the risk of T2DM.

A 10-year study involving a cohort of about 40000 Dutch men and women was performed to investigate the association of coffee or tea consumption with the risk of T2DM. It was found that consumption of at least 3 cups of coffee and/or tea was associated with reduced risk of T2DM, and this association could not be explained

by blood pressure or intake of magnesium, potassium or caffeine (Van Dieren et al., 2009).

There are more and more data supporting the link of being small for gestational age (SGA) and rapid infant "catch-up" growth to higher risk of developing T2DM and metabolic syndrome (reviewed by Vaag, 2009). The "thrifty phenotype hypothesis" or the Barker's Hypothesis states that reduced fetal growth is strongly associated with a number of chronic conditions including coronary heart disease, stroke, diabetes and hypertension later in life and this increased susceptibility results from adaptations of the fetus to in utero environment of limited nutrient supply (Barker, 1997). Other studies also showed that fast postnatal "catch-up" growth may have adverse effects on the metabolic health of SGA babies. The potential state of cellular hunger in SGA infants at birth may facilitate a more efficient nutritional uptake and energy metabolism and therefore a higher postnatal growth rate. However, breastfeeding for 3-6 months is associated with reduced risk of T2DM and metabolic syndrome (Vaag, 2009).

1.1.5 Prevention and treatment

To date, there is no clinically effective measure for preventing T1DM. For the prevention of T2DM, lifestyle interventions like having a diet low in saturated fats and high in polyunsaturated fats and cereal fibers, regular exercise can reduce the long-term risk of developing T2DM and cardiovascular disease (Li et al., 2008;

Risérus et al., 2009). Screening high-risk subjects for diabetes allows early detection and prompt treatment and thus reduces the progression of diabetes and its complications.

For treatment of T1DM, insulin replacement by injection or insulin pump is necessary for survival, together with careful monitoring of blood glucose levels. Pancreas or islet cells transplantation is also an alternative therapy. For treating T2DM, in addition to medications and self-monitoring of blood glucose levels, dietary management and exercises are also important for controlling blood glucose level and prevention of complications.

1.2 The role of adipose tissue in pathophysiology of T2DM

Obesity is a worldwide epidemic and is one of the most important risk factors of T2DM (Hu et al., 2001). It is associated with an increase in adipose tissue mass and regional distribution of fat tissue. Adipose tissue has long been regarded as an inert storage depot for storing dietary energy in the form of triglycerides within lipid droplets in adipocytes. Since the discovery of leptin (Zhang et al., 1994), more and more adipokines have been discovered and the role of adipose tissue as an endocrine organ is now well established. Different hypotheses have been suggested in attempt to explain the link between obesity and insulin resistance and/or T2DM. The "Randle's cycle" introduces the reciprocal relationship between fatty acid oxidation

and glucose oxidation whereas the "ectopic fat storage hypothesis" suggests that the deposition of lipid in insulin-target tissues causes insulin resistance. Other hypotheses include secretion of adipokines by adipose tissues, the oxidative stress and low-grade inflammatory state of obese adipose tissues and endoplasmic reticulum (ER) stress.

1.2.1 Randle's glucose-fatty acid hypothesis

Plasma non-esterified fatty acids (NEFAs) or free fatty acids (FFAs) level is elevated in obese individuals. The adipocyte FFA balance of obese individuals remains negative even postprandially, i.e. the adipocytes fail to uptake dietary fatty acids and keep on releasing NEFA (Frayn et al., 1996). Randle et al. demonstrated that enhanced NEFA oxidation inhibited glucose metabolism in body situations with lipid excess. It was proposed that the increased plasma NEFAs augmented their cellular uptake by mass action and induced their mitochondrial beta-oxidation, winning their competition with glucose as energy source. Clinical study demonstrated that NEFAs excess impaired insulin action on liver (Ferrannini et al., 1983). The utilization of NEFAs might lead to hepatic intracellular accumulation of acetyl-CoA, which stimulates gluconeogenesis and glycogenolysis and thus worsening hyperglycemia. It was also found that prolonged exposure to NEFAs suppressed glucose-induced insulin secretion (GIIS) from pancreatic beta cells (Unger, 1994). The accumulation of acetyl-CoA from beta-oxidation within beta cells may block glycolysis, which

provides to prerequisite ATP for triggering GIIS. Chronic exposure to NEFA excess eventually impairs insulin secretion. Ceramide synthesis due to NEFA utilization also affects beta cell function and promotes apoptosis (Unger, 1994).

1.2.2 Ectopic fat storage hypothesis

Adipose tissue is the lipid storage depot in our body with buffering effect of dietary fat entering the circulation. Adipocytes take up lipids from fat-rich plasma lipoproteins with lipoprotein lipase and store triglycerides by lipogenesis under the effect of insulin but they can also mobilize the stored fat in the form of NEFAs by lipolysis with hormone sensitive lipase when energy is needed elsewhere. In obesity, the buffering capacity of adipocytes decreases as the cells are already overloaded with triglyceride (Goossens, 2008). The energy surplus is then stored as fat in other tissues and organs like liver, skeletal muscles and pancreatic cells (Ravussin et al., 2002).

NEFAs from lipolysis of insulin-resistant adipocytes stimulate lipid flux into liver, enhance lipogenesis and thus result in increased VLDL secretion and hepatocellular lipid accumulation. Glycolysis and beta-oxidation are also enhanced, leading to elevation of acetyl-CoA which enters either TCA cycle or ketogensis and generation of ROS which leads to mitochondrial dysfunction (Szendroedi et al., 2009). The influx of acyl-CoA results in accumulation of signaling intermediates, diacylglycerol (DAG) and ceramide, which inhibits insulin signaling in hepatocytes and thus

promoting gluconeogenesis even under the presence of insulin. Insulin resistance subsequently develops.

NEFAs cause elevation of acetyl-CoA and mitochondrial dysfunction in myocytes of skeletal muscles in a similar way to what they do in hepatocytes. In addition, the rising acyl-CoA pool in myocytes also leads to elevated DAG and ceramide, which blocks insulin signaling resulting in decreased translocation of the GLUT4 and phosphorylation of glucose to glucose-6-phosphate in skeletal muscles. This makes the skeletal muscles fail to take up glucose under insulin action and causes insulin resistance in skeletal muscles.

Excessive triglyceride stores in beta cells and adipocyte infiltration of pancreatic islets are believed to cause insulin secretion defects in T2DM (Szendroedi et al., 2009). It is suggested that hormone sensitive lipase in beta cells promotes the accumulation of ceramide which causes beta cell apoptosis (Unger, 1994).

1.2.3 Adipose tissue as an endocrine organ

Since the discovery of leptin, dozens of adipocyte-secreted factors have been identified and more are yet to be discovered. These adipokines have various effects and some profoundly affect insulin sensitivtiy and might potentially link obesity with T2DM.

Leptin level increases with fat mass. In neuropeptide Y neurons, it regulates appetites by providing a sense of satiety so as to restrict food intake and enhance energy

expenditure. In skeletal muscles, leptin stimulates AMPK-mediated reduction of ectopic lipid content (Winder, 2001). In hepatocytes, leptin is believed to have additive effects with insulin in glycogen storage enhancement and glycogenolysis inhibition by which hyperglycemia is prevented (Aiston et al., 1999). However, in obesity, leptin's action seems to be selectively disturbed, resulting hyperleptinaemia. The central appetite-suppressant effect would be impaired. A direct lipolytic effect of hyperleptinaemia on adipocytes was also shown (Martínez et al., 2000). Prolonged hyperleptinaemia would induce pancreatic beta cells interleukin (IL)-1β production, reducing insulin secretion and enhancing apoptosis of beta cells.

The insulin-sensitizing and anti-inflammatory properties of adiponectin make it a "promising" adipokine secreted by adipocytes. The amelioration of insulin resistance by adiponectin was found to be AMPK-mediated as adiponectin regulates lipid metabolism (increases fuel oxidation and reduces NEFA influx/ectopic fat storage) and carbohydrate metabolism (improves hepatic insulin sensitivity and peripheral glucose uptake) (Yamauchi et al., 2002). Unfortunately, the circulatory level of adiponectin decreases with increasing body mass and visceral fat mass (Weyer et al., 2001) and adiponectin receptors are markedly down regulated under the state of hyperinsulinaemia. It may be due to adipose tissue hypoxia (Hosogai et al., 2007) or the action of other adipokines that are overexpressed in obesity, including TNF-α and IL-6 (Bruun et al., 2003).

Proteins involved in vascular homeostasis, like angiotensinogen and plasminogen activator inhibitor (PAI-1), and proteins involved in lipid metabolism, e.g. retinol binding protein and cholestertyl ester transfer protein, also belong to adipokines. Classical cytokines, like TNF-α, IL-1, IL-6, IL-18, which are associated with immunity and inflammation, were also secreted by adipocytes (Trayhurn, 2005; Badman et al., 2007).

1.2.4 Low-grade inflammation

The identification of TNF-α in adipose tissue of experimental animals (Feinstein et al., 1993; Hotamisligil et al., 1993) and the discovery of macrophage infiltration in adipose tissue in obesity (Weisberg et al., 2003) evidenced the association of inflammation and obesity and insulin resistance. A recent report found a remarkable shift in the pool of adipose tissue macrophages (ATM) from the alternatively-activated, anti-inflammatory M2 type to the classically-activated, proinflammatory M1 type in obese mice due to spatial and temporal differences in the recruitment of distinct ATM subtypes (Lumeng et al., 2008).

Adipose tissue is the site of production of several proinflammatory factors like IL-6 and monocyte chemotactic protein-1 (MCP-1) (Rotter et al., 2003; Sartipy, P., 2003) and the regulation of secretion of these proinflammatory factors by increasing adiposity further substantiated the hypothesis of an on-going low-grade inflammation during the development of obesity and insulin resistance (Karalis et al., 2009). TNF-

α, a well-known cytokine involved in inflammatory response, increases IL-6 production in 3T3-L1 adipocytes (Ruan et al. 2002), inhibits the differentiation of human adipocyte precursor cells and 3T3-L1 cells (Petruschke et al., 1993), induces apoptosis in human pre-adipocytes and mature adipocytes (Prins et al., 1997), increases lipolysis in human and 3T3-L1 adipocytes and promotes release of free fatty acids to the circulation (Zhang et al., 2002), induces insulin-resistance in visceral adipocytes by impairing insulin-stimulated glucose uptake and insulin signaling at the insulin receptor substrate (IRS)-1/AKT level possibly via activation of c-Jun N-terminal kinase (JNK) 1/2 (Fernández-Veledo et al., 2009) and induces peripheral insulin resistance in human in vivo (Plomgaard et al., 2005). IL-6 was also found to stimulate lipolysis and fat oxidation in humans (van Hall et al., 2003), to inhibit insulin-induced glycogen synthesis in human hepatocytes (Senn et al. 2002) and was also suggested to stimulate hepatic production of C-reactive protein (Goossens, 2008).

Some signaling pathways linking insulin resistance to inflammation have been identified. JNK has been regarded as one of the key signaling molecules involved. Elevation of JNK activity was reported in various tissues in T2DM patients and in animal models of obesity and diabetes (Hirosumi et al., 2002; Yang et al., 2008). Activation of JNK increases serine phosphorylation of IRS-1 and impairs the insulin signaling pathway which involves the activation of the phosphatidylinositol 3-kinase

(PI3K pathway). Knockdown of JNK1 in liver of diet-induced-obese mice significantly reduces blood glucose and insulin levels and enhances the insulin signaling (Yang et al., 2007). Activation of glycolysis, mitochondrial biogenesis, fatty acid oxidation, oxidative phosphorylation and TCA cycle was also resulted in genome-wide gene expression analysis after knockdown of hepatic JNK1. Another important signaling molecule involved is IκB kinase β (IKKβ). Activation of IKKβ causes the induction of NFκB, a transcription factor responsible for upregulation of proinflammatory factors. Activation of IKKβ/ NFκB and the subsequent induction of proinflammatory cytokines can be resulted from binding of free fatty acids to toll-like receptor 4 (TLR4) (Song et al., 2006). Blocking NFκB in obese mice with sodium salicylate could reverse insulin resistance (Yuan et al., 2001). High-dose aspirin was found to lower blood glucose in T2DM in clinical observations for many years and its effect is now considered to be mediated by the inhibitory effect of salicylates on IKKβ (Kopp et al., 1994).

1.2.5 Endoplasmic reticulum (ER) stress

The ER is the site of triglyceride (TG) droplet formation (Wolins et al., 2006). Under cellular stress, unfolded protein response (UPR) would be triggered to cope with the misfolded proteins and JNK activation would be resulted, which is linked to inhibition of insulin signaling (Gregor et al., 2007). Adipose tissue of obese ob/ob mice shows signs of ER stress and UPR activation (Ozcan et al., 2006) accompanied

by an increase in JNK activity. Administration of chaperones that block ER stress activation was found to reverse insulin resistance (Ozawa et al., 2005; Ozcan et al., 2006), making the adipocyte ER another promising therapeutic target for obesity- and insulin resistance-related pathologies.

1.3 Use of berberine in the treatment of T2DM

Berberine is a kind of isoquinoline alkaloids found in plants like Berberis, goldenseal (*Hydrastis canadensis*), and *Coptis chinensis*, usually in the roots, rhizomes, stems, and bark. It is the major bioactive component of Chinese medicine Rhizoma Coptidis (Huanglian). As a Traditional Chinese Medicine, the major therapeutic activity of Rhizoma Coptidis is for the treatment of infection, diarrhea and inflammation, as infectious disease were more popular in ancient China (Yin et al., 2008b). The anti-diabetic effect of berberine was found when berberine was used to treat diarrhea of diabetic patients (Yin et al., 2008b) and it has aroused great interest among physicians and researchers.

1.3.1 Efficacy of berberine in treating diabetes

There are substantial reports of the use of berberine in the treatment of diabetes in Chinese literatures in the past 2 decades (Ni, 1988; Xie et al., 2005; Wei et al., 2004). For hypoglycemic effect, one clinical study has shown that administration of berberine (0.5g, three times a day) reduced fasting blood glucose (FBG) (from 10.6 ±

0.9 mmol/l to 6.9 ± 0.5 mmol/l), postprandial blood glucose (PBG) (from 19.8 ± 1.7 to 11.1 ± 0.9 mmol/l) and HbA1c level by 2 % in adult patients with newly-diagnosed T2DM (Yin et al., 2008c). Another group reported that daily administration of 1g berberine for 3 months reduced FBG and PBG from 7.0±0.8 to 5.6±0.9 mmol/l and from 12.0±2.7 to 8.9±2.8 mmol/l, HbA1c from 7.5±1.0% to 6.6±0.7% in adult patients newly diagnosed with T2DM. Body weight reduction was also observed. Mild to moderate constipation was reported in only 5 out of 60 patients in berberine-treated group (Zhang et al., 2008). For the effect on lipid metabolism, plasma triglyceride decreased from 2.51±2.04 to 1.61±1.10 mmol/l with berberine, total cholesterol decreased from 5.31±0.98 to 4.35±0.96 mmol/l and LDL-cholesterol decreased from 3.23±0.81 to 2.55±0.77 mmol/l while the data from placebo group showed no significant changes. It was concluded that berberine is effective and safe for treating T2DM and dyslipidaemia (Zhang et al., 2008).

1.3.2 Berberine on glucose and lipid metabolism of animals

Berberine was shown to lower weight gain, improve insulin sensitivity and reduce blood glucose in both dietary and genetic animal models of T2DM. In obese rats fed on a high-fat diet, berberine was reported to lower body weight, FBG, PBG, fasting insulin and serum triglyceride level (Yin et al., 2008a; Yin et al., 2004). In T2DM rats induced by high-fat diet and low dose of streptozotocin (STZ), berberine significantly decreased FBG and improved insulin tolerance (Yin et al., 2004; Leng

et al., 2004). In db/db mice, body weight and plasma triglyceride was decreased without significant change in food intake and glucose tolerance was improved by berberine. Fat mass of berberine-treated mice also decreased, with reduced adipocyte size (Lee et al, 2006). Berberine was also reported to restore pancreas damage induced by alloxan in Wistar rats (Tang et al., 2006).

1.3.3 Inhibition of adipogenesis

Berberine suppresses adipocytes differentiation and reduces lipid accumulation in 3T3-L1 adipocytes (Lee et al., 2006; Huang et al., 2006; Choi et al., 2006). Berberine treatment suppressed the mRNA expression of several lipogenic genes including PPARγ, C/EBPα, SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, acyl-CoA synthase, lipoprotein lipase, aP2 and CD36. Suppression of PPARγ, C/EBPα and their upstream regulator, C/EBPβ was observed together with inhibition of 3T3-L1 adipocyte differentiation (Huang et al., 2006).

1.3.4 Activation of AMP-Activated Protein Kinase (AMPK)

AMPK is a key regulatory enzyme involved in energy homeostasis. It has been suggested to be a potential therapeutic target in the treatment of obesity and T2DM (Hwang et al., 2009). Various studies reported that berberine activates AMPK by inducing Thr-172 phosphorylation of AMPK in different cells including 3T3-L1 adipocytes and L6 myotubes and it is believed to be responsible for berberine-enhanced glycolysis, glucose consumption and glucose uptake (Yin et al., 2008a; Lee

et al., 2006; Turner et al., 2008). It was also suggested that the berberine-induced AMPK activation is likely to be the consequence of increased AMP/ATP ratio due to mitochondrial inhibition (Yin et al., 2008a).

1.3.5 Mitochondrial inhibition

In addition to its anti-diabetic, anti-infection property, berberine was also shown to induce cell cycle arrest and apoptosis in several malignant cell lines. Pereira et al. showed that berberine is accumulated in the mitochondria of mouse melanoma cell line and inhibits oxygen consumption of mitochondria, especially complex I (Pereira et al., 2007). Yin et al. suggested that berberine inhibited glucose oxidation in mitochondria in 3T3-L1 adipocytes and L6 myotubes as berberine reduced oxygen consumption in both types of cells in a dose-dependent manner with an increase in lactic acid concentration in culture media (Yin et al, 2008a). Turner et al. also showed that berberine inhibits mitochondrial respiratory complex I but not complex II in L6 myotubes (Turner et al., 2008). The mitochondrial inhibitory effect of berberine causes an increase in AMP/ATP ratio, which is causally linked to AMPK activation.

1.4 Introduction of proteomics

The term "proteomics" was coined in 1995 and defined as the large-scale characterization and quantitation of "the entire protein complement expressed by a

genome, or by a cell or tissue type" (Anderson and Anderson, 1996; Wilkins et al., 1995). Its goal is to achieve a global and integrated biological view by studying all the proteins of a cell or individual rather than one by one individually (Graves and Haystead, 2002). Proteomics technology can be divided into two main categories, namely expression proteomics and structural and functional proteomics. Expression proteomics studies the global protein expression in cells/tissues quantitatively. Structural and functional proteomics involves the characterization of protein complexes, investigation of structure and function of proteins, protein-protein interaction studies, protein polymorphisms and modifications and the study of proteins localization in a specific cellular organelle, also known as "cell map" (Blackstock et al., 1999; Meri and Baumann, 2001).

1.4.1 Why proteomics?

Genome sequencing of many organisms had been completed in the last decade. However, genome sequences only tell the static inherited information of an organism but not the dynamic gene expression and cell processes in different cells. It is generally believed that proteins, but not genes, are responsible for cell phenotypes and are the real mediators of physiological functions (Graves and Haystead, 2002; Baggerman et al., 2005). To understand when and where certain genes would be turned on, both mRNA expression and protein abundance are of interest. There have been extensive studies on mRNA expression using technologies like DNA

microarray. However, different studies had shown that there is a poor correlation between intracellular mRNA abundance and the respective protein levels in cells (Anderson and Seilhamer, 1997; Celis et al., 2000). Thus, mRNA expression should not be regarded as a direct reflection of cellular protein content.

In addition, proteome is far more complex and dynamic than both genome and transcriptome, which refers to the total mRNA expression in cells. Many different protein isoforms can be formed from one gene as premature mRNA is subject to posttranscriptional control like alternative splicing, polyadenylation and mRNA editing (Newman, 1998). Translational modifications also regulate the translation of mRNA to protein peptides (Jansen et al., 1995). Proteins formed are then subject to posttranslational modification. It was estimated that there are up to 200 different types of posttranslational protein modification (Krebs, 1994). Proteins levels are also regulated by proteolysis (Kirschner, 1999) and compartmentalization (Colledge and Scott, 1999). Wilkins et al. estimated the average number of protein forms per gene to be one or two in bacteria, three in yeast, and three or more in human (Wilkins et al., 1996). Other group of scientists later estimated that a single mammalian gene can encode on average of six different protein species (Dowsey et al., 2003).

1.4.2 Gel-based proteomics: Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE), originated in the 70's by O'Farrell (O'Farrell, 1975), is the classical technique for separating proteins in biological

samples. Coupled with protein identification by mass spectrometry (MS), 2-DE is still the dominating technique of the field as it allows simultaneous separation, visualization and quantitation of a large number of proteins in a biological sample at one time.

In the first dimension, proteins are separated according to their isoelectric points on an immobilized pH gradient (IPG) (Gorg et al., 2000). Proteins move along the gradient until they reach the pH of their isoelectric point, a point at which the net charge of a protein is zero. This is called isoelectric focusing. Proteins are then further separated in the second dimension, SDS-PAGE, according to their molecular weights (MWs). A distinct distribution of protein spots can be obtained for each kind of biological sample and this protein "map" can act as a biological fingerprint. With different gel sizes and pH gradients, an average of 1000-2500 protein spots can be resolved simultaneously. The number can even be expanded to over 10000 in some conditions after optimization (Carrette et al., 2006).

2-DE has undergone many different modifications since its invention, including the use of pH gradient instead of ampholyte-based gradient in 1970's (Gorg et al., 2000), optimization of protein extraction and solubilizations from different biological samples (Rabilloud et al., 1997; Wang et al., 2008), the development of narrow-overlapping IPG strips for sample prefractionation and higher resolution (Bjellqvist et al., 1993) and improvements in protein detection methods like the use of

fluorescent dyes such as Sypro Ruby and the introduction of two-dimensional fluorescence difference gel analysis technology (2D-DIGE) (Marouga et al., 2005). Despite the great advancement, there are still many drawbacks and limitations in 2-DE. Not all proteins extracted can be completely resolved in a 2D gel. Large or hydrophobic proteins are difficult to solubilize and thus do not enter the IPG strip well. Proteins with isoelectric point below 3 (too acidic) or above 10 (too basic) are not well separated (Gorg et al., 2000). Low-copy proteins are usually under-represented in 2D gels. In a study of yeast mRNA and protein expression, no low-abundance proteins like regulatory proteins were visible in 2D gels while highly abundant proteins with relatively long half-life were overwhelmingly represented (Gygi et al., 1999).

1.4.3 Gel-free proteomics

To enhance coverage of hydrophobic or large proteins, gel-free proteomics separating protein mixtures by liquid chromatography had been developed. Proteins were first prefactionated and digested into peptides by enzymes like trypsin. Peptides were then further separated by liquid chromatography based on two or more parameters. Yates and coworkers performed strong cation exchange chromatography prior to reverse phase chromatography, separating peptides based on two different parameters, net charge and hydrophobicity (Link et al., 1999). This technique was called multidimensional protein identification technology (MudPIT). To reduce

under-sampling of peptides, consequence of sample complexity and the resulting random sampling for MS/MS, targeted approaches like isotope-coded affinity tag (ICAT) (targeting cysteine peptides) (Gygi et al., 1999) and combined fractional diagonal chromatography (COFRADIC) (targeting cysteine, methionine or N-terminal peptides) (Gevaert et al., 2002; Gevaert et al., 2003) were successively developed. These techniques allow peptide quantitation as well as study of posttranslational modifications (Gevaert et al., 2006).

1.4.4 Mass spectrometry

Mass spectrometry (MS) is a powerful tool for protein identification. The separated protein spots from 2-DE usually undergo enzymatic digestion with trypsin into peptides, masses of which can be measured by mass spectrometry. Protein can be identified by its own set of unique peptide masses, called peptide mass fingerprinting (PMF) (Kussmann et al., 2005).

There are three types of MS commonly used for protein identification, namely matrix-assisted laser desorption / ionization (MALDI), Surface-Enhanced Laser Desorption/Ionization (SELDI) and electrospray ionization (ESI) mass spectrometry (MS). In MALDI-MS, a matrix, an organic acid with fairly low molecular weight and strong optical absorption in UV, has to be applied. It co-crystallizes with the digested protein sample and helps the ionization of peptide. When a laser is pulsed onto the crystalline surface, and concomitantly desorbs and ionizes the peptide into

singly-charged peptide ions (Duncan et al., 2008). SELDI-MS is a variation of MALDI-MS. In SELDI-MS, biological samples are spotted on surface with specific affinity, which acts as a solid-phase extractor under chemical and/or biochemical modifications. The selective retention of proteins makes SELDI-MS advantageous in biomarker discovery and clinical proteomics (Tang et al., 2004). In ESI-MS, electrospray disperses liquid sample into fine aerosol. Peptides are multiply protonated and then dispersed from solution to gas phase yielding multiple charged ions (Kussmann et al., 2005). After ionization, determination of peptide mass is by measuring the time for peptide ions flying to the detector (time-of-flight detector, ToF).

By comparing the PMF of a protein sample with the theoretical PMFs in database, known proteins can be identified. However, for novel protein samples without a sequence record in database, MS/MS approach can be adopted to read out the partial amino acid sequence by selectively fragmentizing the peptides isolated in MS (Kussmann et al., 2005).

1.4.5 Proteomics as tool for diabetes research

With the advancement of proteomic technology, increasing number of researches focusing on protein profiling of diabetes-related organs/tissues have been performed, including but not limited to insulin target tissues: adipose tissue, liver and skeletal muscles, plasma or sera of diabetic subjects/models and even saliva.

As insulin target tissues, adipose tissue, muscle cells and hepatocytes are all by different means involved in T2DM and insulin resistance. In order to detect the effect and markers of high fat diet and insulin resistance, Schmid et al. performed a 2-DE study (Schmid et al., 2004). Muscle, white and brown adipose tissues and liver were taken from normal mice fed with a high fat or a normal chow diet. Many differentially expressed proteins between obese and lean mice were detected, out of which more than half were found in brown adipose tissue. Several of these proteins were stress and redox proteins. In addition, enzymes involved in glycolysis and respiratory chain, such as α-enolase and ubiquinol-cytochrome C reductase complex core protein 1, were down-regulated in high fat fed animals. This hinted at a competition between fatty acid and glucose as an oxidative fuel source under high fat diet. The authors concluded that high fat fed animals increase their energy expenditure to defend against weight gain by regulating the brown adipose tissue proteome, which in turn leads to energy dissipation.

To unravel the molecular basis of the relationship between mitochondrial dysfunction, lipolytic responsiveness, and insulin resistance in adipocytes, Cho et al. lipolytically stimulated mouse adipocyte cell line, 3T3-L1, with isoproterenol or TNF- α and isolated the mitochondrial fractions for 2-DE and Western blot study (Cho et al., 2009). Isoproterenol treatment up-regulated several proteins involved in fatty acid oxidation, TCA cycle and oxidative phosphorylation while the up-

regulating effect of TNF-α on those proteins was less significant. However, both isoproterenol and TNF-α treatment significantly down-regulated proteins involved in oxidative stress dissipation, suggesting that both isoproterenol and TNF- α-treated mitochondria experience similar type of oxidative damage from lipolytic stimulation. But isoproterenol-treated mitochondria may overcome this damage through the high expression of prohibitin, which was not detected in TNF-α-treated mitochondria.

Analysis of liver protein expression profiles connected to hepatic insulin resistance was performed using an insulin resistant hamster model (Morand et al., 2005). In their search for mechanisms linking development of hepatic insulin resistance and overproduction of atherogenic lipoproteins the authors analysed the proteome of the liver ER. By 2-DE, 34 differentially expressed proteins were identified. Hepatic ER proteins ER60, ERp46, ERp29, glutamate dehydrogenase and TAP1 were all decreased, while alpha-glucosidase, P-glycoprotein, fibrinogen, protein disulfide isomerase, GRP94 and apolipoprotein E were all increased in the insulin resistant animal.

To characterize the metabolic features of mice fed with high-fat diet (HFD) and investigate the impact of leptin upon lipogenesis, Jiang et al. performed 2-DE and compared the protein profiles of white adipose tissues (WAT) of mice fed with standard chow and their littermates fed with HFD (Jiang et al., 2009). Hyperleptinemia was observed at an early stage during the progressive accumulation

of fat mass in HFD-fed mice. In WAT, HFD down-regulated several proteins involved in lipid, fatty acid and carbohydrate metabolism and up-regulated several stress response- or detoxification-related proteins like heat shock cognate 71 kDa protein. Lipogenesis-related enzymes, including ATP-citrate lyase, fatty acid synthase, transketolase and malic enzyme 1, displayed prominent suppression in response to HFD. Western blot results also showed reduced expression of ATP-citrate lyase and fatty acid synthase in liver of HFD-fed mice. However, this suppression of hepatic proteins expression was not observed in HFD-fed db/db mice, which suffer from leptin receptor deficiency and are thus leptin-resistant. The authors suggested the involvement of leptin in adaptive responses to overnutrition by suppressing lipogenesis.

Skeletal muscle plays a role in glucose homeostasis by taking up and utilising glucose in an insulin dependent manner. Hittel et al. characterized cytosolic skeletal muscle proteins in lean and obese individuals. They identified increased levels of adenylate kinase, glyceraldehyde-3-phosphate dehydrogenase and aldolase A in skeletal muscles of obese women compared with lean women (Hittel et al., 2005). Hojlund et al. have recently characterised the human skeletal muscle proteome from healthy subjects by combining 1DGE and HPLC electrospray ionisation tandem mass spectrometry (Hojlund et al., 2008). Nearly a thousand different proteins were identified. Beside muscle contractile proteins, many identified proteins were

involved in glucose and lipid metabolism. Mitochondrial proteins accounted for a fairly large percentage (22%) of all proteins identified. Enzymes involved in cell signaling and calcium homeostasis pathways were also found.

Since all tissues are in contact with blood, proteins secreted or leaking from the different tissues are reflected in the circulation. Blood sample is relatively easy and safe to collect, making a good source to look for biomarkers for diagnosis or screening. Haptoglobin level was found to be elevated in serum of T2DM patients when compared to control serum. Several proteins involved in the inflammatory response, like α -2 macroglobulin, fibrinogen, complement C3 and C1 inhibitor, were also altered in serum of patients with insulin resistance (Zhang et al., 2004). Increased serum level of Apolipoprotein CIII was found in T2DM patients in a recently study (Sundsten et al., 2008) and is also considered as a cardiovascular risk factor (Gervaise et al., 2000).

To identify salivary biomarkers for T2DM, Rao et al. separated salivary proteins with strong cation-exchange chromatography followed by LC-MS/MS (Rao et al., 2009). Quantitation was based on spectral counting, i.e., the total number of tandem mass spectra matched to the protein. Out of 487 proteins identified, a majority of the differently abundant proteins are predicted to have functions in metabolism, followed by the functional categories of development, cell organization and biogenesis, immune function, etc. Inflammatory factors like protease inhibitors CysC, leukocyte

elastase inhibitor, and uteroglobin were increased in saliva of T2DM patients than that of healthy subjects and it is consistent with the chronic inflammatory state in progression of diabetes and metabolic syndrome. A2MG was also elevated in diabetic saliva and Western blot analysis showed progressive elevation of A2MG level in different diabetic stages: with impaired glucose tolerance, with both impaired glucose tolerance and impaired fasting glucose and diagnosed diabetes. It was suggested that A2MG variation could be adopted as a candidate biomarker in prediabetic saliva for screening of preclinical diabetes.

1.5 Objective and significance

Current hypoglycemic agents for treating diabetes have various side effects including like nausea and vomiting for metformin, hypoglycemia for sulphonylureas and unwanted weight gain and even potential heart or liver failure for thiazolidinedione. Berberine, with promising hypoglycemic and weight-lowering effect, appears to have fewer side effects (only mild constipation was reported in clinical cases (Yin et al., 2008c)). This project aimed at understanding the underlying mechanisms of the anti-diabetic effect of berberine in the hope of revealing good therapeutic targets for treating diabetes. In this project, the effect of berberine on visceral adipose tissue of diabetic db/db mice and 3T3-L1 adipocytes was investigated in proteomic approach. Among various target sites of action of berberine, adipose tissue deserves an in-depth

investigation as fat mass reduction was accounted for the weight-lowering effect of berberine. The role of adipose tissue as an endocrine organ and its causal role in the pathophysiology of diabetes are widely recognized with substantial evidences (reviewed above). Metabolic alterations in both in vivo and in vitro models were investigated, followed by proteomic study of the visceral adipose tissues (in vivo) or 3T3-L1 adipocytes (in vitro) and Western blotting, in an attempt to studying the changes in the metabolic pathways and other proteins after berberine treatment.

2. Materials and Methods

2.1 Drug preparation

Berberine hydrochloride (BBR) tablets 100mg (Wah Kin Pharmaceutical Products) were ground and dissolved in warm Milli-Q water (Millipore) to a final concentration 2mg/mL. The solution was filtered through a filter of 0.22μm porosity and stored at -20°C for up to 1 week. The BBR solution prepared was for both animal and cell culture experiment.

2.2 Animal experiment

12-week-old obese db/db mice and lean m+/db mice were obtained from the Laboratory Animal Services Centre of The Chinese University of Hong Kong. The animals were maintained on 12h/12h dark/light cycle and received standard chow (Labdiet) and tap water *ad libitum* during the study unless otherwise specified.

For estimating a safe dose of BBR for db/db mice, a preliminary treatment with different doses of BBR by gastric feeding was performed. Preliminary results showed that high dose of BBR (100mg/kg body weight or above) killed the mice in 2 weeks (data not shown). Yellow BBR powder was found in the gut of dead mice and probably blocked the alimentary canal and killed the mice. Mice treated with

50mg/kg bodyweight BBR appeared healthy and normal with observable weight loss and thus, 50mg/kg body weight was chosen as the optimal dose for the treatment in further experiment. For BBR treatment, 20 obese db/db mice were divided into 2 groups, BBR-treated group and control group. Each mouse in the BBR-treated group was administered with 50mg/kg body weight BBR daily by gastric feeding. Water was fed to the mice in the control group instead. The treatment lasted for 7 weeks. Body weight was measured weekly. In the morning of the last day of treatment period, BBR was administered by gastric feeding as usual. Thirty minutes later (t=0min), fasting blood glucose level of the mice was measured, immediately followed by intraperitoneal injection of D-glucose solution (1g/kg body weight). Blood sample was collected from tail and glucose level was monitored with Bayer Elite Glucometer (Bayer Healthcare). Blood glucose levels were measured at t=45min, t=90min and t=120min respectively. For comparison of protein profiles of the visceral adipose tissues, all the mice were sacrificed by cervical dislocation the next day to collect their visceral white adipose tissues. As the mice were fasted overnight before glucose tolerance test, the mice were allowed access to food one more day so that the visceral adipose tissues collected would be at normal but not fasted state. The tissue samples were ground in liquid nitrogen and stored at -80°C until use. For the comparison of proteome of the visceral adipose tissue of obese db/db mice and that of lean m+/db mice, tissues samples from 5 mice of each group

were collected and pooled together. For the comparison of the visceral adipose tissue of BBR-treated and untreated db/db mice, tissue samples from 8 mice of each group were collected and pooled together.

2.3 Comparison of proteome of visceral white adipose tissue: obese db/db mice vs lean m+/db mice and BBR-treated vs control db/db mice

2.3.1 Protein sample preparation from adipose tissue

Ice-cool n-hexane (Labscan) was added to the ground adipose tissues. The samples were sonicated for 15min with ultrasonic cleaner (Model 5210, Branson) and centrifuged at 13,000g for 10min (Tabletop High-speed Microcentrifuge, Hitachi Koki). The supernatants and fat pads at the top were discarded. Ice-cool 20% trichloroacetic acid (TCA)/acetone was added to resuspend the tissue pellets and the samples were incubated overnight at -20°C for protein precipitation. After incubation, the samples were centrifuged at 15,000g for 10min at 4°C (Tabletop High-speed Microcentrifuge, Hitachi Koki). The protein pellets were washed thrice with ice-cool acetone and briefly dried with SpeedVac (LABCONCO). The protein pellets were resolubilized in rehydration buffer containing 8M urea (PlusOne, GE Healthcare), 2M thiourea, 2% (w/v) CHAPS and 0.002% (w/v) bromophenol blue.

The samples were centrifuged at 15,000rpm for 10min Tabletop High-speed Microcentrifuge, Hitachi Koki) and the supernatants were transferred to another 1.5mL microcentrifuge tube for storage at -80°C before use.

2.3.2 Protein quantitation

Protein concentration of each sample was assayed using PlusOne 2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions. A standard curve was prepared for every quantitation using the 2mg/mL bovine serum albumin (BSA) standard solution provided. The assay range is 0.5-50μg. The standard curve was plotted using protein amount against absorbance at 480nm and the amount of the unknown protein samples can be found out from the standard curve with their absorbance accordingly.

2.3.3 2D Gel electrophoresis

Isoelectric focusing (IEF) was performed using Ettan IPGphor III isoelectric focusing system (GE Healthcare). The samples were quantified by PlusOne 2-D Quant Kit as mentioned and normalized to 100μg. Rehydration buffer and 2% (v/v) Pharmalyte (GE Healthcare) of corresponding pH range were added to bring up to a total volume of 125uL. The prepared sample was applied to the Immobiline DryStrip (7cm, linear pH3-10) by rehydration loading overnight. The first dimension (IEF) was performed at 13500Vhr.

Before the second dimension, the IPG strip was equilibrated at room temperature for 15min in the SDS equilibration buffer containing 50mM Tris-HCl pH8.8, 6M urea (USB), 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue. 1% (w/v) DTT was added prior to use. The strip was then briefly washed with Milli-Q water (Millipore) and further equilibrated at room temperature for another 15 minutes in the SDS equilibration buffer containing 50mM Tris-HCl pH8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue and 2.5% (w/v) iodoacetamide.

The strip was washed with Milli-Q water (Millipore) briefly again and put onto the 4% stacking gels and 12% separating gels with 1mm gel thickness. The strip was covered with 400μL agarose sealing solution with 0.5% (w/v) agarose in SDS electrophoresis buffer containing 25mM Tris-base, 192mM glycine, 0.1% (w/v) SDS. The agarose sealing solution was allowed to polymerize at room temperature. The gels were then run at 80V for 30 with the SDS electrophoresis buffer mentioned above using the electrophoresis system (XCell Surelock mini-vertical electrophoresis system, Invitrogen Life Technologies).

After the second dimension run, the gels were washed with Milli-Q water and then stained with 50mL Coomassie staining solution which contains 45% (v/v) methanol, 10% (v/v) acetic acid, 0.15% (w/v) Coomassie Brilliant Blue R-250 (Sigma) for 1-

2h. The gels were destained in destaining solution containing 45% (v/v) methanol, 10% (v/v) acetic acid in Milli-Q water.

2.3.4 Image analysis

The gels were scanned under visible light at 300dpi using ImageScanner (Amersham Biosciences). Analysis was performed using the computer program, ImageMaster 2D Platinum 5.0 (Amersham Biosciences). Spots were detected automatically with the following parameters: smooth 2, minimum area 6 and saliency 1.5. Manual spot editing was performed if necessary. The same distinct, well-focused spot was marked as "landmark" in all gels. Auto-matching and pair-up was performed using the software, followed by necessary manual editing (addition or deletion of pairs). Spot pairs with over 1.5-fold change in %vol and all-or-none spots without pair on the other gel were identified.

2.3.5 In-gel digestion and protein identification by MALDI-ToF MS

Spot pairs with over 1.5-fold change in %vol and all-or-none spots without pair were excised after image analysis. The spots were first destained with spot-destaining solution containing 50mM ammonium bicarbonate and 50% (v/v) methanol and then dehydrated with acetonitrile (ACN). The ACN liquid was then removed and the spots were completely dried with SpeedVac (LABCONCO). 5μL of 50mM ammonium bicarbonate containing 20ng/μL Sequencing Grade modified trypsin (Promega) was added to each spot for rehydration and protein digestion. The samples

were incubated overnight at 37°C. 4μL extraction buffer (75% v/v ACN and 2.5% v/v TFA) was added to the spots, which were then sonicated for 10min with ultrasonic cleaner (Model 5210, Branson). 0.4μL digested protein sample was then spotted onto the mass spectrometry plate (Applied Biosystems) twice or thrice, depending on the size of the gel spot, and then 0.4μL matrix (x-cyano-4-hydroxycinnamic acid) was spotted once onto the air-dried plate. Mass spectrometry was performed with the 4700 proteomics analyzer (Applied Biosystems). Mass tolerance of 0.5 atomic mass unit and peptide mass tolerance of 50ppm was set in all searches Oxidation of methionines was taken into account for the modification. The peptide mass fingerprint data collected were then searched against the NCBI database with the *Mus* taxonomy selection. The proteins with C.I.% larger than 99 were regarded as confirmed match.

2.4 Cell culture experiment

Stock of mouse pre-adipocytes, 3T3-L1, was a kind gift from Professor Cheung Wing Tai in The Department of Biochemistry of The Chinese University of Hong Kong. The cell line was grown in high-glucose DMEM (Invitrogen Life Technologies) supplemented with 4.93% (v/v) sodium bicarbonate (Invitrogen Life Technologies), 10% (v/v) FBS (Hyclone) and penicillin (100U/mL) (Invitrogen Life Technologies). The cell line was kept in an incubator of 5% CO_2 at 37°C in

humidified atmosphere. For adipocyte differentiation and BBR treatment, 3T3-L1 cells were seeded into 35mm x 10mm tissue culture dishes (Nunclon) or 24-well tissue culture plates (Nunclon). Two days post-confluence (Day 0), cells were grown in differentiation medium with 0.5mM isobutylmethylxanthine (Sigma), 1μM dexamethasone (Sigma) and 10μg/mL insulin (Sigma) dissolved in "standard" DMEM medium for 2 days. Then the cells were changed to insulin medium with 10μg/mL insulin (Sigma) dissolved in "standard" DMEM medium for 2 more days. After that, the cells were grown and re-fed with the "standard" DMEM medium every 2 days. On day 10, >90% of the cells exhibited adipocyte phenotype and were regarded as mature.

In an attempt to investigate whether the in vivo weight-lowering effect of BBR was due to the loss of triglyceride accumulated in mature adipocytes or the inhibition of differentiation in pre-mature adipocytes, both prolonged treatment on mature adipocytes and short-term treatment on pre-mature adipocytes were performed. The former aimed at mimicking the in vivo situation of visceral adipose tissues exposed to long term daily BBR treatment in human, while the latter revealed the inhibitory effect of BBR on differentiation and lipid accumulation of pre-mature adipocytes, as reported in previous studies (Huang et al., 2006; Hu et al., 2009). For prolonged experiments of mature adipocytes, berberine treatment began on day 10 for 72h. For short-term experiments of pre-mature adipocytes, berberine treatment was performed

on day 4 for 24h. 5uM BBR solution was added to DMEM medium for treating both mature and pre-mature adipocytes.

2.5 Oil Red O staining

Oil Red O, with sharp red color and readily binds to lipid, can be used to stain and measure intracellular triglyceride content (Choi et al., 2006). The cells were washed with PBS twice and fixed with 10% formalin at room temperature for 1h. 60% isopropanol (v/v) in Milli-Q water was used to wash the fixed cells, which were then completely air-dried. The cells were then stained with filtered Oil Red O staining solution containing 0.18% (w/v) Oil Red O (Sigma) and 60% (v/v) isopropanol in Milli-Q water at room temperature with gentle shaking for 10min. The staining solution was then removed and the cells were washed in running distilled water for at least 4 times. The red dye retained in the cells was eluted into 100% isopropanol and the absorbance at 540nm of the eluted dye was measured as a semi-quantitative assay of intracellular triglyceride level.

2.6 Glycerol determination

To estimate the rate of lipolysis of the cells after BBR treatment, glycerol content of the culture medium was determined. Higher glycerol content in the culture medium indicates a higher rate of lipolysis of cells. Media of BBR-treated and control 3T3-L1

cells were collected after treatment. The Free Glycerol Reagent (Sigma) with Glycerol Standard solution from the same company (Sigma) as standard was used according to manufacturer's instructions.

2.7 In vitro comparison of proteomes of BBR-treated and control 3T3-L1 adipocytes

2.7.1 Protein sample preparation from 3T3-L1 cells

The cells were washed thrice with PBS and scraped out with a cell scraper (Corning). Ice-cool 20% TCA/acetone was added to the cells and the samples were ultra-sonicated on ice for 10s before overnight incubation at -20°C for protein precipitation. The samples centrifuged at 15000g for 10min at 4°C (Tabletop High-speed Microcentrifuge, Hitachi Koki). The protein pellets were washed thrice with ice-cool acetone and briefly dried with SpeedVac (LABCONCO). The protein pellets were resolubilized in rehydration buffer containing 8M urea (PlusOne, GE Healthcare), 2M thiourea, 2% (w/v) CHAPS and 0.002% (w/v) bromophenol blue. The samples were centrifuged at 15,000g for 10min (Tabletop High-speed Microcentrifuge, Hitachi Koki) and the supernatants were transferred to new 1.5mL microcentrifuge tubes for storage at -80°C before use.

2.7.2 Protein quantitation

Same as section 2.3.2.

2.7.3 2D Gel electrophoresis

Same as section 2.3.3.

2.7.4 Image analysis

Same as section 2.3.4.

2.7.5 In-gel digestion and MALDI-ToF MS

Same as section 2.3.5.

2.8 Western Immunoblotting

2.8.1 Protein sample preparation of BBR-treated and control 3T3-L1

Same as section 2.7.1.

2.8.2 SDS-PAGE

A 10-well polyacrylamide gel containing 4% stacking gels and 12% separating gels with 1mm gel thickness was set. 40μg of the protein samples were mixed with loading dye and heated at 99°C for 5min before being loaded into each well. 4μL of biotinylated protein ladder (Cell signaling technology) was used for each gel. The gels were run in a electrophoresis system (XCell Surelock mini-vertical electrophoresis system, Invitrogen Life Technologies) and run with 80V for 30min and then 180V for 1h using the SDS electrophoresis buffer mentioned in section 2.3.3.

2.8.3 Protein blotting

After running, the gel was washed with Milli-Q water, and placed inside the iBlot™ Dry Blotting System (Invitrogen Life Technologies) together with the provided iBlot Anode Stack (containing the PVDF membrane, 0.2μm, low fluorescence), filter paper soaked in Milli-Q water and the iBlot™ Cathode Stack. Any trapped air bubble that may interfere with efficient protein transfer was carefully removed using the debubbling Roller. Default protocol P3 was used for 7 minutes in order to perform the blotting.

2.8.4 Membrane blocking and antibody incubations

The membrane was incubated in 25mL blocking buffer (TBST containing 2mM Tri-HCl, 15mM NaCl, pH7.5, 0.1% v/v Tween-20 with 5% w/v bovine serum albumin) for 1h at room temperature with gentle shaking. Then, the membrane was washed for 5 minutes with 15mL TBST (2mM Tri-HCl, 15mM NaCl, pH7.5, 0.1% Tween-20) for three times. The membrane was then incubated with primary antibody (1:1000 dilution) (Cell signaling technology) in 5mL TBST with gentle agitation overnight at 4°C. After that, the membrane was washed three times for 5 minutes with 15mL TBST. HRP-conjugated secondary antibody (1:2000 dilution) (Cell Signaling Technology) and HRP-conjugated anti-biotin antibody (1:1000 dilution) (Cell Signaling Technology) in 5mL TBST was then added to detect biotinylated protein markers and the primary antibody. Gentle agitation for 1 hour was allowed at room

temperature. The membrane was washed again for three times for 5 minutes with 15mL TBST.

2.8.5 Detection of Proteins

The membrane was shaked in 4mL LumiGLO® (200µL 20X LumiGLO® and 200µL 20X peroxide in 3600µL Milli-Q water) (Cell Signaling Technology) at room temperature for 2min. The membrane was then wrapped in plastic wrap. Chemiluminescence image detection was performed for 3min using Lumi-Imager F1 (Roche). Detection of band intensity was performed using the computer program, ImageMaster 2D Platinum 5.0 (Amersham Biosciences).

2.9 Statistical analysis

Data were presented as mean±SD. Wherever p-value was provided, paired t-tests were performed and results with p-value<0.05 were considered statistically significant.

3. Results

3.1 Comparison of total protein profiles of visceral adipose tissue of obese db/db and lean m+/db mice

Visceral adipose tissues were collected from 5 obese db/db mice and 5 lean m+/db mice, respectively and pooled together for protein extraction. Four replicates of 2-DE gel were run and proteins showing differential abundance of over 1.5 fold in all 4 sets of gel were picked for mass spectrometry analysis. 2D gel patterns were shown in Fig. 3.1a and 3.1b and proteins of differential abundance were listed in Table 3.1. Several proteins involved in glycolysis were up-regulated in adipose tissue of obese db/db mice compared with that of lean m+/db mice while proteins involved in TCA cycle and oxidative phosphorylation were down-regulated. Proteins involved in lipolysis, including lipase, hormone sensitive isoform 2 and carboxylesterase 3, were both down-regulated. Down-regulation of stress-related proteins, superoxide dismutase 1 and carbonic anhydrase 3, may indicate oxidative stress in adipose tissues of obese mice.

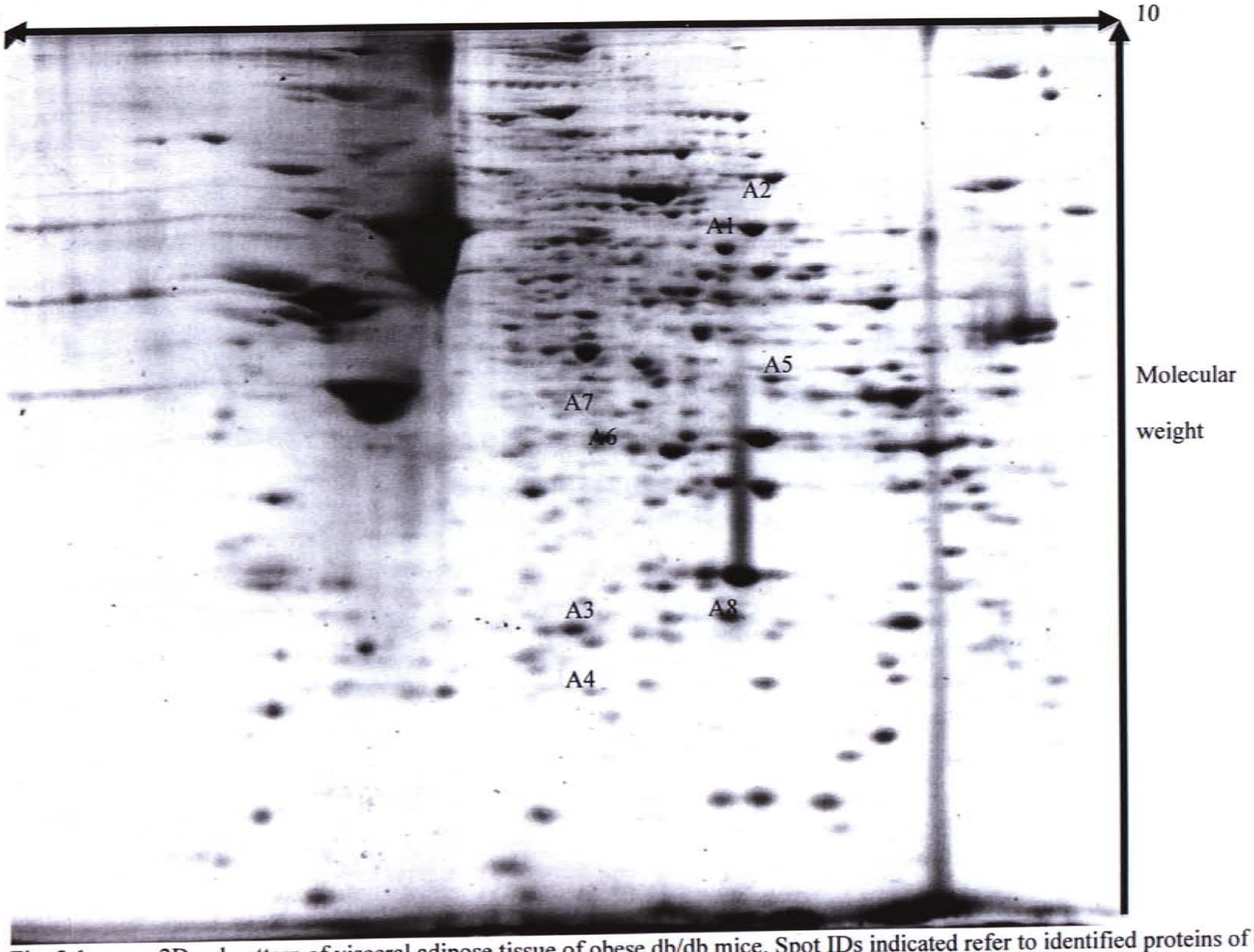


Fig. 3.1a 2D gel pattern of visceral adipose tissue of obese db/db mice. Spot IDs indicated refer to identified proteins of differential abundance.

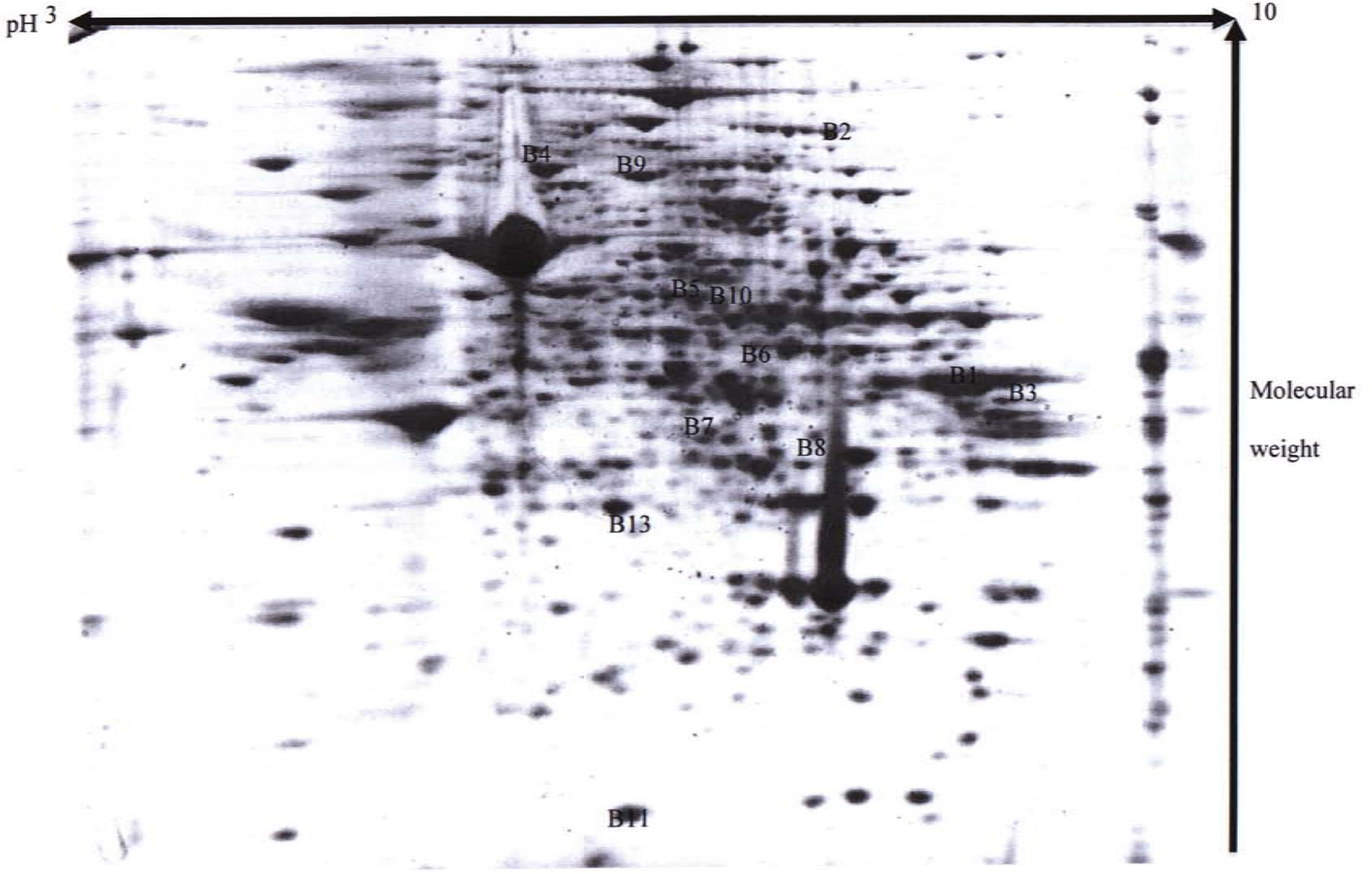


Fig. 3.1b 2D gel pattern of visceral adipose tissue of lean m+/db mice. Spot IDs indicated refer to identified proteins of differential abundance.

Table 3.1 Proteins of differential abundance between visceral adipose tissue of obese db/db mice and that of lean m+/db mice.
Spot IDs correspond to those shown in annotated gel image (Fig. 3.1a and 3.1b). * Regulation refers to the fold difference between %vol of spots. Proteins with fold difference over 1.5 are presented. %vol db / %vol m+ is shown as positive whereas %vol m+ / %vol db is shown as negative. A/N refers to all-or-none difference.

Spot ID	Fucntion / pathways	Regulation*	Protein name	Accession no.	MW	pI	Peptide count	Protein score	Protein score C.I.%	Best ion score	Best ion C.I. %
Carbohydrate metabolism											
A1	Glycolysis	2.06	pyruvate kinase M [Mus musculus]	gi|551295	57824	7.58	17	408	100	69	100
A2	Glycolysis	1.59	glucose phosphate isomerase 1 [Mus musculus]	gi|6680067	62,767	8.49	7	145	100	77	99.818
A3	Glycolysis	2.15	triosephosphate isomerase 1 [Mus musculus]	gi|6678413	26695.8	6.9	12	320	100	94	100
A4	Glycolysis	2.44	Enolase 1, alpha non-neuron [Mus musculus]	gi|19353272	12,936	6.43	4	288	100	92	100
B1	TCA cycle	-2.53	Isocitrate dehydrogenase [NADP], mitochondrial precursor (Oxalosuccinate decarboxylase)	gi|158518416	50873.9	8.88	14	220	100	65	99.998
B2	TCA cycle	-2.35	iron response element binding protein [Mus musculus] (Aconitase A)	gi|52736	98139.62	7.23	6	339	100	78	100
B3	TCA cycle	-1.87	malate dehydrogenase	gi|387129	36454.1	6.16	7	295	100	74	100
B4	TCA cycle	-2.11	pyruvate carboxylase [Mus musculus]	gi|6679237	90868.21	5.79	4	95	99.4	77	100
B5	Oxidative phosphorylat	-2.13	NADH dehydrogenase (ubiquinone) Fe-S protein	gi|21704020	79748.74	5.51	3	247	100	66	100

	ion		1[Mus musculus]								
B6	Oxidative phosphorylation	-1.59	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial precursor (ETF-QO)	gi|52000730	68090.93	7.34	4	87	99.987	50	99.991
B7	Others	-4.49	sorbitol dehydrogenase [Mus musculus]	gi|22128627	38224.7	6.56	6	128	100	36	99.871
Lipid metabolism/Adipocyte-related											
A5	Beta-oxidation	1.57	Acyl-Coenzyme A dehydrogenase, medium chain [Mus musculus]	gi|15488707	46407.6	8.56	14	340	100	58	99.999
B8	Beta-oxidation	-1.62	enoyl Coenzyme A hydratase, short chain, 1, mitochondrial [Mus musculus]	gi|29789289	31454.2	8.76	2	101	100	87	100
B9	Lipolysis	-2.3	lipase, hormone sensitive isoform 2 [Mus musculus]	gi|87239972	83347.52	6.49	6	226	100	85	100
B10	Lipolysis	-2.63	carboxylesterase 3 [Mus musculus]	gi|117553604	61748.7	6.17	7	180	100	50	99.993
A6		1.70	annexin A1 [Mus musculus]	gi|124517663	38710	6.97	10	337	100	68	100
Amino acid metabolism											
A7		1.53	glutamate-ammonia ligase	gi|483918	42161.3	6.64	8	222	100	137	100
Stress-related											
A8		1.67	Phospholipid hydroperoxide	gi|13124257	22166.9	8.74	6	108	100	35	99.8

		glutathione peroxidase, mitochondrial precursor (PHGPx) (GPX-4)								08
A9	1.84	peroxiredoxin 1 [Mus musculus]	gi|6754976	22162.3	8.26	5	101	100	25	96.5 14
B11	-2.04	superoxide dismutase 1, soluble [Mus musculus]	gi|45597447	15932.8	6.02	6	198	100	72	100
B12	-2.6	carbonic anhydrase 3 [Mus musculus]	gi|31982861	29347.6	6.89	11	290	100	65	100
Others										
B13	-1.56	transferrin [Mus musculus]	gi|17046471	76677.73	6.92	7	188	100	83	100

3.2 Effect of berberine on glucose metabolism of obese db/db mice

After 49-day treatment of berberine (BBR) (gastric feeding 50mg/kg body weight per day), body weight, fasting blood glucose level of the db/db mice were measured and glucose tolerance test was performed.

Body weight of the BBR-treated db/db mice was reduced by 25.33±2.93% whereas body weight of water-treated control decreased by 14.00±3.75% only ($p<0.05$). Figure 3.2.1 shows the change in body weight of both BBR-treated and control mice throughout the whole treatment period.

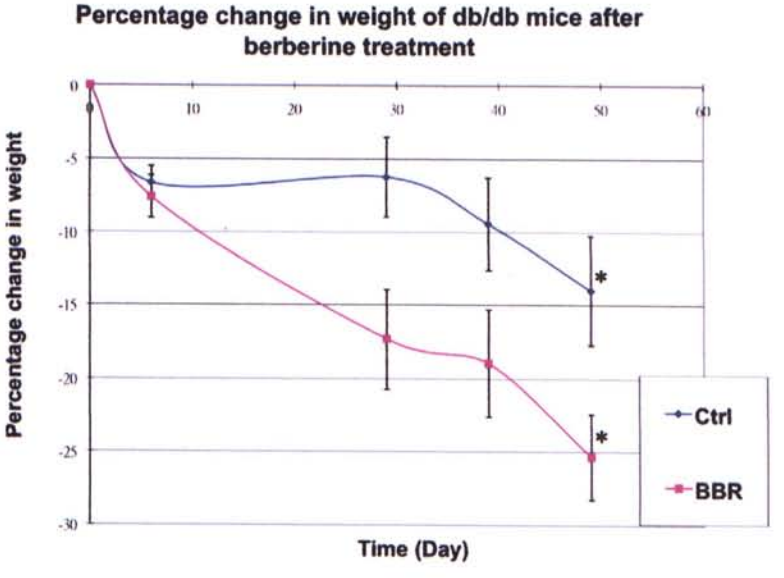


Fig. 3.2.1 Body weight of BBR-treated mice dropped more drastically than that of control db/db mice. n=8 in both BBR-treated and control groups. *$p<0.05$.

At the end of the treatment period, the average fasting blood glucose level of control db/db mice was 466.83±38.34mg/dL and that of BBR-treated mice was 266.80±36.0mg/dL. The difference was statistically significant with $p=0.000151$.

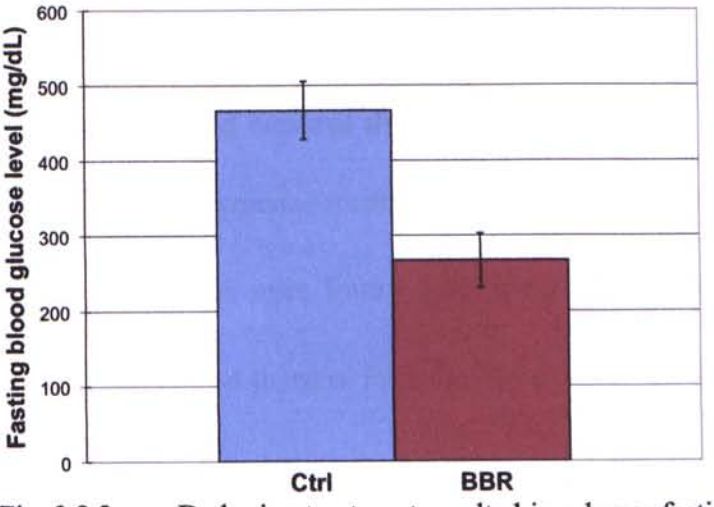


Fig. 3.2.2 Berberine treatment resulted in a lower fasting blood glucose level in db/db mice.

Glucose tolerance test indicates how quickly the body can clear glucose intake to the blood. 120 minutes after intraperitoneal glucose injection, the average blood glucose level of the control db/db mice was 567.43±31.53mg/dL and that of BBR-treated mice was 466.60±53.36mg/dL. Though the difference was not large, it is still statistically significant with p-value=0.0028. Figure 3.2.3 shows the change of blood glucose of the mice after glucose injection.

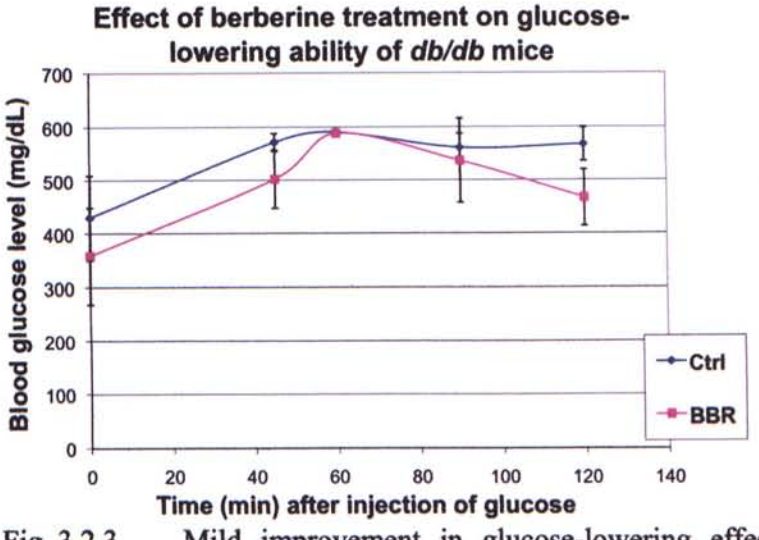


Fig. 3.2.3 Mild improvement in glucose-lowering effect was resulted after berberine treatment.

3.3 Comparison of the protein profiles of visceral adipose tissue of BBR-treated and control db/db mice

After 7 weeks of berberine treatment, the mice were sacrificed (8 mice from BBR-treated group and 8 mice from control group) and visceral adipose tissues were collected and pooled together for 2-DE. Four replicates were run and protein spots with differential abundance over 1.5 fold in all 4 sets of replicates were picked for mass spectrometry analysis. 2D gel patterns were shown in Fig. 3.3a and 3.3b and proteins of differential abundance were listed in Table 3.3.

Several enzymes involved in glycolysis were up-regulated. Proteins responsible for beta-oxidation and lipolysis also increased after berberine treatment. Three stress-related protein increased as well, including carbonic anhydrase 3, calreticulin and annexin A1. The increase of mitogen activated protein kinase 1 (MAPK1), the signaling molecule involved in MAPK/ERK pathway, is also of special interest.

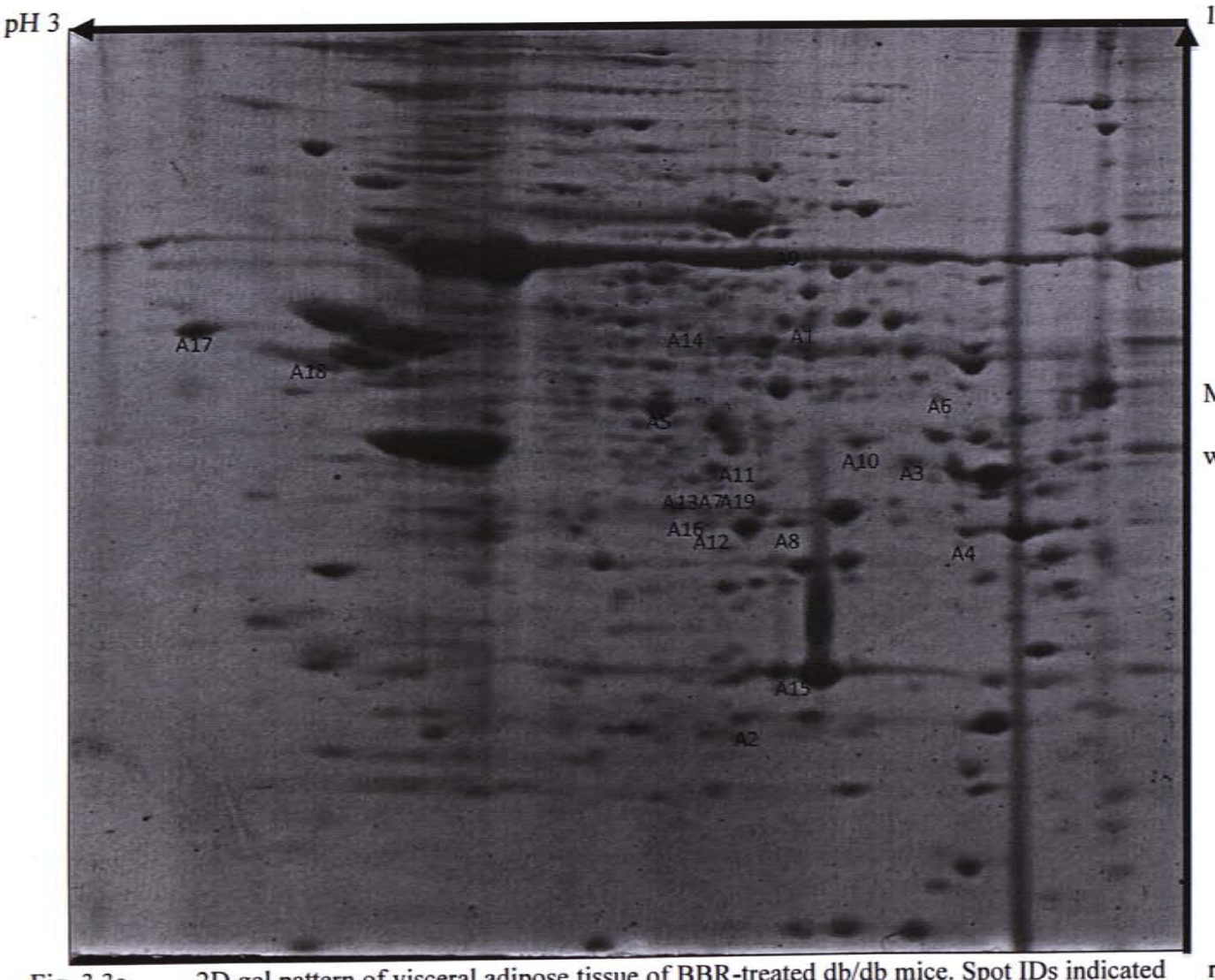


Fig. 3.3a 2D gel pattern of visceral adipose tissue of BBR-treated db/db mice. Spot IDs indicated refer to identified proteins of differential abundance.

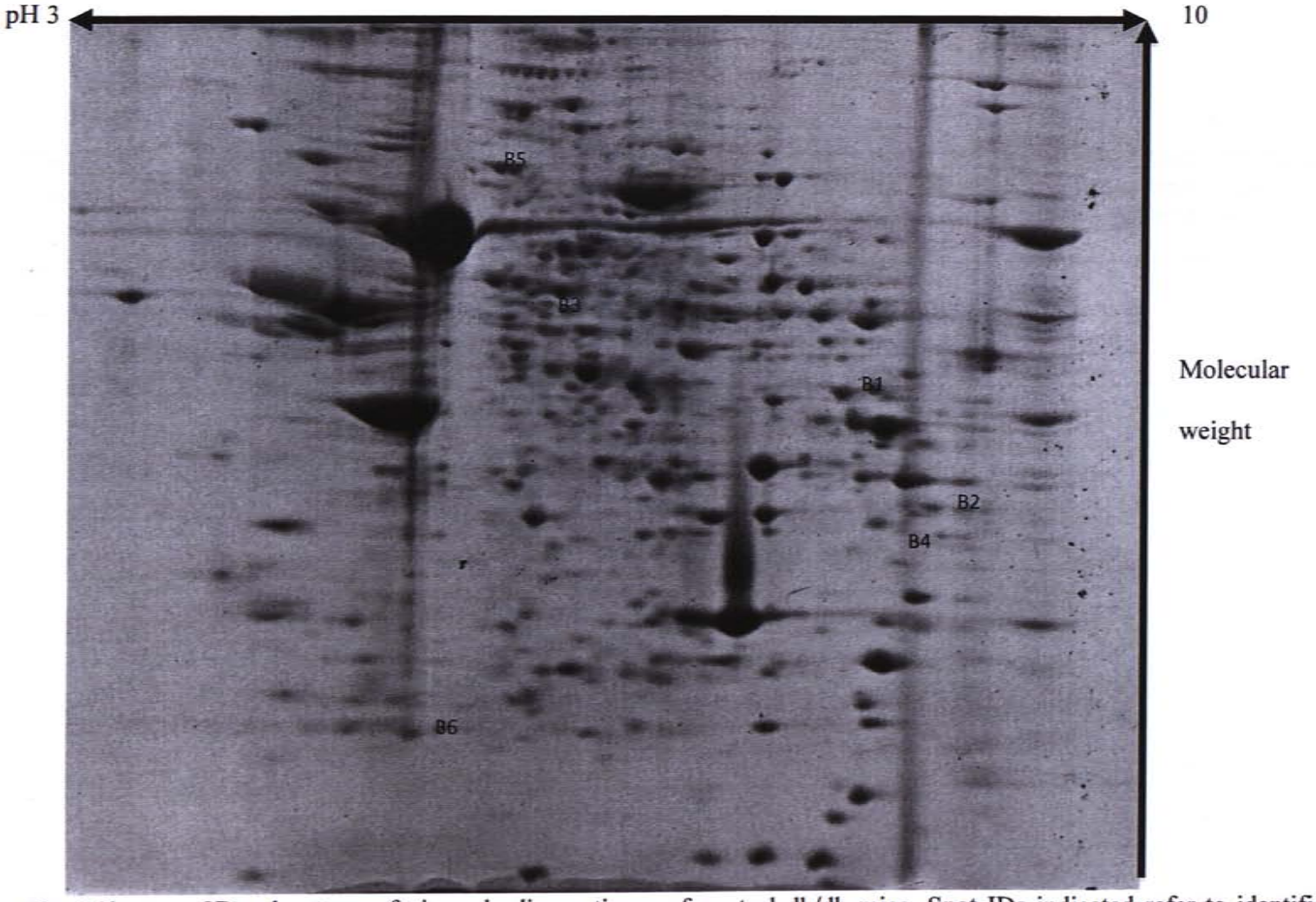


Fig. 3.3b 2D gel pattern of visceral adipose tissue of control db/db mice. Spot IDs indicated refer to identified proteins of differential abundance.

Table 3.3 Proteins of differential abundance between visceral adipose tissue of BBR-treated db/db mice and that of control db/db mice.
Spot IDs correspond to those shown in annotated gel image (Fig. 3.3a and 3.3b). * Regulation refers to the fold difference between %vol of spots. Proteins with fold difference over 1.5 are presented. %vol BBR / %vol Ctrl is shown as positive whereas %vol Ctrl / %vol BBR is shown as negative. A/N refers to all-or-none difference.

Spot ID	Function/ Pathway	Regulation*	Protein name	Accession no.	MW	Protein pI	Peptide count	Protein score	Protein score C.I.%	Best ion score	Best ion C.I.%
Carbohydrate metabolism or carbohydrate-related											
A1	Glycolysis	1.8	M2-type pyruvate kinase [Mus musculus]	gi|1405933	57878.1	7.18	13	272	100	170	100
A2	Glycolysis	2.08	triosephosphate isomerase [Mus musculus]	gi|54855	26678.8	6.9	6	181	100	89	100
A3	Glycolysis	3.69	aldolase A [Mus musculus]	gi|7548322	39525.8	8.55	12	291	100	158	100
A4	Glycolysis	1.51	glyceraldehyde-3-phosphate dehydrogenase	gi|6679937	35787.2	8.44	7	158	100	88	100
A5	Glycolysis	2.32	hypothetical protein LOC433182 [Mus musculus] (enolase 1-like)	gi|70794816	47111.2	6.37	13	402	100	272	100
A6	TCA cycle	2.23	RecName: Full=Fumarate hydratase, mitochondrial	gi|21431774	54336.1	9.12	5	95	99.998	50	99.987
B1	TCA cycle	-2.41	citrate synthase [Mus musculus]	gi|13385942	51703.4	8.72	7	267	100	205	100
B2	TCA cycle	-2.05	malate dehydrogenase 2, NAD (mitochondrial) precursor	gi|31982186	35588.8	8.93	4	291	100	250	100
B3	Pentose phosphate pathway	-1.71	glucose-6-phosphate dehydrogenase X-linked [Mus musculus]	gi|6996917	59224.8	6.06	15	295	100	151	100

	and fatty acid synthesis										
A7		2.52	sorbitol dehydrogenase [Mus musculus]	gi|22128627	38224.7	6.56	4	73	99.678	36	99.929
A8		2.18	Aldo-keto reductase family 1, member A4 (aldehyde reductase) [Mus musculus]	gi|28386164			345	100	237	100	
B4		-1.52	galectin 3 [Mus musculus]	gi|33859580			380	100	283	100	
Lipid metabolism or lipid-related											
A9		1.58	Long-chain-fatty-acid-CoA ligase 1	gi|729927	77873.3	6.81	9	196	100	108	100
A10	Beta-oxidation	1.68	Acyl-Coenzyme A dehydrogenase, medium chain [Mus musculus]	gi|15488707	46407.6	8.56	14	508	100	325	100
A11	Beta-oxidation	1.72	Long-chain specific acyl-CoA dehydrogenase	gi|32130423	47877.5	8.53	6	229	100	175	100
A12		1.82	glycerol-3-phosphate dehydrogenase 1 (soluble) [Mus musculus]	gi|6753966	37548.4	6.75	6	122	100	68	100
A13	Beta-oxidation	1.57	acyl-Coenzyme A dehydrogenase, short chain	gi|31982522	44861.1	8.68	7	151	100	99.965	100
A14	Lipolysis	1.66	carboxylesterase 3	gi|117553604	61748.7	6.17	7	180	100	50	99.993

Stress-related										
A15	1.98	carbonic anhydrase 3 [Mus musculus]	gi|31982861			297	100	142	100	
A16	2.11	annexin A1 [Mus musculus]	gi|124517663			674	100	433	100	
A17	1.9	calreticulin [Mus musculus]	gi|6680836			310	100	198	100	
Structural protein										
B5	-1.85	gelsolin [Mus musculus]	gi|28916693			291	100	149	100	
A18	1.96	vimentin [Mus musculus]	gi|2078001			394	100	157	100	
Others										
A19	1.51	mitogen activated protein kinase 1 [Mus musculus]	gi|6754632	41249.2	6.5	4	113	99.259	35	99.6
B6	-2.02	ferritin light chain 1 [Mus musculus]	gi|114326466	20759.5	5.66	13	662	100	121	100

3.4 Effect of berberine treatment on 3T3-L1 adipocytes

Mature 3T3-L1 adipocytes (day 10) grown in 24-well plates were treated with various concentrations of berberine in medium for 72h (prolonged treatment) in order to study the effect of berberine on lipid metabolism of adipocytes. A 72h treatment was performed as previous report showed that prolonged treatment with berberine could significantly reduce the size of lipid droplets (Lee et al., 2006). To study the effect of short-term berberine treatment on lipid-accumulating pre-mature adipocytes, 3T3-L1 adipocytes (day 4) grown in 24-well plates were treated with 5μM berberine for 24h. Oil Red O staining was performed to estimate effect of berberine treatment on the intracellular accumulation and glycerol concentration in the media was determined after treatment so as to estimate the rate of lipolysis.

3.4.1 Berberine treatment inhibited intracellular triglyceride accumulation in both mature and pre-mature 3T3-L1 adipocytes

For the prolonged (72h) BBR treatment in mature 3T3-L1 adipocytes, the average absorbance of untreated 3T3-L1 cells at 540nm was 1.10 ± 0.065 and that of cells treated with 1μM, 5μM and 15μM berberine (n=8 in all 4 groups) was 0.93 ± 0.044, 0.75 ± 0.046 and 0.76 ± 0.034 ($p<0.05$) respectively as shown in Figure 3.4.1.1 and 3.4.1.3. Prolonged BBR treatment at 1μM, 5μM and 15μM could reduce intracellular

accumulation of triglyceride in mature adipocytes by 15.3%, 31.9% and 31.6% respectively.

For short-term BBR (24h) treatment in lipid-accumulating pre-mature 3T3-L1 adipocytes, the average absorbance of untreated 3T3-L1 cells at 540nm was 0.15 ± 0.0125 and that of cells treated with 5μM berberine was 0.13 ± 0.0048 (n=4 in both groups, $p<0.05$) as shown in Figure 3.4.1.2. Short-term BBR treatment at 5μM could inhibit triglyceride accumulation in pre-mature adipocytes by 17.5%.

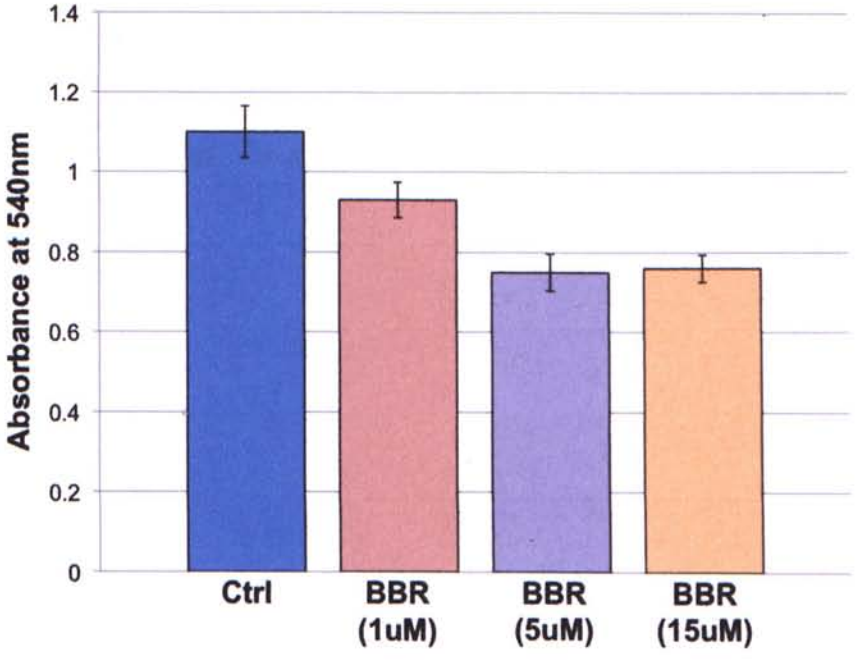


Fig. 3.4.1.1 Berberine inhibited intracellular lipid accumulation in mature 3T3-L1 adipocytes. The absorbance of Oil Red O stain at 540nm was lower in mature adipocytes treated with berberine for 72h in a dose-dependent manner in a lower dose (<5μM). The inhibitory effect was similar in higher dose (5μM and 15μM).

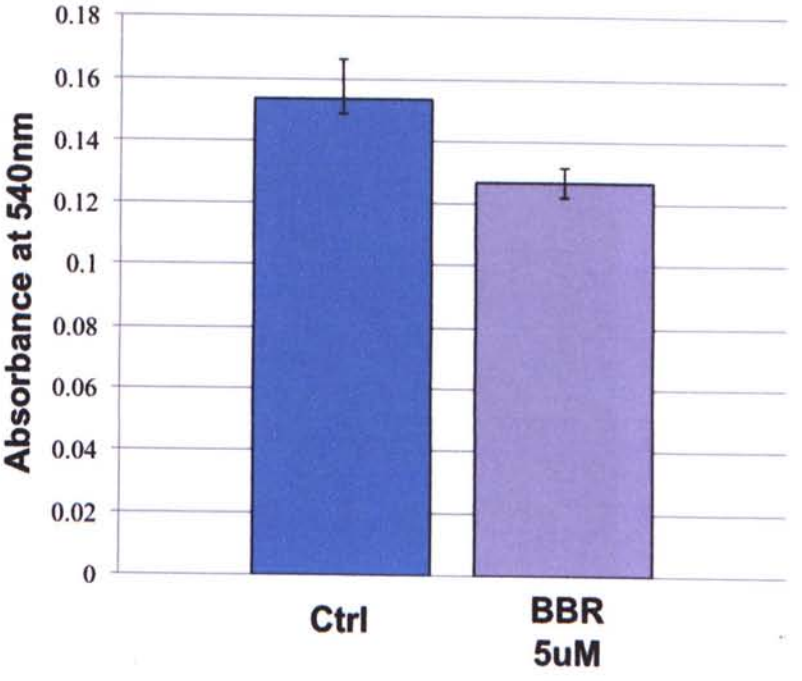
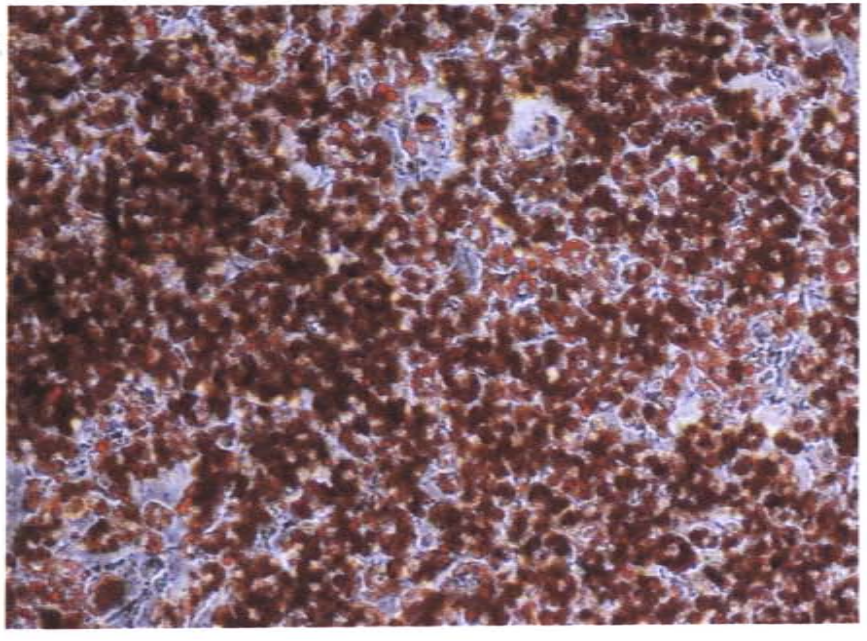


Fig. 3.4.1.2 Berberine inhibited intracellular lipid accumulation in pre-mature 3T3-L1 adipocytes. The absorbance of Oil Red O stain at 540nm was lower in pre-mature adipocytes treated with berberine for 24h.

(A)



(B)

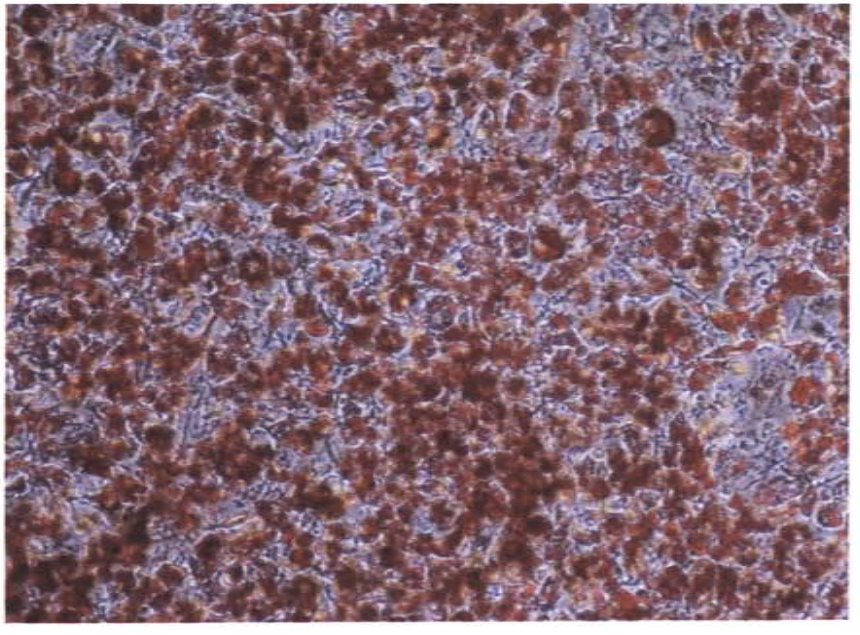


Fig. 3.4.1.3 Mature 3T3-L1 adipocytes stained with Oil Red O Staining (magnification, X100). (A) Control cells; (B) Cells treated with 5μM berberine.

3.4.2 Berberine treatment enhanced lipolysis in mature 3T3-L1 adipocytes but inhibited lipolysis in pre-mature 3T3-L1 adipocytes

For the prolonged (72h) BBR treatment in mature 3T3-L1 adipocytes, the average glycerol concentration of culture media from untreated 3T3-L1 adipocytes was 0.042 ± 0.0057 mg/mL and that of cells treated with 1μM, 5μM and 15μM berberine (n=3 in all 4 groups) was 0.059 ± 0.0024 mg/mL, 0.056 ± 0.0054 mg/mL and 0.058 ± 0.0024 mg/mL, respectively ($p<0.05$) as shown in Figure 3.4.2.1.

For short-term BBR (24h) treatment in lipid-accumulating pre-mature 3T3-L1 adipocytes, the average glycerol concentration of culture media from untreated 3T3-L1 cells was 0.015 ± 0.0009 mg/mL and that of cells treated with 5μM berberine was 0.010 ± 0.0007 mg/mL (n=3 in both groups, p-value=0.0015<0.05) as shown in Figure 3.4.2.2.

3.4.3 Color change in culture media after berberine treatment

It was observed that culture media of 3T3-L1 adipocytes treated with berberine turned orange or even yellow after 3-day treatment. It may indicate an increase in energy metabolism or change to anaerobic respiration.

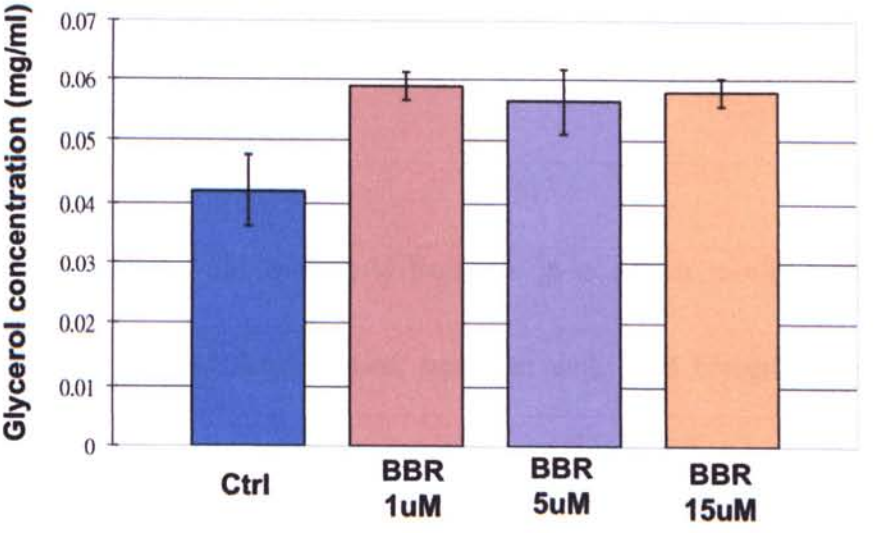


Fig. 3.4.2.1 Glycerol concentration of culture media from mature 3T3-L1 adipocytes increased after berberine treatment for 72h, indicating an increase in the rate of lipolysis.

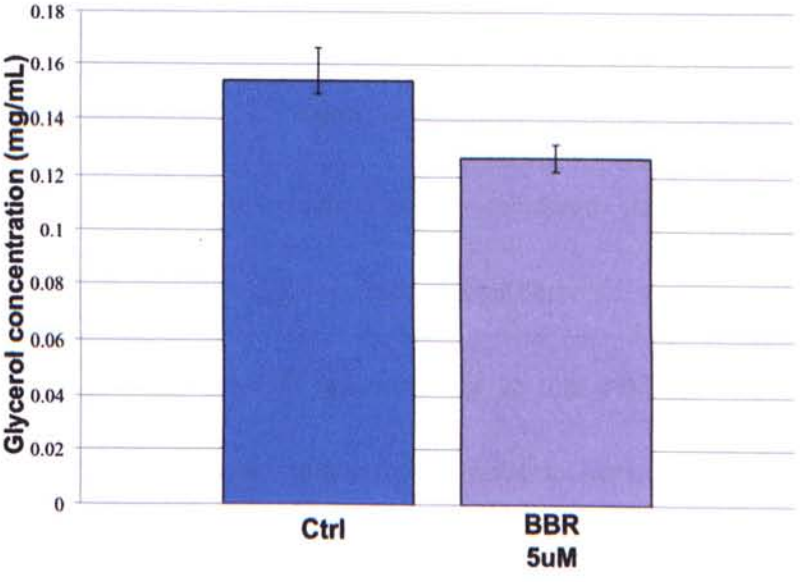


Fig. 3.4.2.2 Glycerol concentration of culture media from pre-mature 3T3-L1 adipocytes decreased after treated with 5μM berberine for 24h, indicating a decrease in the rate of lipolysis.

3.4.4. Comparison of protein profiles between berberine-treated and control 3T3-L1 adipocytes

As treatment with 5μM and 15μM berberine gave similar results in Oil Red O Staining and glycerol determination, treatment with 5μM berberine for 72h was chosen for 2-DE protein profile comparison. Four replicates were run and protein spots with differential abundance over 1.5 fold in all 4 sets of replicates were picked for mass spectrometry analysis. 2D gel patterns were shown in Fig. 3.4a and 3.4b and proteins of differential abundance were listed in Table 3.4.

Proteins involved in carbohydrate and lipid metabolism were altered. Carnitine O-palmitoyltransferase 2, which is responsible for the transport of fatty acid into mitochondria for beta-oxidation, was up-regulated. Triacylglycerol hydrolase, which is involved in lipolysis, also increased after berberine treatment. It is also noteworthy that several stress-related proteins were up-regulated by berberine, including heat shock protein 90, calreticulin and protein-disulphide isomerase.

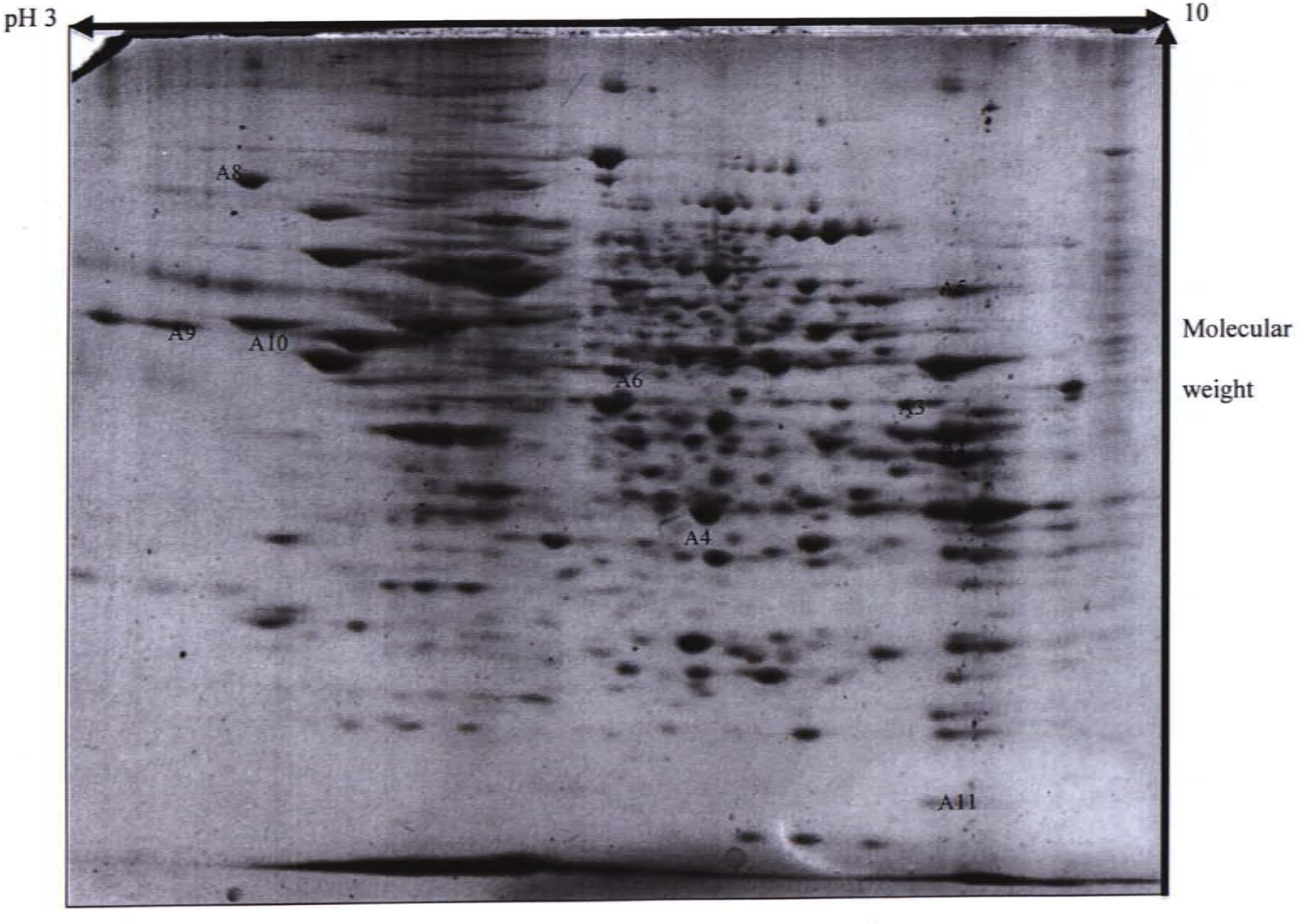


Fig. 3.4a 2D gel pattern of mature 3T3-L1 adipocytes treated with 5μM berberine for 72h. Spot IDs indicated refer to identified proteins with differential abundance.

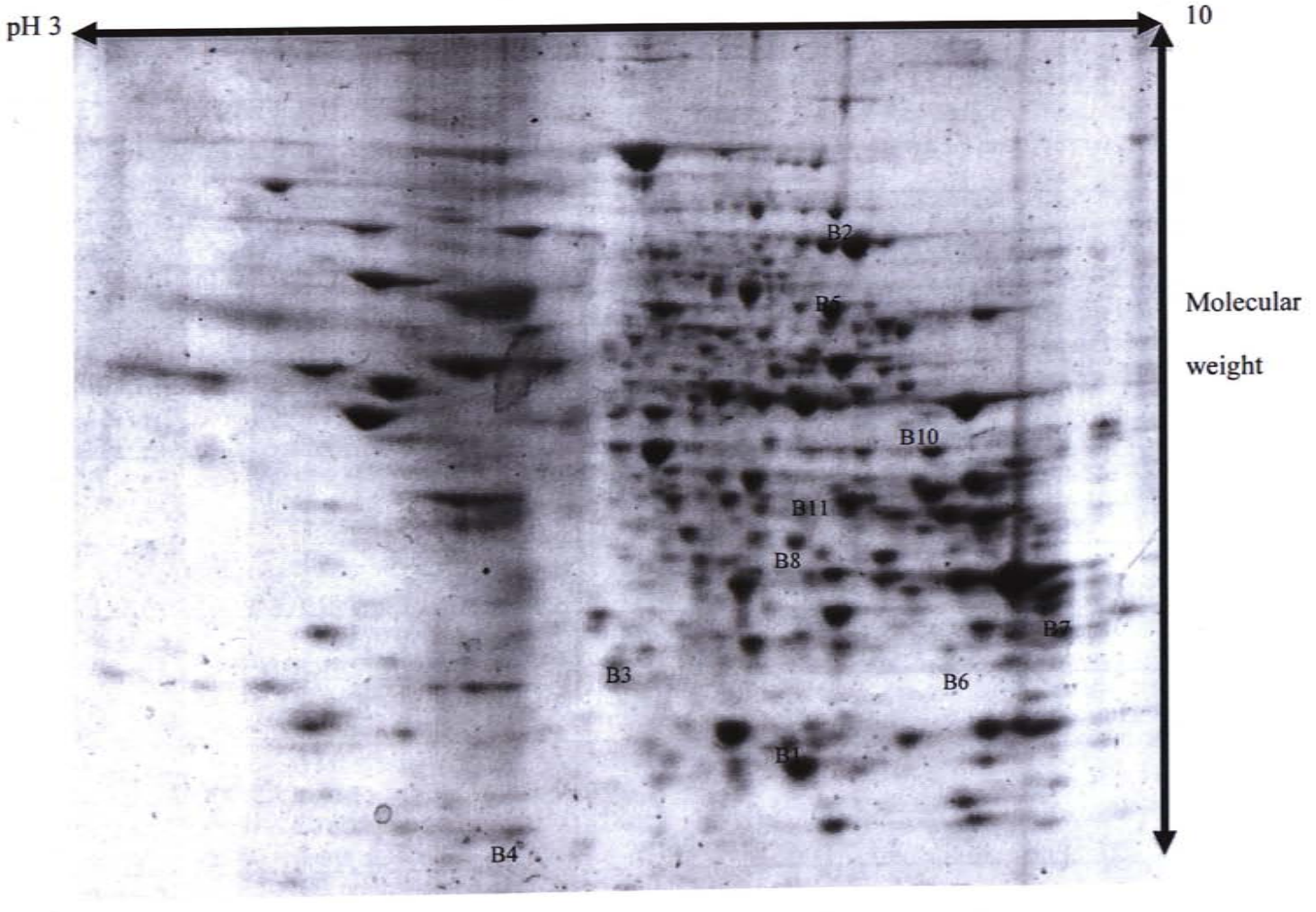


Fig. 3.4b 2D gel pattern of control mature 3T3-L1 adipocytes. Spot IDs indicated refer to identified proteins of differential abundance.

Table 3.4 Proteins of differential abundance between BBR-treated and control 3T3-L1 adipocytes.

Spot IDs correspond to those shown in annotated gel image (Fig. 3.4a and 3.4b). * Regulation refers to the fold difference between %vol of spots. Proteins with fold difference over 1.5 are presented. %vol BBR / %vol Ctrl is shown as positive whereas %vol Ctrl / %vol BBR is shown as negative. A/N refers to all-or-none difference.

Spot ID	Function/ Pathway	Regulation*	Protein name	Accession no.	MW	Protein pI	Peptide count	Protein score	Protein score C.I.%	Best ion score	Best ion C.I.%
Carbohydrate metabolism or carbohydrate related											
A1	Glycolysis	2.25	fructose-bisphosphate aldolase A [Mus musculus]	gi|6671539	39331.3	8.31	14	380	100	252	100
B1	Glycolysis	-1.92	triosephosphate isomerase [Mus musculus]	gi|54855	26678.8	6.9	8	131	100	43	99.991
B2	TCA cycle	-1.91	iron response element binding protein [Mus musculus] (Aconitase)	gi|52736	98077.5	7.23	28	573	100	289	100
B3	TCA cycle	-1.72	isocitrate dehydrogenase 3, beta subunit	gi|18700024	42167.6	8.76	4	108	99.897	34	100
A2	TCA Cycle	1.89	citrate synthase [Mus musculus]	gi|13385942	51703.4	8.72	9	237	100	173	100
A3	TCA Cycle	2.03	fumarate hydratase 1 [Mus musculus]	gi|33859554	54336.1	9.12	5	95	99.998	50	99.987
B4	Oxidative phosphorylation	-2.09	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d [Mus musculus]	gi|16741459	18751.6	5.52	6	156	100	86	100
B5		-2.17	transketolase [Mus musculus]	gi|11066098	60545.1	6.54	21	487	100	267	100
B6		A/N (Ctrl)	carbonyl reductase 1 [Mus musculus]	gi|113680352	30622	8.53	9	175	100	72	100

B7		-1.64	Galectin-3	gi|126679	27497.5	8.46	8	236	100	91	100
Lipid metabolism or lipid related											
B8	Fatty acid metabolism	-1.88	unnamed protein product [Mus musculus] (Acetyl-CoA acetyltransferase)	gi|12850542	38180.6	7.13	6	209	100	156	100
A4		1.94	Gpd1 protein [Mus musculus] (Glycerol-3-phosphate dehydrogenase	gi|13543176	36934.1	6.34	16	339	100	194	100
A5	Transport of fatty acid	1.52	RecName: Full=Carnitine O-palmitoyltransferase 2, mitochondrial	gi|1706111	73880.5	8.46	11	201	100	65	100
A6	Lipolysis	1.84	triacylglycerol hydrolase [Mus musculus]	gi|14269427	61847.8	6.3	13	238	100	79	100
A7		1.73	fatty acid binding protein 5, epidermal [Mus musculus]	gi|6754450	15127.4	6.14	2	99	100	79	100
Stress-related											
A8		2.28	Heat shock protein 90, beta (Grp94), member 1 [Mus musculus]	gi|14714615	92432.3	4.74	18	352	100	166	100
A9		1.9	calreticulin [Mus musculus]	gi|6680836	47964.9	4.33	10	313	100	220	100
A10	Unfolded protein response	1.64	RecName: Full=Protein disulfide-isomerase; Short=PDI	gi|129729	57107.8	4.79	24	582	100	317	100
B9		-1.69	superoxide dismutase 1, soluble [Mus musculus]	gi|45597447	15932.8	6.02	6	324	100	253	100

Amino acid metabolism										
B10	-2.17	serine hydroxymethyltransferase 2 (mitochondrial) [Mus musculus]	gi|21312298	55723.6	8.73	11	137	100	29	99.361
B11	-1.54	epsilon-trimethyllysine 2-oxoglutarate dioxygenase	gi|15529963	47473.9	7.96	10	152	100	92	100
Structural protein										
A12	1.78	cofilin 1, non-muscle [Mus musculus]	gi|6680924	18547.7	8.22	5	133	100	77	100

3.4.5 Western blotting

Total proteins were extracted from BBR-treated (72h) or control mature 3T3-L1 adipocytes. All western blotting experiments were performed three times.

To confirm activation of AMPK by BBR, AMPK-alpha and phospho-AMPK-alpha (Thr172) were detected. After normalization with the intensity of AMPK-alpha, level of phospho-AMPK-alpha (Thr172) of BBR-treated 3T3-L1 was 2.34-fold of that of control 3T3-L1 cells as shown in Fig. 3.4.5.1.

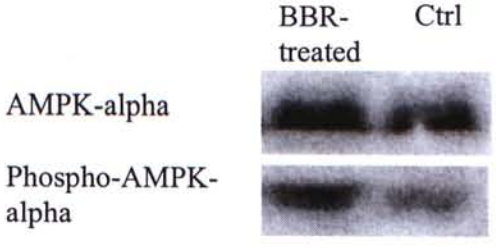


Fig. 3.4.5.1 Berberine treatment enhanced the activation of AMPK-alpha in mature 3T3-L1 adipocytes.

To investigate whether inhibition of lipid accumulation in mature 3T3-L1 adipocytes by berberine is associated with the JNK pathway, SAPK/JNK and phospho-SAPK/JNK (Thr183/Tyr185) were also detected. After normalization with the intensity of JNK/SAPK, level of phospho-SAPK/JNK (Thr183/Tyr185) of BBR-treated 3T3-L1 was found to be -1.36-fold of that of control 3T3-L1 cells as shown in Fig. 3.4.5.2.

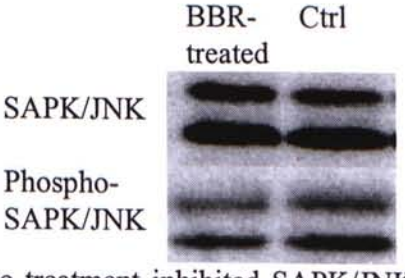


Fig. 3.4.5.2 Berberine treatment inhibited SAPK/JNK activity in mature 3T3-L1 adipocytes.

To investigate whether the berberine induced AMPK activation is due to increased adiponectin secretion from the cells, adiponectin was detected. However, berberine treatment resulted in no significant change in the level of adiponectin in mature 3T3-L1 adipocytes. Protein disulphide isomerase (PDI), which was up-regulated by berberine in 2-DE, was also detected for confirmation. Level of PDI in BBR-treated cells was 1.8-fold higher than that in control cells. β-actin was detected as control. Fig. 3.4.5.3 shows the photos of the western blot of these 3 proteins.

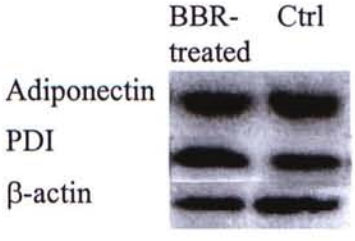


Fig. 3.4.5.3 Berberine treatment increased PDI expression but did not alter the expression of adiponectin in mature 3T3-L1 adipocytes.

4. Discussion

4.1 Comparison of total protein profiles of visceral adipose tissue of obese db/db and lean m+/db mice

All four proteins involved in glycolysis with differential abundance were upregulated in visceral adipose tissues (VAT) of obese db/db mice, including pyruvate kinase M, glucose phosphate isomerase 1, triosephosphate isomerase 1 and enolase 1, alpha non-neuron, indicating an increase in the rate of glycolysis. The down-regulation of proteins involved in TCA cycle and oxidative phosphorylation, including isocitrate dehydrogenase [NADP], mitochondrial precursor, iron response element binding protein, malate dehydrogenase, pyruvate carboxylase, NADH dehydrogenase (ubiquinone) Fe-S protein 1 and electron transfer flavoprotein-ubiquinone oxidoreductase, suggested an inhibition of TCA cycle and oxidative phoshorylation. The increased abundance of glycolytic proteins went along with the increase in expression of glycolysis enzyme encoding genes in human adipose tissues of obese patients (Baranova et al., 2005). The enhancement of glycolysis and the inhibition of TCA cycle and oxidative phosphorylation could be caused by the hypoxic conditions of adipose tissues in obese state as hypoxia can inhibit mitochondria respiration and biogenesis (Ye, 2009). Adipose tissue hypoxia could be caused by reduction in

adipose tissue blood flow in obesity (Karpe et al., 2002) and increase in adipocyte size. A large adipocyte can be over 150μm, far beyond the interstitial oxygen diffusion limit of about 120μm. The pyruvate produced should have undergone anaerobic respiration. It was reported that in adipose tissue of obese mice, the lactate concentration was 1.7-fold higher than that of lean control mice (Hosogai et al., 2007). Sorbitol dehydrogenase was also significantly down-regulated. It is a mitochondrial protein involved in the polyol pathway, catalyzing the conversion of sorbitol to fructose. Accumulation of sorbitol is known to contribute to various diabetic complications such as cataract and diabetic neuropathay (Chung et al., 2003). Two enzymes involved in lipolysis, carboxylesterase 3 (also known as triglycerol hydrolase) and lipase, hormone sensitive isoform 2, were also down-regulated in VAT of db/db mice. This indicates a reduction in the rate of lipolysis, which may be accounted for the large adipocyte size in obesity. A recent study reported that the expression of hormone sensitive lipase was down-regulated in both subcutaneous and omental fat depot of obese women (Ray et al., 2009). Two enzymes involved in beta-oxidation were also altered. Acyl-Coenzyme A dehydrogenase, medium chain was up-regulated while enoyl Coenzyme A hydratase was down-regulated. However, reduced expression of other enzymes involved in beta-oxidation was detected by microarray in omental adipocytes isolated from obese patients (Walewski et al., 2009), suggesting a reduction in the rate of beta-oxidation in adipose tissues in obese

states. The inhibition of both lipolysis and beta-oxiation suggest a possible loss of lipid turnover in VAT of obese db/db mice, which could be causally linked to ectopic fat storage causing fatty liver of db/db mice. Up-regulation of annexin A1 (ANAX1) was also detected in VAT of db/db mice. Beside its more well-known anti-inflammatory property, annexin A1 was recently reported to regulate adipose tissue mass and IL-6 release (Warne et al., 2006). A significant reduction of epididymal fat mass without significant change in mean adipocyte size was observed in ANAX1-null mice, reflecting a reduction in adipocyte cell number. This suggested the role of annexin A1 in adipogenesis and regulation of adipocyte cell number. Glycerol and IL-6 release were also lowered in adipocytes isolated from ANAX1-null mice. The up-regulation of annexin A1 in VAT of db/db mice may explain the obesity and adiposity of the mice.

Stress-related proteins were also altered. Superoxide dismutase 1 and carbonic anhydrase 3 were down-regulated (both >2-fold) while phospholipid hydroperoxide glutathione peroxidase and peroxiredoxin 1 were up-regulated (1.67-fold and 1.84-fold respectively). The reduction of superoxide dismutase 1, an important anti-oxidant in cells, and carbonic anhydrase 3 may indicate the oxidative stress in VAT of obese db/db mice. Phospholipid hydroperoxide glutathione peroxidase catalyzes the conversion of glutathione and lipid hydroperoxide to glutathione disulphide, lipid and water, protecting the cells from lipid peroxidation. Besides, the reduction of

transferrin (-1.56-fold), which is involved in iron transport and metabolism, may affect cellular iron balance and cause oxidative stress.

4.2 Berberine lowers body weight, reduces fasting blood glucose level and improves glucose-lowering ability of db/db mice

After 49 days of treatment, body weight of the BBR-treated db/db mice was reduced by 25.33%±2.93% whereas body weight of water-treated control decreased by 14.00%±3.75% only ($p<0.05$). Average fasting blood glucose level of control db/db mice was 466.83±38.34 mg/dL and that of BBR-treated mice was 266.80±36.07 mg/dL whereas 2h-post-load glucose level in glucose tolerance test of control db/db mice was 567.43±31.53 mg/dL and that of BBR-treated mice was 466.60±53.36 mg/dL. The significant body weight reduction of BBR-treated mice should be due to reduction of fat mass, which was shown by computer tomography in BBR-treated db/db mice performed by another group (Lee et al., 2006). Milder reduction of body weight was also observed in control group. This may be an unintentional weight loss observed in patients with severe diabetes. This undesirable unintentional weight loss may be due to dehydration resulting from frequent urination or muscle breakdown under hyperglycemia. The reduction of fasting blood glucose level indicates an improvement in hepatic homeostasis as circulatory glucose after overnight fasting

mainly comes from hepatic glucose production. The lower 2h-post-load glucose level in BBR-treated db/db mice also indicates an improved glucose-lowering ability.

4.3 Comparison of the protein profiles of visceral adipose tissue of BBR-treated and control db/db mice

After BBR treatment, several but one proteins involved in glycolysis were up-regulated in VAT of db/db mice, including triosephosphate isomerase, aldolase A, glyceraldehyde-3-phosphate dehydrogenase, enolase and pyruvate kinase. This indicates an up-regulation in glycolysis. For TCA cycle, two enzymes, citrate synthase and malate dehydrogenase 2, were down-regulated whereas fumarate hydratase was up-regulated. This indicates an up-regulation in glycolysis and down-regulation in TCA cycle. Increase in glycolysis can be resulted from AMPK activation, which stimulates energy providing pathways like glycolysis, glucose uptake and beta-oxidation (Viollet et al., 2009). Induction of glycolysis and activation of AMPK in adipocytes by berberine was also reported previously (Yin et al., 2008a; Lee et al., 2006). The down-regulation of TCA cycle could also be due to mitochondrial inhibitory effect of AMPK activation.

BBR treatment also led to decrease of glucose-6-phosphate dehydrogenase level. Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme in the pentose phosphate pathway. It converts glucose-6-phosphate to 6-phosphogluconolactone

with the concomitant production of $NADPH+H^+$. The $NADPH+H^+$ produced during this reaction provides 50-75% reducing equivalents necessary for fatty acid biosynthesis in liver. Thus, glucose-6-phosphate dehydrogenase is also regarded as a lipogenic enzyme. It was recently reported that the inhibition of glucose-6-phosphate dehydrogenase in hepatocytes by arachidonic acid involves activation of both AMPK and p38 MAPK (Kohan et al., 2009). The inhibition of glucose-6-phosphate dehydrogenase in VAT of BBR-treated db/db mice may also be due to AMPK activation and/or p38 MAPK activation.

Sorbitol dehydrogenase level, which decreased in adipose tissue of db/db mice compared to lean m+/db mice, increased after BBR treatment. This may alleviate the accumulation of sorbitol, which is associated with various diabetic complications. Aldo-keto reductase family 1, member A4, also known as aldehyde reductase or aldose reductase (AR), was also up-regulated. The role of AR is quite controversial. AR is also involved in the polyol pathway by converting aldehyde form of glucose into sorbitol together with the conversion of $NADPH+H^+$ to $NADP^+$. Sorbitol produced is then converted to fructose, a more effective glycating agent than glucose, by sorbitol dehydrogenase. The involvement of the polyol pathway in oxidative stress and diabetic complications makes inhibition of AR a potential therapeutic strategy against diabetic complications (Corso et al., 2008). However, AR also plays a role in detoxification of various toxic aldehydes (Kang et al., 2007). It was shown

that the up-regulation of AR via phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway by curcumin, a polyphenolic antioxidant, is necessary for the cytoprotective effect of the polyphenolic compound (Kang et al., 2007). Up-regulation of AR may also contribute to inhibition of fatty acid biosynthesis by depletion of $NADPH+H^+$.

Galectin-3 (Gal-3) level was also lowered after BBR treatment. This lectin is involved in a wide variety of biological activities such as cell adhesion, induction of pre-mRNA splicing, regulation of apoptosis, modulation of inflammation and cell proliferation (Kiwaki et al., 2007). It was recently found that Gal-3-null mice are less susceptible to streptozotocin-induced diabetes (Mensah-Brown et al., 2009). Macrophages infiltrated into the pancreas produced less TNF-α and nitric oxide in Gal-3-null mice and thus protecting the mice from immune-mediated beta cell damage. It was also found that galectin-3 stimulates human primary preadipocyte proliferation and up-regulation of galectin-3 was associated with growth of adipose tissue induced by high-fat diet in mice (Kiwaki et al., 2007). However, the role of galectin-3 in adiposity and obesity is still unknown. In this project, we found that a decrease in galectin-3 level was associated with body weight loss and reduced FBG in BBR-treated db/db mice, supporting the relationship between galectin-3 level and adiposity.

Proteins involved in beta-oxidation, including acyl-Coenzyme A dehydrogenase (medium chain), long-chain specific acyl-CoA dehydrogenase, acyl-Coenzyme A

dehydrogenase (short chain) were all up-regulated after BBR treatment. This indicates an increase in the rate of beta-oxidation, which should be due to the activation of AMPK by berberine. Long-chain-fatty-acid-CoA ligase 1, also known as long chain fatty acyl-CoA synthetase, catalyzes the activation of a long fatty acid chain to a fatty acyl-CoA. Activation of fatty acid by esterification to fatty acyl-CoA is required before further utilization, usually beta-oxidation or lipid synthesis (Digel et al., 2009). Recent report stated that suppression of long chain acyl-CoA synthetase 3 in hepatocytes lowered *de novo* fatty acid synthesis (Bu et al., 2009). Up-regulation of long chain fatty acid CoA ligase 1 by berberine may enhance activation of fatty acids and help reduce the amount of free fatty acids released from VAT. Whether the activated fatty acyl CoA would undergo beta-oxidation or lipid synthesis and whether the up-regulation of the fatty acyl CoA synthetase could inhibit de novo fatty acid synthesis in VAT is still uncertain. Carboxylesterase 3, which was down-regulated in VAT of db/db mice compared with that of m+/db mice, was up-regulated after BBR treatment. Though lipolysis should have been inhibited by AMPK activation, the up-regulation of carboxylesterase 3, a lipolytic enzyme, may just restore its level back to normal and allow a normal turnover of lipids, fatty acids and glycerol in the adipocytes. Glycerol-3-phosphate dehydrogenase 1 (soluble) was also up-regulated. It catalyzes the reduction of dihydroxyacetone phosphate into glycerol-3-phosphate together with the conversion of $NADH+H^+$ to NAD^+. Several

compounds or plant extracts ameliorating lipid accumulation or dyslipidaemia were shown to reduce glycerol-3-phosphate dehydrogenase activity (Kim et al., 2009; Lee et al., 2009; Kubota et al., 2009). Up-regulation of glycerol-3-phosphate by berberine may contribute to the recycling of NAD^+ required for the enhanced glycolysis.

Two stress-related proteins were up-regulated. Carbonic anhydrase 3, which was down-regulated in VAT of obese db/db mice compared with that of m+/db mice, was up-regulated by berberine. Calreticulin, a multifunctional Ca^{2+} buffering molecular chaperone and modulator of gene expression (Michalak et al., 2009), was also up-regulated. Recent study found that adipogenesis is promoted in calreticulin-deficient embryonic stem cells. Overexpression of [P+C] domain, which is responsible for the Ca^{2+} buffering function, of calreticulin in 3T3-L1 preadipocytes also inhibits lipid accumulation (Szabo et al., 2008), indicating the effect of Ca^{2+} content modulation in adipogenesis. The up-regulation of calreticulin by berberine may play a role in inhibition of adipogenesis in VAT of db/db mice. However, it may also indicate presence of stress in endoplasmic reticulum.

Mitogen activated protein kinase 1 (MAPK1), also known as extracellular signal-regulated kinase 2 (ERK2), was up-regulated by berberine. This protein is known to be involved in the control of cell proliferation and it was recently reported that it is involved in lipid droplet formation (Boström et al., 2009).

4.4 Berberine inhibited lipid accumulation in mature and pre-mature 3T3-L1 adipocytes

Prolonged berberine treatment (72h) at 5μM reduced the intracellular accumulation of triglyceride in mature adipocytes by 31.9% whereas short-term berberine treatment (24h) at 5μM reduced the intracellular accumulation of triglyceride in pre-mature adipocytes by 17.5%. The reduction of triglyceride accumulated in mature adipocytes and inhibition of lipid accumulation in pre-mature, differentiating adipocytes may be accounted for the body weight loss in BBR-treated db/db mice. But the former one should have played a more important role in weight loss and the adipocytes in the obese db/db mice are mostly mature and overload with triglyceride.

4.5 Berberine enhanced lipolysis in mature 3T3-L1 adipocytes but inhibited lipolysis in pre-mature 3T3-L1 adipocytes

Prolonged (72h) berberine treatment at 5μM in mature 3T3-L1 adipocytes increased the glycerol concentration in culture media by 33% whereas short-term treatment (24h) reduced the glycerol concentration in culture media by almost 50%. It was previously reported that 18h-treatment of 5μM BBR in mature 3T3-L1 adipocytes could reduce glycerol secretion by 41% (Choi et al., 2006), similar to our short-term treatment results. Decrease in secreted glycerol level indicates inhibition of lipolysis by berberine, at least for short-term. The increase in secreted glycerol level after

prolonged berberine treatment may be attributed to the possible increase in beta-oxidation induced by berberine, which may in turn drive lipolysis. For in vivo situation, it is speculated that an increase in lipolysis after long term daily BBR treatment is more likely to take place as the adipocytes in adult individuals are mostly mature and the treatment is usually long term. This is also supported by the loss of intracellular triglyceride accumulated in mature 3T3-L1 adipocytes shown in Oil Red O staining. The loss of triglyceride already accumulated in the mature adipocytes should be due to an increase in lipolysis.

4.6 Comparison of the protein profiles of BBR-treated and control 3T3-L1 adipocytes

Glycolytic enzyme, aldolase A, which catalyzes the conversion of fructose 1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, was up-regulated after berberine treatment. This may indicate the enhancement of glycolysis by berberine, which was also reported previously (Yin et al., 2008a). Triosephsophate isomerase, unlike the result from BBR-treated db/db mice, was down-regulated. Triosephosphate isomerase is responsible for catalyzing the conversion of dihydroxyacetone phosphate into glyceraldehyde-3-phosphate which proceeds in glycolytic processes. Down-regulation of this enzyme may lead to accumulation of dihydroxyacetone phosphate, which is the substrate for the up-regulated glycerol-3-phosphate dehydrogenase. The conversion of dihydroxyacetone

phosphate to glycerol-3-phosphate may therefore be enhanced. This may allow the recycling of NAD^+ from $NADH+H^+$ for glycolysis. Enzymes involved in TCA cycle, including aconitase and isocitrate dehydrogenase, were down-regulated. This may be due to the mitochondrial inhibitory effect of AMPK activation induced by BBR. Citrate synthase and fumarate hydratase were, however, up-regulated. The up-regulation of fumarate hydratase was even found in both VAT of BBR-treated db/db mice and BBR-treated 3T3-L1 adipocytes. Fumarate can inhibit prolyl hydroxylase enzymes, which are responsible for degradation of hypoxia-inducible factors (HIFs), in cytosol (King et al., 2006). HIFs enhance glycolysis, angiogenesis and other mechanisms under hypoxic conditions for cell survival. Up-regulation of fumarate hydratase by berberine may prevent the accumulation of fumarate and alleviate the subsequent prolyl hydroxylase inhibition and thus enhance degradation of HIFs. Hence, the increase in glycolysis induced by berberine is not likely due to hypoxia in obesity, but should be due to AMPK activation. It was also reported that berberine can inhibit HIF-1α expression via enhanced proteolysis in human gastric adenocarcinoma SC-M1 cell line (Lin et al., 2004). Galectin-3, which was down-regulated in the VAT of BBR-treated db/db mice, was also down-regulated in BBR-treated 3T3-L1 adipocytes.

Down-regulation of acetyl-CoA acetyltransferase, which is responsible for formation of acetoacetyl-CoA and thus cholesterol synthesis, may help reduce cholesterol.

Carnitine O-palmitoyltransferase, which is responsible for the transport of acyl-CoA from cytosol into mitochondrial matrix for beta-oxidation, was up-regulated. This may enhance beta-oxidation. There may be increase in lipolysis as indicated by the up-regulation of triacylglycerol hydrolase (carboxyesterase 3). Up-regulation of glycerol-3-phosphate dehydrogenase may be responsible for providing the NAD^+ required in enhanced glycolysis. Enhanced lipolysis, beta-oxidation and formation of glycerol-3-phosphate (lipogenesis) may show that the cells have a better turnover of fatty acid and lipids and have gained a better elasticity for energy storage.

Unexpectedly, several endoplasmic reticulum (ER) stress-related proteins were up-regulated after BBR treatment, including heat shock protein 90 (HSP90), calreticulin and protein disulphide-isomerase (PDI). HSP90 is a molecular chaperone for the folding, stabilization, activation and assembly of other "client" proteins. Calreticulin is another stress-induced molecular chaperone with Ca^{2+} buffering action. Up-regulation of calreticulin may also inhibit lipid accumulation and adiogenesis as mentioned above. PDI is involved in unfolded protein response (UPR). As ER stress is positively associated with diabetes and obesity and administration of chaperones that block ER stress activation could to reverse insulin resistance (Ozawa et al., 2005; Ozcan et al., 2006), the up-regulation of UPR- and ER stress-related proteins is quite unexpected. However, a recent study states that berberine can induce G2/M arrest and apoptosis in C6 rat glioma cells in an ER-dependent manner, with an

increase in level of glucose-regulated protein 78, a regulator of UPR. Production of reactive oxygen species is also promoted (Chen et al., 2009). This may explain the down-regulation of superoxide dismutase.

Trimethyllysine 2-oxoglutarate dioxygenase is involved in lysine degradation and carnitine biosynthesis. It catalyzes the conversion of trimethyllysine to 3-hydroxy-trimethyllysine, together with the conversion of α-ketoglutarate to succinate. Succinate, like fumarate as mentioned above, can inhibit prolyl hydroxylase enzymes, which are responsible for degradation of hypoxia-inducible factors (HIFs), in cytosol (King et al., 2006). Down-regulation of trimethyllysine 2-oxoglutarate dioxygenase by berberine treatment may prevent succinate accumulation and thus ameliorate hypoxia-induced cellular responses.

4.7 Western blotting

Level of phospho-AMPK-alpha (Thr172) of BBR-treated 3T3-L1 was 2.37-fold of that of control cells. This confirmed that berberine enhanced AMPK activity in 3T3-L1 adipocytes as previously reported by others (Lee et al., 2006; Yin et al., 2008a). However, this AMPK activation was not due to increased adiponectin secretion as level of adiponectin did not alter significantly after berberine treatment.

Level of phospho-SAPK/JNK (Thr183/Tyr185) of BBR-treated 3T3-L1 was found to be -1.36-fold of that of control cells, revealing that berberine inhibited JNK activity in 3T3-L1 adipocytes. As JNK leads to serine phosphorylation of IRS-1 and impairs

the insulin signaling pathway which involves the activation of phosphatidylinositol 3-kinase (PI3K pathway) and MAP kinase pathway, inhibition of JNK activity may be another mechanism for the anti-diabetic effect and inhibition of lipid accumulation of berberine. Although Kim et al. reported that berberine does not affect insulin-responsive signal transduction pathway (Kim et al., 2007), they just showed that berberine does not augment tyrosine phosphorylation of IRS-1 and effect of berberine on 3T3-L1 is not via PI3K pathway.

Increased expression of PDI after berberine treatment observed in 2-DE was confirmed in western blotting. This suggested that berberine might cause ER stress in 3T3-L1 adipocytes.

4.8 General discussion

Berberine treatment lowered body weight and fasting blood glucose level of obese, diabetic db/db mice. It also improved glucose-lowering ability of db/db mice in glucose tolerance test. The reduction of body weight may be due to reduction of triglyceride accumulation in adipose tissues of the mice. In vitro study showed that both short-term (24h) and prolonged (72h) treatment of berberine could reduce triglyceride accumulation in 3T3-L1 adipocytes.

Proteomic study of VAT of BBR-treated db/db mice and BBR-treated 3T3-L1 adipocytes suggested there was increase in glycolysis, lipolysis and beta-oxidation

but also inhibition of TCA cycle and oxidative phosphorylation. The increase in glycolysis and beta-oxidation as well as the inhibition of TCA cycle and oxidation phosphorylation should be owing to activation of AMPK by berberine. But AMPK activation does not enhance lipolysis. Increased expression of glycerol-3-phosphate dehydrogenase in both VAT of BBR-treated db/db mice and BBR-treated 3T3-L1 adipocytes also hinted an increase of glycerol-3-phosphate level in the adipocytes, suggesting a possible restoration of a "healthy" turnover of fatty acids and triglyceride in the adipocytes. Similar proteomic changes in these metabolic pathways in both in vivo and in vitro model suggested that the proteomic changes in the visceral adipose tissue of BBR-treated db/db mice were largely due to direct drug action but not mainly due to the metabolic alterations produced by the drug action. Western blotting showed that berberine inhibited SAPK/JNK in 3T3-L1 adipocytes. It was recently reported that silencing of JNK1 accelerates basal lipolysis as well as enhances re-esterification of fatty acids in 3T3-L1 (Rozo et al., 2008). Therefore, the increased expression of carboxyesterase 3 and triglycerol hydrolase, in 2-DE of BBR-treated VAT of db/db mice and BBR-treated 3T3-L1 adipocytes respectively, may be due to JNK inhibition. The link of berberine to JNK pathway may worth further investigation.

The association between ER stress and obesity has been supported in this decade. Berberine treatment, unexpectedly, increased PDI level in both VAT of db/db mice

and 3T3-L1 adipocytes, suggesting that berberine might enhance ER stress in adipocytes.

Though generally regarded as an anti-oxidant, berberine did not increase the expression of many anti-oxidizing proteins or ROS scavengers in 2-DE. However, the increased expression of fumarate hydratase in both VAT of db/db mice and 3T3-L1 adipocytes and the decreased expression of trimethyllysine 2-oxoglutarate dioxygenase hinted possible inhibition of HIFs and amelioration of hypoxic conditions in adipocytes. But further experiments will be needed for confirmation.

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