PUNICIC ACID, A PARTIAL PPAR GAMMA AGONIST, ENHANCES INSULIN SENSITIVITY IN 3T3-L1 ADIPOCYTES BY SAFEGUARDING MITOCHONDRIA AND AMELIORATING INFLAMMATION

Thesis submitted to the University of Kerala
for the award of the degree of
Doctor of Philosophy in Biochemistry

By

ANUSREE S S

Agroprocessing and Natural Products Division
Council of Scientific and Industrial Research - National Institute for Interdisciplinary Science and Technology (CSIR - NIIST)
Thiruvananthapuram-695019, Kerala, India

March 2016

DECLARATION

I hereby declare that the thesis entitled **"PUNICIC ACID, A PARTIAL PPAR GAMMA AGONIST, ENHANCES INSULIN SENSITIVITY IN 3T3-L1 ADIPOCYTES BY SAFEGUARDING MITOCHONDRIA AND AMELIORATING INFLAMMATION"** embodies the results of investigations carried out by me for the award of the degree of Doctor of Philosophy in Biochemistry at Agroprocessing and Natural Products Division, Council of Scientific and Industrial Research - National Institute for Interdisciplinary Science and Technology (CSIR - NIIST), Thiruvananthapuram as a full time research scholar under the supervision of Dr. K. G. Raghu and the same has not been submitted elsewhere for any other degree. In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators.

Anusree S S

Thiruvananthapuram,
March 2016

COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH
NATIONAL INSTITUTE FOR INTERDISCIPLINARY SCIENCE & TECHNOLOGY
GOVERNMENT OF INDIA
Thiruvananthapuram-695 019, Kerala, India

Telephone: 91-471-2515486
Fax: 91-471-2491712

Dr. K. G. Raghu
Principal Scientist
Agroprocessing and Natural Products Division

CERTIFICATE

This is to certify that the work embodied in the thesis entitled "**PUNICIC ACID, A PARTIAL PPAR GAMMA AGONIST, ENHANCES INSULIN SENSITIVITY IN 3T3-L1 ADIPOCYTES BY SAFEGUARDING MITOCHONDRIA AND AMELIORATING INFLAMMATION**" has been carried out by **Ms. Anusree S S**, under my supervision and guidance at Agroprocessing and Natural Products Division of Council of Scientific and Industrial Research-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Biochemistry under the Faculty of Science, University of Kerala, Thiruvananthapuram, Kerala, India and the same has not been submitted elsewhere for any other degree. All the relevant corrections, modifications and recommendations suggested during the pre-synopsis presentation of Ms Anusree S S have been incorporated in the thesis.

K. G. Raghu
(Thesis Supervisor)

Thiruvananthapuram,
March 2016

e-mail: raghukgopal2009@gmail.com; raghukgopal@rediffmail.com

ACKNOWLEDGEMENTS

It is with immense pleasure that I express my deep sense of gratitude to my research supervisor Dr. K. G Raghu for suggesting the research topic and his guidance, encouragement and constant support throughout my research.

I wish to thank Dr. A. Ajayaghosh, Director, CSIR-NIIST and Dr. Suresh Das, Former Director, for providing necessary facilities for carrying out the work. I would like to express my gratitude to Mr. M. M. Sreekumar (Chief Scientist & Head, Agroprocessing and Natural Products Division), CSIR-NIIST and Dr. A. Sundaresan (Chief Scientist & Former Head, Agroprocessing and Natural Products Division) for their support and encouragement extended to my work.

I wish to extend my sincere thanks to Dr. P. Jayamurthy, Dr. S. Priya, Dr. P. Nisha, Dr. B. S. Dileep Kumar, Smt. M. V. Reshma, Mr. V.V Venugopalan, Dr. Beena Joy and Mr. D. R. Soban Kumar for their help and support in one or other way. All the former and present members of Agroprocessing and Natural Products Division have been extremely helpful and co-operative and I am thankful to one and all for their kind gesture.

I am obliged to Dr. Annie Abraham (Professor & Head, Dept. of Biochemistry, University of Kerala), Dr. G. Muraleedhara Kurup (Former Head, Dept. of Biochemistry, University of Kerala), Dr. S. Mini, Prof. (Dr.) M. Indira, Dr. A. Helen, Dr. Arun A Rauf, Dr. P. Biju and Dr. Saja (Faculty members, Dept. of Biochemistry, University of Kerala) for all their help and support.

I gratefully acknowledge the timely help and support received from my colleagues Ms. Nisha V.M., Dr. Priyanka A., Dr. Soumya R.S., Dr. Priya Rani, Dr. Prathapan A., Dr. Vineetha V.P., Dr. Shyni G.L., Dr. Vandana Sankar, Mr. Salin Raj, Dr. Riya M.P., Ms. Reshma P.L., Dr. Antu K. Antony, Ms. Preetha Rani, Ms. Kavitha Sasidharan, Ms. Anupama Nair, Ms. Sreelekshmi and Dr. Sindhu G. I would also like to acknowledge all the support from Ms. Saranya, Ms. Janu Chandran, Mr. Pratheesh S. Nair, Ms. Shamla L., Ms. Shilpa, Mr. Arun K.B., Dr. Nishanth Kumar, Ms. Shyama H.P., Ms. Dhanya R., Ms. Sithara Thomas, Ms. Sini and all other friends of Agroprocessing and Natural products Division, CSIR-NIIST. I also express my sincere thanks to my friend Ms. Arya Das, for her help and support.

It is my pleasure to thank all the members of library, administration, academic programme committee and technical staff of NIIST for their help and support.

I extent my sincere thanks to my seniors and all friends in other divisions of NIIST for their help and support. I express my sincere gratitude to all my teachers for their care and support.

I am also indebted to University Grant Commission (UGC) for financial assistance in the form of research fellowship and Council of Scientific & Industrial Research (CSIR) 12th five year plan project 'NaPAHA' and 'UNDO' for financial assistance.

I owe my deep sense of gratitude and regard to my parents, husband, daughter, brother and my in-laws for their prayers, affection, encouragements, inspiration, patience and support which smoothly paved my path towards the successful completion of the research work.

Finally, I humbly bow before the almighty God for showering his blessings upon me and giving me the strength, wisdom and patience to reach this important milestone in my academic life.

Anusree S S

.....TO MY FAMILY

CONTENTS

Title **Page No**

Chapter 2: Materials and methods

Chapter 5: Punicic acid safeguards mitochondria and ER function during insulin resistance by TNF-α in 3T3-L1 adipocytes

PREFACE

Insulin resistance is the major underlying pathophysiology of metabolic syndrome. The role of adipocytes in insulin resistance in metabolic syndrome is well known. The two main approaches for the management of insulin resistance include life style modifications and pharmacological interventions. The pharmacological means to improve insulin resistance utilizes drugs that are antihyperglycaemic agents which enhances insulin sensitivity. Peroxisome proliferator-activated receptor gamma (PPARγ) agonists are an important class of insulin sensitizers, the thiazolidinediones group of drugs, which belong to the nuclear receptor super family. Example of this class of drug is pioglitazone. But these synthetic and full PPARγ agonists have many adverse effects including cardiac failure. So there is an increasing requirement for natural partial PPARγ agonist with fewer side effects. In the present study, punicic acid (PA), a naturally occurring PPARγ agonist, which is found in pomegranate seed oil, was tested for its efficiency to improve insulin sensitivity in 3T3-L1 adipocytes.

The entire thesis is divided into six chapters including summary and conclusion. Chapter 1 comprises a general introduction on metabolic syndrome, insulin resistance, its prevalence and pathophysiology, the cross talk between insulin resistance, inflammation, mitochondrial and endoplasmic reticulum (ER) dysfunctions in adipose tissue. The chapter also describes various pharmacological interventions including PPARγ agonists for the management of insulin resistance and the importance of naturally occurring PPARγ agonists as treatment option for insulin resistance. Chapter 2 describes materials and methods used in the study.

Chapter 3 characterises PA as a partial PPARγ agonist, using various *in vitro* methods like adipocyte differentiation, triglyceride estimation and determination of GPDH activity. The chapter also describes the effect of PA on various adipocyte differentiation markers like C/EBPα, CD36 etc.and various insulin sensitivity markers like IRS2, PI3K and pAkt. Effect of PA on the translocation of GLUT4 in 3T3-L1 adipocyte was also studied. PA was characterized as a partial PPARγ agonist by TR-FRET assay and molecular docking study.

Chapter 4 describes the crosstalk betweenTNF-α induced insulin resistance and inflammation in 3T3-L1 adipocytes. TNF-α induced insulin resistance in 3T3-L1 adipocytes was found to be associated with an increase in the expression of SOCS3, PTP1B and serine phosphorylated IRS1. TNF-α also increased JNK phosphorylation, NFκB translocation to the nucleus and secretion of various inflammatory cytokines in 3T3-L1 adipocytes. TNF-α induced insulin resistance was also found to be associated with a decrease in the expression of insulin responsive genes like GLUT4 and PPARγ. PA was found to ameliorate TNF-α induced inflammation and insulin resistance in 3T3-L1 adipocytes.

Chapter 5 describes oxidative stress, mitochondrial dysfunction and ER stress associated with TNF-α induced insulin resistance in 3T3-L1 adipocytes. In addition, the chapter explains how PA improved mitochondrial dysfunction and associated ER stress in insulin resistance by studying various parameters like ROS production, mitochondrial membrane potential, mitochondrial superoxide production, ATP content, oxygen consumption, mitochondrial dynamics (fission and fusion), mitochondrial biogenesis etc. ER stress associated with TNF-α induced insulin resistance was monitored by studying the expression of various ER stress markers (GRP78, ERO1-α, PERK, IRE-1α, ATF-6 & CHOP) and intracellular Ca^{2+} homeostasis.

Chapter 6 is the overall summary and conclusion. The study established that PA is a partial PPARγ agonist with insulin sensitizing property. Since PA behaves as a partial PPARγ agonist and ameliorates insulin resistance and associated complications, it can be considered as an effective lead for the management of metabolic syndrome and insulin resistance.

List of Tables Page No

List of Figures Page No

LIST OF ABBREVIATIONS

2-NBDG	: 2-(N-(7-Nitr1o,b3e-dniza-z2o-lo-x4a-y- l)Amino)-2-Deoxyglucose
ap2	: Adipocyte protein 2
ADD1	: Adipocyte determination and differentiation factor-1
AMPK	: Adenosine monophosphate-activated protein kinase
ATCC	: American Type Culture Collection
ATF-6	: Activating transcription factor-6
ATP	: Adinosine triphosphate
BCA	: Bicinchoninic acid
BMI	: Body mass index
cAMP	: Cyclic AMP
CAT	: Catalase
CHOP	: CCAT enhance-binding protein homologous protein
CRP	: C-reactive protein
DAB	: 3, 3'-diaminobenzidine
DMEM	: Dulbecco's modified eagle medium
DRP1	: Dynamin related protein 1
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetraacetic acid
eIF2α	: Eukaryotic Initiation Factor 2 alpha
ELISA	: Enzyme-linked immunosorbent assay
FAT	: Fatty acid translocase
FATP	: Fatty acid transport protein
FBS	: Foetal bovine serum
FDA	: Food and Drug Administration
FFA	: Free fatty acid
FIS 1	: Fission protein 1
GLP1	: Glucagon-like peptide-1
GLUT4	: Glucose transporter-4
GPx	: Glutathione peroxidase
GR	: Glutathione reductase

GRP78 : Glucose-regulated protein 78

GSH : Glutathione

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IGT : Impaired glucose tolerance

IFN-γ : Interferon-gamma

IL-1β : Interleukin-1beta

IL-6 : Interleuki- 6

IRE-1α : Inositol-requiring enzyme 1alpha

IR : Insulin resistance

IRS1 : Insulin receptor substrate-1

JNK : c-Jun N-terminal kinase

LPL : Lipoprotein lipase

MCP1 : Monocyte chemotactic protein-1

MFN1 : Mitofusin-1

MFN2 : Mitofusin-2

MS : Metabolic syndrome

mtDNA : Mitochondrial DNA

MTT : 3-(4, 5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide

NAFLD : Non-alcoholic fatty liver disease

NBT : Nitro blue tetrazolium

NCEP-ATP III: National cholesterol education program adult treatment programme III

NF-κB : Nuclear factor kappa-light-chain-enhancer of activated B

NHANES : National health and nutrition examination survey

NRF1 : Nuclear respiratory factor 1

OPA1 : Optic atrophy 1

PA : Punicic acid

PBS : Phosphate buffered saline

PDI : Protein disulfide isomerise

PEPCK : Phosphoenolpyruvate carboxykinase

PERK : Protein kinase RNA-like endoplasmic reticulum kinase

PGJ2 : Prostaglandin J2

PI3K : Phosphatidylinositide 3-kinases

PKB : Protein kinase B

PKC : Protein kinase C

PPARα : Peroxisome proliferator-activated receptor alpha

PPARβ/δ : Peroxisome proliferator-activated receptor beta/delta

PPARγ : Peroxisome proliferator-activated receptor gamma

PVDF : Polyvinylidene difluoride

qRT-PCR : quantitative real time polymerase chain reaction

RIPA buffer : Radioimmunoprecipitation buffer

ROS : Reactive oxygen species

RXR : Retinoid X receptor

SCD1 : Stearoyl-CoA desaturase-1

SDS : Sodium dodecyl sulphate

SOD : Superoxide dismutase

SPPARγM : Selective PPARγ modulator

SREBP1 : Sterol regulatory element binding protein-1

STAT : Signal transducer and activator of transcription

T2DM : Type 2 diabetes mellitus

TG : Triglyceride

TBST : Tris buffered saline-tween 20

TCA : Tricarboxylic acid

Tfam : Transcription factor A, mitochondrial

TMB : 3, 3' -5, 5' Tetramethylbenzidine

TNF-α : Tumor necrosis factor alpha

TZDs : Thiazolidinediones

UCP1 : Uncoupling protein-1

UPR : Unfolded protein response

VLDL : Very low density lipoprotein

WAT : White adipose tissue

WHO : World Health Organization

XBP1 : X-box binding protein-1

$\Delta\psi m$: Mitochondrial membrane potential

Chapter 1

Introduction

1.1. Metabolic syndrome

Metabolic syndrome (MS) is now being considered as a major worldwide epidemic. MS represents a cluster of abnormalities including abdominal obesity, insulin resistance (IR), hyperlipidemia, type 2 diabetes mellitus (T2DM) and cardiovascular diseases. MS was variously named in time; the X syndrome, the cardiovascular metabolic syndrome, the IR-dyslipidemia syndrome, the atherogenic metabolic syndrome etc. (Huang, 2009). World health organization (WHO), in 1999 defined MS as impaired glucose tolerance (IGT) or diabetes mellitus, and/or IR, together with two or more of the components listed below (Parikh and Mohan, 2012):

1. Raised arterial pressure, i.e., ≥140/90 mm of Hg
2. Raised plasma triglyceride (≥ 150 mg/dl) and/or low HDL-C (<35 mg/dl in men and <39 mg/dl in women)
3. Central obesity, i.e., waist/hip ratio (WHR) >0.9 in men and >0.85 in women and/or body mass index (BMI) >30 kg/m^2
4. Microalbuminuria, i.e., urinary albumin excretion rate ≥ 20 μg/minute or albumin/creatinine ratio ≥ 30 μg/mg.

The risk of development of MS increases with increase in the occurrence of number of factors contributing to its development (Andreadis et al., 2007). The co-occurrence of many of these factors increases the risk of development of cardiovascular abnormalities in MS (Reilly and Rader, 2003). People affected by MS are reported to have greater risk of developing T2DM and cardiovascular diseases which are the major pathologies from non-communicable diseases contributing to mortality and morbidity worldwide (Buttar et al., 2005; Wilborn et al., 2005). Also, MS is associated with chronic low grade tissue inflammation leading to fatty liver, polycystic ovary syndrome, asthma and even some types of cancer (Buttar et al., 2005; Ouchi et al., 2011).

1.1.1. Pathophysiology of MS

MS is a complex disorder affected by genetic and environmental factors (Kaur, 2014). The predominant risk factors for the development of metabolic syndrome are visceral obesity and IR. MS is characterized by an accumulation of several interrelated abnormalities including, IR, visceral obesity, atherogenic dyslipidemia, thrombophilia, hyperuricaemia, hypertension etc. (Bonora and Targher, 2012). Obesity, especially visceral obesity, in MS is associated with hypertrophy and hyperplasia in adipose tissue (Halberg et al., 2008). Since adipose tissue is composed of different cell types like adipocytes, stromal pre-adipocytes, immune cells, and endothelium, a hypertrophy of adipose tissue can cause an infiltration of macrophages into the adipose tissue leading to the overproduction of various proinflammatory cytokines like tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), plasminogen activator inhibitor 1 (PAI-1) and C-reactive protein (CRP) (Lau et al., 2005). Hypertrophy and hyperplasia of adipose tissue also causes an increased release of free fatty acids into the circulation, leading to an increased production of very low density lipoprotein (VLDL) into the circulation, causing metabolic dyslipidemia (Bamba and Rader, 2007). The dyslipidemia and increased concentrations of VLDL and triglyceride (TG) in the circulation cause the development of hypertension (Laaksonen et al., 2008). MS is also associated with oxidative stress, hyperuricemia, thrombophilia and non-alcoholic fatty liver diseases (NAFLD). The pathophysiological conditions associated with MS are represented diagrammatically in Figure 1.1.

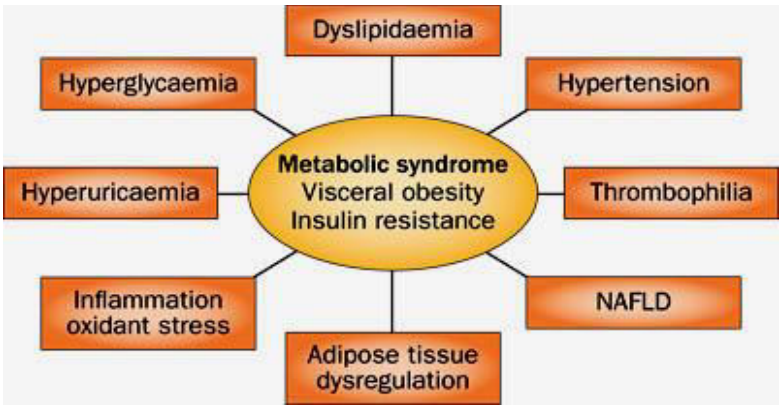


Figure 1.1. Pathophysiology of metabolic syndrome: Insulin resistance and visceral obesity are the predominant risk factors associated with MS (Bonora and Targher, 2012). NAFLD- non alcoholic fatty liver disease.

1.1.2. Prevalence of MS

The prevalence of MS is increasing worldwide due to sedentary lifestyle and consumption of high calories (Kaur, 2014). According to International Diabetic Federation (IDF), around one-quarter of the world's adult population is affected with MS (The IDF consensus worldwide definition of the MS, 2006). Depending upon the region, environment (rural or urban), sex and age of the population, MS ranges from <10% to about 84% (Desroches and Lamarche, 2007). The prevalence of MS is affected by various factors including diet, genetic background, physical activity and family history of diabetes (Cameron et al., 2004). According to National Health and Nutrition Examination Survey (NHANES), the prevalence of MS was found to be 5% among the subjects of normal weight and 22% among the overweight. For obese subjects, the prevalence was found to be 60% (Park et al., 2003).The prevalence of MS also increases with age. In individuals in the age group of 20-29, it increases to 10%, 20% in individuals aged of 40–49, and 45% in individuals aged 60–69 (Ford et al., 2002). According to National Cholesterol Education Program- Adult Treatment Programme III (NCEP-ATP III) 2001 criteria, the prevalence of MS varied from 8% to 43% in men and from 7% to 56% in women around the world (Cameron et al., 2004).

In India, the prevalence of MS was found to be higher in urban area as compared with rural area, and increases further with increase in the socio-economic status (Mohan et al., 2001; Mohan et al., 2005; Ramachandran et al., 1998). Epidemiological studies showed that the prevalence of MS is significantly lower in rural subjects and varies from 9% to 12%, and in urban South Asians, it ranges from 30-50% (Gupta et al., 2004; Enas et al., 2007). South Asians have a distinct obesity phenotype, which is characterised by abdominal obesity, excess body fat, fat deposition in ectopic sites, including liver which contribute to the increased prevalence of MS (Misra and Khurana, 2009).

1.2. Insulin resistance (IR)

IR is the major underlying pathophysiology of MS (Guo, 2014). IR is a condition where a normal or elevated level of insulin produces an attenuated biological response (Cefalu, 2001). In IR, there is an impairment in insulin- mediated glucose disposal and the cells are not able to respond to the normal circulating levels of insulin.

The major metabolic consequence of IR is hyperglycemia, which results from the failure of insulin to decrease hepatic glucose output and also glucose disposal by other peripheral tissues like skeletal muscles (Wilcox, 2005). So the pancreatic β-cells secrete more insulin to maintain the normal plasma level of glucose and hyperinsulinemia results. So, over time, when IR progresses, pancreatic β-cells are no longer able to secrete the adequate amount of insulin, leading to the development of T2DM (Reaven, 1995). The metabolic staging of IR and T2DM is represented in Figure 1.2.

Obesity associated expansion of the adipocyte, as well as the deficiency of adipocyte (lipodystrophy) are associated with the development of IR. Both the conditions cause an increased influx of TG into the circulation, leading to the accumulation of excess fat in other insulin responsive tissues, leading to systemic IR. Thus, adipose tissue plays an important role in the development of IR (Frayn, 2001).

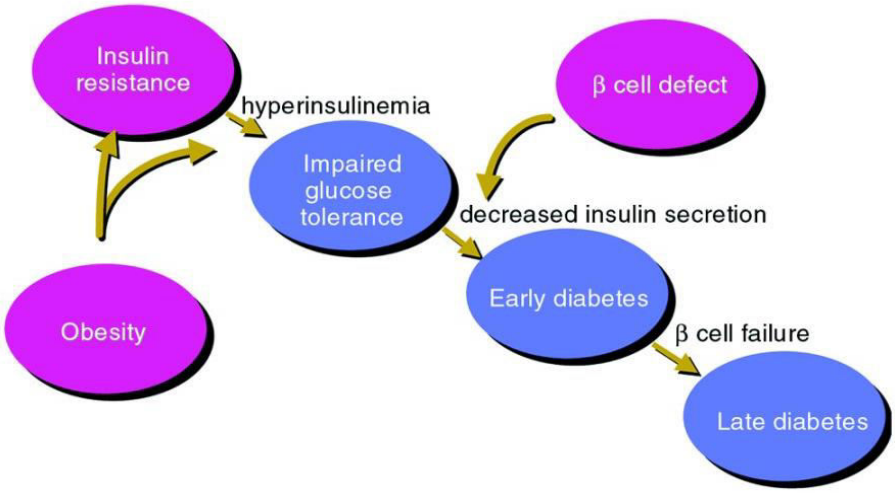


Figure 1.2. Metabolic staging of insulin resistance and type 2 diabetes mellitus: Hyperglycemia and hyperinsulinemia causes pancreatic β-cell dysfunction leading to T2DM (Saltiel, 2000).

1.3. White adipose tissue -Physiological importance

There are mainly three types of adipose tissue, white, brown and beige (brite) (Giralt and Villarroya, 2013). White adipose tissue (WAT) is a loose connective tissue. It is mainly of two types, subcutaneous WAT, which is located under the skin and visceral WAT, which is found around the internal organs (Gil et al., 2011). The primary functions of white adipose include heat insulation, mechanical cushion and most importantly, it act as a source of energy. WAT helps to store excess energy in the

form of TG. Brown adipose tissue (BAT) is mainly found in newborn infants and foetuses and helps in thermogenesis through mitochondrial uncoupling protein 1 (UCP1) (Fonseca-Alaniz et al., 2007). Beige adipose tissues are BAT appearing in the region of WAT. Like WAT, they have low basal level expression of UCP1, but like the BAT, on stimulation with cyclic AMP (cAMP), there is a high expression of UCP1 and respiration rate (Wu et al., 2012).

WAT is a metabolically active organ that affects a number of functions including glucose and lipid metabolism, thermogenesis, immunity, cardiovascular function and neuroendocrine function (Trujillo and Scherer, 2006). In early 1987, adipose tissue was identified as the site of production of adipsin, an endocrine factor, which is remarkably down-regulated in obesity (Flier et al., 1987). In 1994, another hormone called leptin was identified, which confirms the role of adipose tissue as an endocrine organ (Zhang et al., 1994). Adipose tissue is now being considered as a major endocrine organ which secretes a number of bioactive peptides known as adipokines. Adipose tissue also expresses a number of receptors for hormones from traditional hormone system and central nervous system (Kershaw and Flier, 2004). Besides adipocytes, adipose tissue contains connective tissue, stromovascular cells, nerve tissues and immune cells (Frayn et al., 2003). These components function as an integral unit and make adipose tissue an endocrine organ.

The major cytokines secreted by adipocytes include adiponectin, leptin, TNF-α, MCP-1, IL-6, MCP-1, PAI-1 etc. These adipokines can affect adipocyte function in an autocrine or paracrine way or can be released into the circulation and affect one more metabolic pathways in an endocrine way (Ronti et al., 2006; Chaldakov et al., 2003).

1.3.1. Insulin signaling in WAT

Insulin signaling cascade in adipocytes is initiated by the binding of insulin to its receptor. The binding of insulin to the receptor causes autophosphorylation of the receptor, causing the activation of receptor tyrosine kinases leading to the tyrosine phosphorylation of the insulin receptor substrates (IRSs). Phosphorylation of IRS promotes the activation of various serine/threonine kinases, phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B (PKB) and protein kinase C (PKC) (Sale and Sale 2008; Scott et al., 1998). Phosphorylated/activated Akt initiates the translocation

of glucose transporter-4 (GLUT4) from the cytoplasm to the surface of the cell allowing glucose to enter down its concentration gradient (Olson, 2012). A Schematic representation of insulin signaling pathways in adipocytes is given in Figure 1.3.

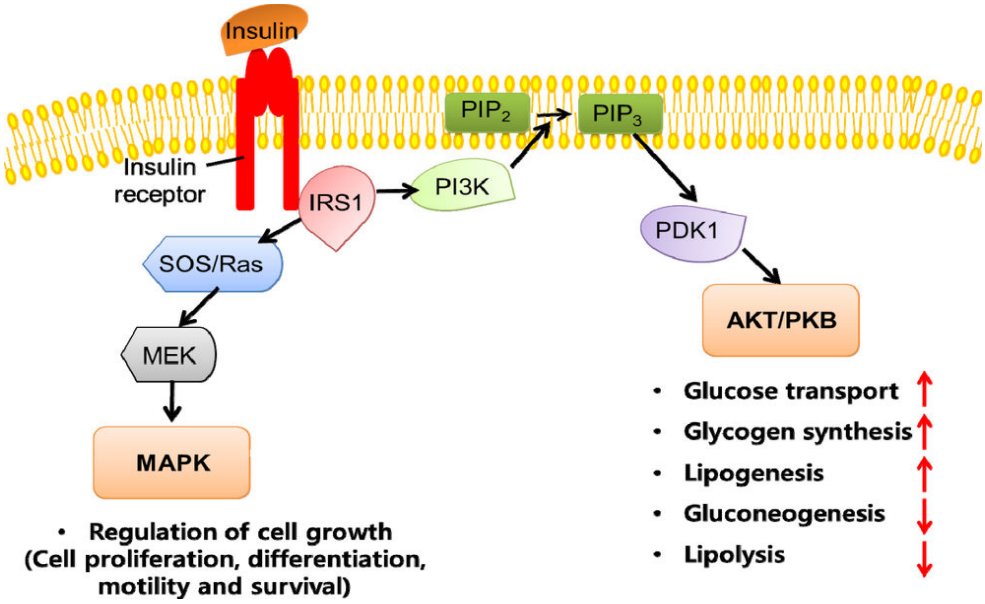


Figure 1.3. Schematic view of insulin signaling pathway in adipose tissue: PI3/Akt pathway leads to the regulation of glucose and lipid metabolism. The Ras-mitogen-activated protein kinase pathway leads to the activation of genes which are involved in the regulation of cell growth, proliferation, differentiation and survival (Jung and Choi, 2014). IRS1, insulin receptor substrate 1; MAPK, mitogen-activated protein kinase; PDK, phosphoinositide-dependent protein kinase 1; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B.

1.3.2. Adipocyte differentiation

Adipocyte differentiation or adipogenesis is the process which is a highly coordinated signaling pathway, by which, pre-adipocytes are converted into adipocytes. It is accompanied by morphological changes and changes in gene expression. Peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT/Enhancer binding protein α (C/EBPα) are the key transcription factors which are involved in the process of adipocyte differentiation, which act synergistically leading to the development of insulin responsive fully differentiated adipocytes (Camp et al., 2002).

The stages of adipocyte differentiation include a determination and terminal differentiation phase. In the determination phase, stem cells are converted into preadipocytes, which though morphologically indistinguishable, have lost the potential

to differentiate to other cell types (Otto and Lane, 2005). Initially, there occurs a growth arrest phase of pre-adipocytes, which is normally achieved by contact inhibition, followed by a clonal expansion of pre-adipocytes. Initially there is a transient increase in the expression of transcriptional factors, C/EBPβ and C/EBPδ followed by PPARγ and C/EBPα, which enhances the expression of proteins, which contribute to the characteristic adipocyte phenotype. In terminal differentiation phase, pre-adipocytes take the characteristics features of adipocytes and adipocytes increases in size. During the terminal phase, there is increased synthesis of proteins required for lipid transport and synthesis, insulin sensitivity and adipocyte specific proteins (Rosen and MacDougald, 2006).

1.3.2.1. Transcriptional regulation of adipocyte differentiation

Adipocyte differentiation includes a coordinated regulation of adipocyte specific genes. PPAR and C/EBP family of transcription factors function cooperatively to transactivate adipocyte genes leading to the differentiation process.

PPARγ is the key regulator of adipogenesis. It is important for promotion of differentiation to the mature adipocyte and for maintenance of the differentiated state (Rosen and MacDougald, 2006). Among the three isoforms of PPARγ, PPARγ1 and PPARγ2, which are mainly expressed in adipose tissue is considered as the important type required for adipogenesis (Otto and Lane, 2005). It is responsible for activation of adipogenic genes such as adipocyte-selective fatty acid binding protein (aP2), lipoprotein lipase (LPL), acyl CoA synthase and phosphoenolpyruvate carboxykinase (PEPCK). These genes are involved in fatty acid transport, uptake and storage in adipocytes (Rosen and MacDougald, 2006; Tontonoz et al., 1995).

C/EBP class of transcription factor is the other important transcription factor involved in adipocyte development. It has six isoforms, α, β, γ, δ, ζ and ε. All except the ε form is involved in adipogenesis (Otto and Lane, 2005). During adipogenesis, C/EBPβ and C/EBPδ are detected first, followed by PPARγ, which in turn activates C/EBPα (Rosen et al., 2000). C/EPB-α can activate the expression of adipocyte differentiation genes like aP2, stearoyl CoA desaturase-1 (SCD1), GLUT4, PEPCK, leptin, and the insulin receptor (Hwang et al., 1996). Both C/EBPβ and C/EBPδ directly induce expression of PPARγ and C/EBPα, the key transcriptional regulators of

adipocyte differentiation. C/EBP isoforms like C/EBPγ and C/EBPζ are considered as anti-adipogenic (Rosen and MacDougald, 2006).

Adipocyte determination and differentiation factor 1/ sterol regulatory element binding protein-1 (ADD1/SREBP1) is another important transcription factor involved in adipogenesis. It can activate PPARγ by inducing its expression as well as by promoting the production of an endogenous PPARγ ligand (Rosen et al., 2000). In addition to these, several other transcription factors are also found to be involved in the process of adipocyte differentiation, including PPARδ, the orphan nuclear receptors RORγ, various signal transducer and activator of transcription (STAT) proteins etc. (Amri et al., 1995; Austin et al., 1998; Stephens et al., 1999). A representation of transcriptional regulation of adipogenesis is given in Figure 1.4

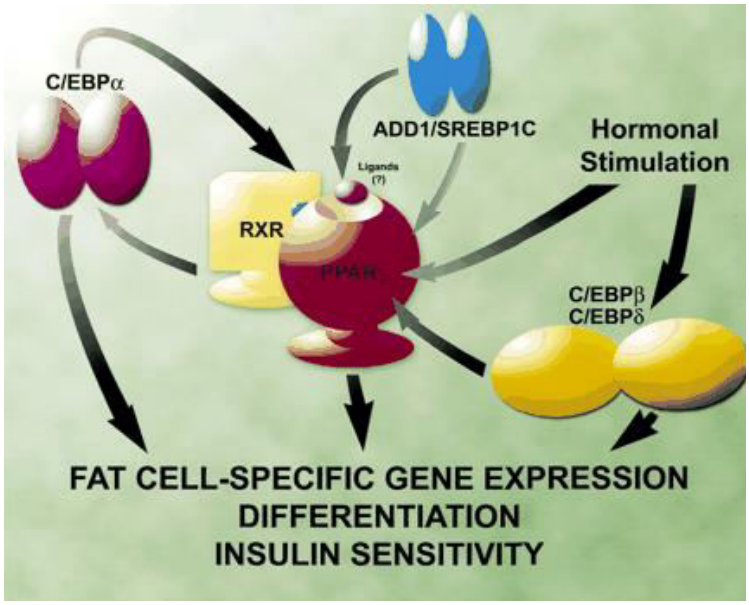


Figure 1.4. Transcriptional regulation of adipogenesis: Adipocyte differentiation is a highly coordinated pathway which is regulated by numerous transcription factors like C/EBPα, β, δ, PPARγ and ADD1/SREBP1C (Rosen et al., 2000).

1.3.2.2. Cell culture models to study adipocyte differentiation

Both primary culture and cell lines can be used as models to study the process of adipocyte differentiation (Hwang et al., 1997). Primary cultures are isolated from vascular stroma of adipose tissue from animal models. They are expensive, difficult to isolate and are heterogeneous, which limit their use (Ntambi and Kim, 2000). The cell line models mainly used are multipotent fibroblastic cell lines or established preadipocyte cell lines. The multipotent fibroblastic cells are not committed, but are

capable of differentiating. Examples include NIH-3T3, Balb/c3T3, C3H 10T1/2 etc. Preadipocyte cell lines include 3T3-L1, Ob1771, 3T3F442A, TA1, Ob17 etc., which has the same morphological and biochemical properties of *in vivo* adipocytes and are more homogeneous and easy to maintain in culture (Hwang et al., 1997).

1.3.2.2.1. 3T3-L1 preadipocytes

The murine 3T3-L1 preadipocyte cell line is a widely used model to investigate the adipocyte differentiation process. They are derived from cloned subline of Swiss 3T3 mouse embryo fibroblasts (Todaro and Green, 1963). They can undergo differentiation in culture and exhibit morphology and biochemical properties similar to adipocytes. In culture, 3T3-L1 preadipocytes can grow at high rate and reach growth arrest and steady stage (Todaro and Green, 1963). The post confluent 3T3-L1 preadipocytes when treated to a hormonal cocktail of differentiation inducers consisting of dexamethasone (Dex), 3-isobutyl-1-methylxanthine (IBMX) and insulin, there is an increase in cAMP level, which can differentiate preadipocytes to mature adipocytes in 6 to 8 days (Pantoja et al., 2008).

1.4. Adipose tissue inflammation in obesity and IR

Obesity related MS was found to be associated with chronic low-grade inflammation in adipose tissue and the accumulating evidences suggest the role of adipocytes in the development of IR in MS (Frayn 2001; Guilherme et al., 2008; Romeo et al., 2012). Adipose tissue has an important role in buffering the daily influx of fat in our body (Goossens, 2008). In obesity, this buffering is disturbed and causes an increase in the secretion of various pro-inflammatory cytokines and free fatty acids into the circulation, leading to a state of inflammation in adipocytes (Guilherme et al., 2008). Since there is increased concentration of free fatty acids in the circulation during obesity, it leads to the accumulation of fat in non-adipose tissue including skeletal muscle, liver and pancreatic islets, which cause a local IR in these sites (Boden, 1997; Eckel et al., 2005). Increased accumulation of fat in liver causes increased glucose output and reduced glucose clearance by liver, leading to systemic IR (Fanelli et al., 1993; Ferrannini et al., 1983).

Obesity associated chronic, low-grade inflammation in adipocyte is considered as an important factor in the pathogenesis of IR (Cawthorn and Sethi, 2008; Makki et al., 2013; Shoelson et al., 2006). As the size and mass of adipocytes are increased in obesity, macrophage content in adipose tissue is also increased (Weisberg et al., 2003), and there is a phenotypic switch of these macrophages from antiinflammatory M2 polarization to proinflammatory M1 phenotype causing increased secretion of pro-inflammatory cytokines including TNF-α (Lumeng et al., 2007). In addition to M1 macrophages, levels of multiple pro-inflammatory immune cells, such as interferon (IFN)-γ+ T helper type 1 cells and CD8+ T cells, are increased in adipose tissue in obesity (Schipper et al., 2012) and on the other hand, in obesity, the secretion of insulin-sensitizing adipocytes with anti-inflammatory activity such as adiponectin is decreased contributing further to IR and inflammation (Figure. 1.5). The inflammatory pathways are also developed in other insulin sensitive tissues like liver and skeletal muscles, suggesting a close connection between inflammation and IR in MS (Figure. 1.6). The two important inflammatory pathways activated in adipocytes during inflammation are c-Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Hirosumi et al., 2002; Yuan et al., 2001).

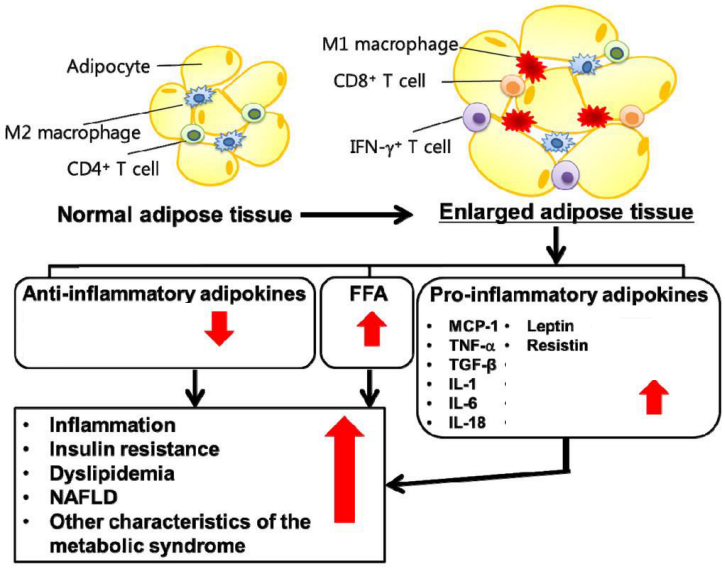


Figure 1.5. Secretion of adipokines from adipose tissue in normal and obese state: Enlargement of adipose tissue as in the case of obesity causes increased secretion of various proinflammatory cytokines and decreased secretion of anti-inflammatory cytokines leading to the development of inflammation and insulin resistance (Jung and Choi, 2014). NAFLD-non-alcoholic fatty liver disease

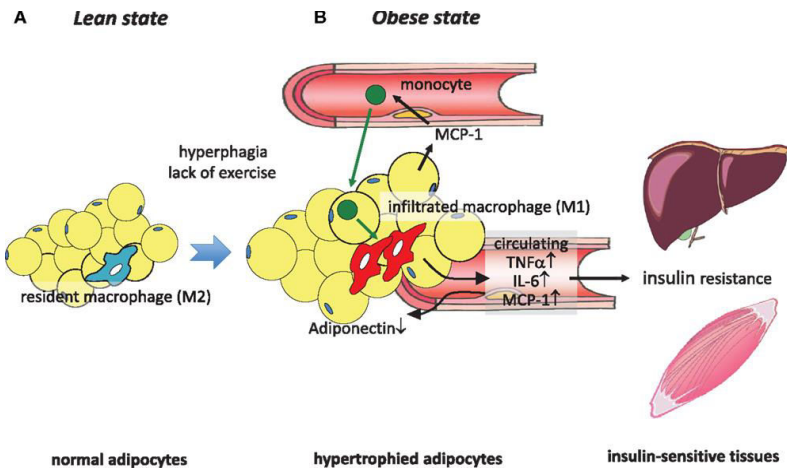


Figure 1.6. Adipose tissue hypertrophy leading to IR in peripheral tissues in obesity: In obesity, the level of pro-inflammatory cytokines like TNFα, IL-6, MCP-1 are increased, leading to IR in liver and muscles (Tateya et al., 2013)

1.4.1. IKKβ/NF-κB Pathway

IKKβ/NF-κB pathway is the most dominant inflammatory pathway. NF-κB is involved in a series of pathological processes including inflammation and immune responses (Rahman and McFadden, 2011). NF-κB is a transcription factor, found in the cytoplasm in association with IκB-α protein in normal conditions. On activation with several intracellular signals including ROS and inflammatory cytokines, the two subunits of IκB kinase (IKK) complex get activated, leading to the phosphorylation of IκBα on Serine 32 and 36, causing the degradation of IκBα and translocation of NF-κB to the nucleus (Han et al., 2009). The translocation of NF-κB to the nucleus causes the upregulation of various target genes and increased the expression of various inflammatory mediators including TNF-α, IL-1β, and IL-6 (Chen et al., 2015).

1.4.2. c-Jun N-terminal kinase (JNK) Pathway

JNK belongs to p38 mitogen-activated protein kinases (MAPK) family and there are mainly three different isoforms of JNK (JNK 1, 2 and 3). JNK activation can lead to the production of proinflammatory cytokines leading to inflammation and IR (Han et al., 2013). Endoplasmic reticulum (ER) stress can cause an activation of JNK, leading to serine phosphorylation of IRS1 and IR. Activated JNK can inhibit the secretion of insulin from pancreatic β-cells, thereby enhancing IR (Lanuza-Masdeu et

al., 2013). Suppression of JNK pathway is reported to improve IR and inhibition of JNK is reported to reduce the release of proinflammatory cytokines such as TNF-α and MCP-1 and improves insulin sensitivity (Hsu et al., 2012; Jin et al., 2011; Nakatani et al., 2004).

1.4.3. Adipokines and insulin resistance

TNF-α is an important cytokine that can regulate many physiological processes including inflammation, differentiation, apoptosis and energy metabolism (Cawthorn and Sethi, 2008). It can affect glucose homeostasis in adipocytes and can promote lipolysis in cultured adipocytes (Stephens and Pekala, 1991). TNF-α is found to be elevated in obese diabetic rodents and acts as a mediator in obesity related IR and T2DM (Hotamisligil et al., 1993). It has been shown that it causes IR by increasing the serine phosphorylation of IRS1 (Shoelson et al., 2006) via activation of JNK1/2 (Chen et al., 2015). The levels of TNF-α was found to be higher in obese people compared to lean subjects, indicating that obese people are more prone to the development of IR and other associated complications (El-Haggar and Mostafa, 2015). In addition, TNF-α down-regulates the expression of many insulin sensitive proteins like GLUT4 and PPARγ (Cawthorn and Sethi, 2008). It can also downregulate the expression of PPARγ target gene C/EBPα (Jain et al., 1999). On the other hand TNF-α can activate NFκB and NF-κB target gene expression, accelerating inflammation and associated complications (Cawthorn and Sethi, 2008). TNF-α inhibits tyrosine phosphorylation of IRS1 via suppressor of cytokine signaling 3 (SOCS3) gene and promote its serine phosphorylation (Emanuelli et al., 2001; Hotamisligil et al., 1996).

In hyperglycemia and T2DM , the level of the proinflammatory cytokine, interleukin-1β (IL-1β) is found to be increased. IL-1β plays an important role in the development of inflammation induced organ dysfunction in T2DM (Grant and Dixit, 2013). IL-1β impairs insulin signaling in peripheral insulin sensitive tissues and macrophages, and reduced insulin sensitivity in pancreatic β-cells, leading to impaired insulin production (Chen et al., 2015).

IL-6 is another important proinflammatory cytokine secreted by adipocytes and it causes IR by decreasing the expression of GLUT4 and IRS1, by increasing the expression of SOCS3 and activating Janus kinase-signal transducer and activator of

transcription (JAK-STAT) signaling pathway (Serrano-Marco et al., 2012). IL-6 can also block PI3K pathway, contributing further to the development of IR (Chen et al., 2015).

Leptin is another important adipokines secreted by adipose tissue, which is involved in the suppression of appetite and energy homeostasis. The leptin-signaling pathway can activate SOCS3 pathway which in turn inhibit insulin signaling (Howard and Flier, 2006). A condition called leptin resistance, which is characterized by increased plasma leptin concentration, is found in obese conditions (Chen et al., 2015). Increased plasma leptin concentration is associated with hyperinsulinemia and IR (Martin et al., 2008). Increased leptin concentration can induce IR by decreasing insulin secretion from pancreatic β cells through direct action on leptin receptors on pancreatic β cells (Seufert et al., 1999).

Resistin is another important cytokine produced by adipocytes. The concentrations of resistin is found to be increased with increase in the levels of inflammatory markers and its concentrations increase concurrently with the levels of inflammatory mediators (Reilly et al., 2005). The levels of leptin were found to be elevated during obesity and IR (Szulinska et al., 2014). Resistin can increase the expression of various proinflammatory cytokines like TNF-α and IL-6, through NF-κB pathway, and also by activating toll like receptor-4 (TLR4) (Benomar et al., 2013).

MCP-1 is a proinflammatory chemokine produced by adipocytes, macrophages, and endothelial cells, which recruits the immune cells to the site of inflammation (Kanda et al., 2006). In obesity, there is an increase in the expression of MCP-1, contributing to the pathogenesis of IR (Kanda et al., 2006). It plays a role in the pathogenesis of IR by regulating the inflammatory response, macrophage polarization and infiltration, and the phosphorylation of extracellular signal-regulated kinase-1/2 (ERK-1/2) and p38 MAPK (Nio et al., 2012).

Adiponectin is an important adipokines produced by WAT, the amount of which is found to be reduced in obesity, IR and T2DM. There are three major isoforms of adiponectin; low molecular weight (LMW), formed from three adiponectin monomers, middle-molecular weight (MMW) which is an octomer, and high-molecular weight (HMW) consisting of 12 or more monomers (Magkos and Sidossis, 2007). The adiponectin receptor 1 (Adipo R1) is involved in the amelioration of IR by

reducing the expression of genes involved in hepatic gluconeogenesis, and by activating adenosine monophosphate-activated protein kinase (AMPK). Adiponectin receptor 2 (AdipoR2) increases glucose consumption by activating PPARα signaling in the liver (Crimmins and Martin, 2007; Yamauchi et al., 2007).

1.5. Insulin resistance and oxidative stress in adipocytes

Cellular redox homeostasis is maintained by innate antioxidant system which includes enzymatic and non-enzymatic antioxidants. The major enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) and the non-enzymatic antioxidants include β-carotene, reduced glutathione (GSH), vitamins E, C etc. (Gomes et al., 2012). Decreases in the activity of these antioxidant enzymes are reported in high fat fed diabetic mice models (Furukawa et al., 2004; Lee et al., 2006).

Oxidative stress in adipose tissue is considered as an early event in the development of MS. Excess nutrient intake and expansion of visceral adipose tissue causes an increased generation of ROS, leading to increased production of inflammatory cytokines from adipose tissue, leading to the development of IR in adipose tissue as well as in peripheral tissues (Aroor and DeMarco, 2014). Also, the increased production of pro-inflammatory cytokines in obesity can lead to the development of oxidative stress in adipocytes. The increased production of ROS and decreased activity of antioxidant enzymes causes oxidative stress in adipocytes and this pro-oxidant state may impair insulin signaling and leading to the development of IR and T2DM (Roberts and Sindhu, 2009).

1.6. Insulin resistance and mitochondrial dysfunctions in adipocytes

Since glucose and lipid metabolism is the main energy sources of the cell, there is a pivotal role for mitochondria in these biochemical processes. Accordingly mitochondrial dysfunction has been reported in the genesis of IR and associated complications (Kim et al., 2008). Mitochondria has been considered as a global regulator of energy homeostasis and factors like nutrient excess, inflammation, oxidative stress etc. contribute to mitochondrial dysfunction and lead to the development of IR (Kusminski and Scherer, 2012). Impairment in mitochondrial ATP

production, respiration rate, membrane potential and increased mitochondrial ROS production are the hallmarks of mitochondrial dysfunction (Brand and Nicholls, 2011).

Mitochondrial biogenesis involves the coordinated action of both nuclear and mitochondrial encoded genomes. Mitochondrial biogenesis is regulated by various factors like PPARγ coactivator-1α (PGC-1α), nuclear respiratory factors (NRFs) and mitochondrial transcription factor A (Tfam) (Liu et al., 2009). The key regulatory molecules modulating mitochondrial biogenesis like PGC-1α, NRFs and Tfam are decreased in IR (Hao et al., 2010; Heilbronn et al., 2007). Role of WAT in mitochondrial dysfunction is illustrated in Figure 1.7.

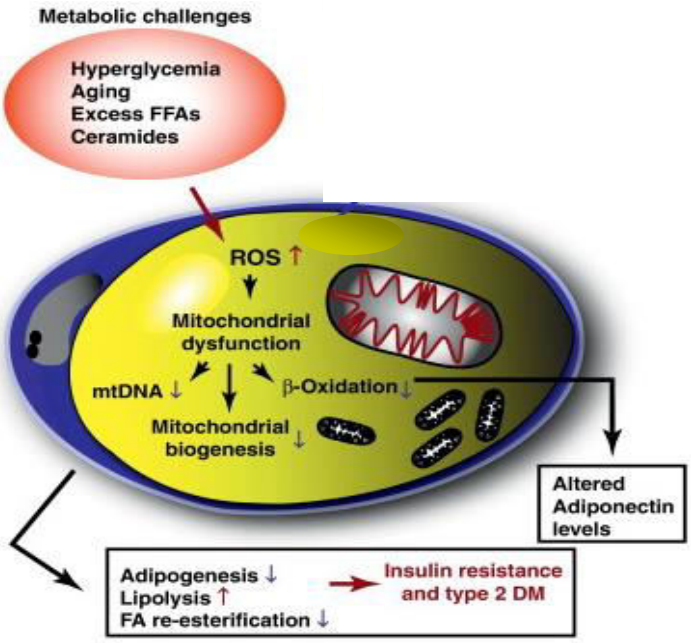


Figure 1.7 Mitochondrial dysfunction and IR in WAT: Metabolic challenges like hyperglycemia increases the development of ROS leading mitochondrial dysfunction and insulin resistance (Kusminski and Scherer, 2012). FFA, free fatty acid; TZDs, thiazolidinediones

In addition, IR is found to be associated with altered mitochondrial dynamics (Hahn et al., 2014; Chen et al., 2010; Jheng et al., 2012). Mitochondria are dynamic organelle that undergo continuous fission and fusion in a balanced way to maintain mitochondrial morphology and function. The rates of mitochondrial fission and fusion are finely regulated by mitochondrial fission proteins and fusion proteins. An altered mitochondrial dynamics can lead to mitophagy leading to a reduction in the number of functional mitochondria (Seo et al., 2010). Mitochondrial fusion proteins include mitofusins 1 and 2 (MFN1 and MFN2), which are involved in the fusion of outer

mitochondrial membrane, and optic atrophy 1 (OPA1), which is involved in the fusion of inner mitochondrial membrane (Escobar-Henriques and Anton, 2013). The mitochondrial fission proteins are dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1). The fission protein DRP1 is mostly found in the cytoplasm, and it is translocated into the mitochondrial surface for the process of fission with the help of proteins called FIS1, mitochondrial fission factor (Mff) and mitochondrial dynamics proteins of 49 and 51 (MiD49 and MiD51) (Archer, 2013; Lee et al., 2004). An illustration of mitochondrial fission and fusion is given in Figure 1.8.

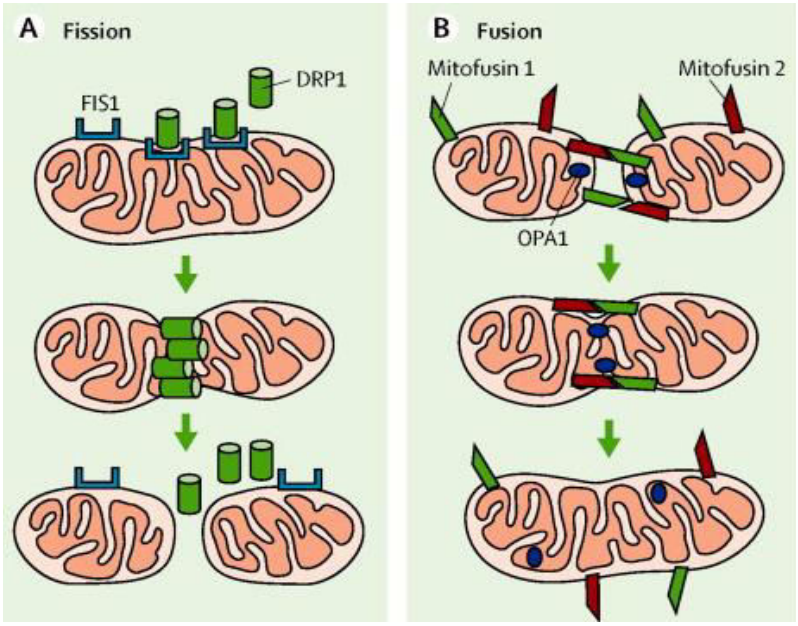


Figure 1.8. Proteins associated with mitochondrial fission and fusion: Mitochondrial fission is controlled by mitochondrial fission protein 1(FIS1) and dynamin-related protein 1 (DRP1). Mitochondrial fusion is mainly controlled by fusion proteins mitofusins 1 and 2 (MFN1 and MFN2) and optic atrophy 1 (OPA1) (Hagberg et al., 2014).

A balance between mitochondrial fission and fusion is required for the normal functioning of the mitochondria. A high mitochondrial fission rate can lead to the development of highly fragmented mitochondria which are nonfunctional and get engulfed by a phagophore. A possible relationship between mitochondrial fission, fusion, degradation and mitophagy are represented in Figure 1.9. IR is found to be associated with an altered mitochondrial dynamics in cultured adipocytes as well as in muscle cells (Chen et al., 2010; Jheng et al., 2012)

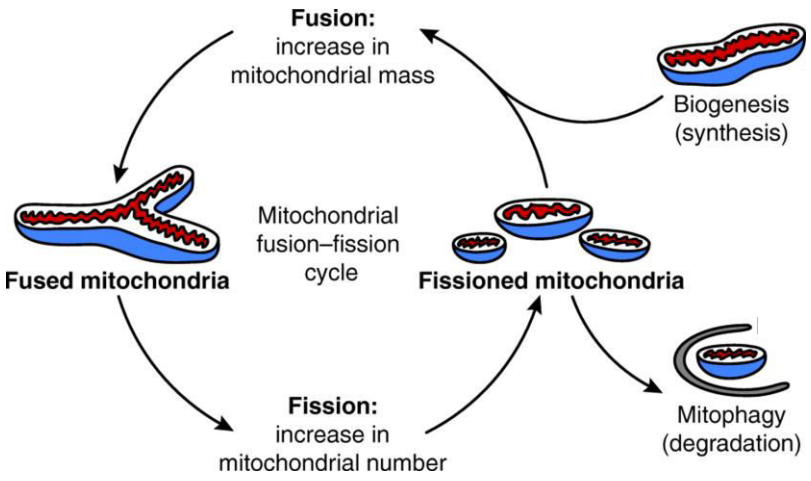


Figure 1.9. Possible relationship between mitochondrial fusion, fission, degradation and mitophagy: A high rate of mitochondrial fission leads to the formation of highly fragmented mitochondria, which are removed by mitophagy (Seo et al., 2010).

1.7. Insulin resistance and endoplasmic reticulum (ER) stress

ER stress is developed as an early consequence of over/excess nutrient intake and act as a cause for the development of IR and inflammation (Boden, 2008). Excess nutrient intake seems to be an important factor contributing to the development of ER stress in obesity. Adipose tissue is the site, where excess energy is stored in the form of fat, and, so it needs to synthesize many proteins to cope up with this situation. Also, the expansion of adipose tissue requires an increased synthesis of many structural proteins and can lead to the development of ER stress. It has been shown recently that in obese human subjects, ER stress is increased in the subcutaneous fat (Boden et al., 2008). Over expression of some chaperone genes or administration of chaperones has been found to ameliorate ER stress, inflammation and IR (Gregor and Hotamisligil, 2007). In obesity and overnutrition, the amount of free fatty acids in the circulation is increased and this circulating fatty acid can cause ER stress in pancreatic β-cells and cultured hepatocytes (Lai et al., 2008).

ER stress can contribute to the development of IR by triggering the activation of different serine/threonine kinases like JNK and IKK. ER stress interferes with insulin signaling by the activation of JNK leading to serine phosphorylation of IRS1 and 2 (van der Kallen et al., 2009). Considering these facts, it can be said that

ameliorating ER stress in obesity has an important role in maintaining the pathogenesis of IR. The association between ER stress and IR is illustrated in Figure 1.10.

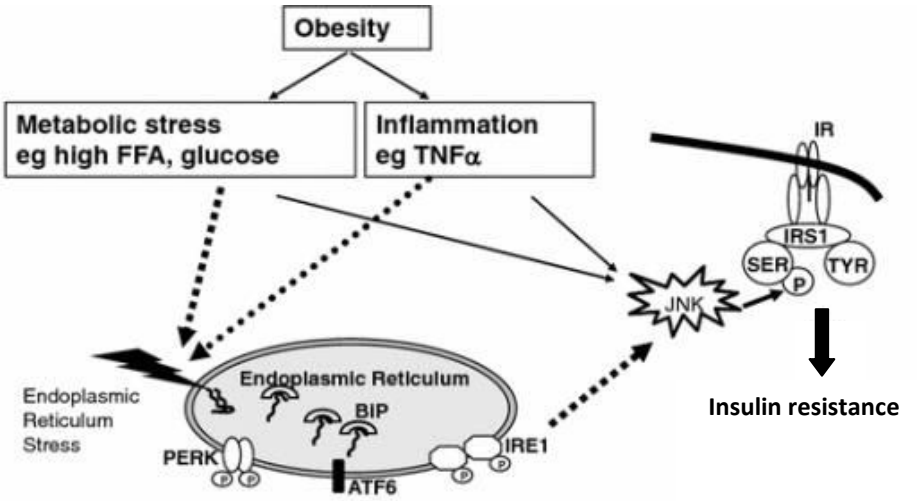


Figure 1.10. The role of ER stress in obesity related IR: ER stress causes the phosphorylation of JNK leading to serine phosphorylation of IRS-1 and IR (van der Kallen et al., 2009).

1.7.1. Endoplasmic reticulum stress and unfolded protein response (UPR)

ER is a major site for protein synthesis and folding. The newly synthesised proteins from ribosomes are trafficked to the ER lumen and the molecular chaperons and foldases found in the ER lumen help in the post translational modifications and proper folding of these nascent peptides (Schroder and Kaufman, 2005). The properly folded proteins are then transported to the Golgi apparatus, where they are further modified and then trafficked to their destinations. If there is an increased influx of unfolded or misfolded proteins to the ER membrane, which is beyond the capacity of ER, ER stress is implicated, activating signaling network called the unfolded protein response (UPR) (Figure 1.11).

UPR mainly respond via three transducers, namely, inositol-requiring enzyme-1 (IRE-1), PKR-like ER-regulating kinase (PERK), and activating transcription factor-6 (ATF-6). The UPR sensors transmit signals of ER stress from the ER to the cytoplasm or nucleus, and activate three pathways: i) suppression of protein translation to avoid the generation of more unfolded proteins; ii) induction of genes encoding ER molecular chaperones to facilitate protein folding; and iii) activation of ER-associated degradation (ERAD) to reduce unfolded protein accumulation in the ER. In normal

conditions, PERK binds to the molecular chaperon, GRP78 in normal conditions. When the load of unfolded proteins increases, GRP78 get dissociated, allowing PERK to get oligomerize and autophosphorylate (Xu et al., 2005). Activation of PERK causes the phosphorylation of the α-subunit of eukaryotic translation initiation factor-2 (eIF2α), leading to decreased rate of translation initiation in the cell (Harding et al., 1999).

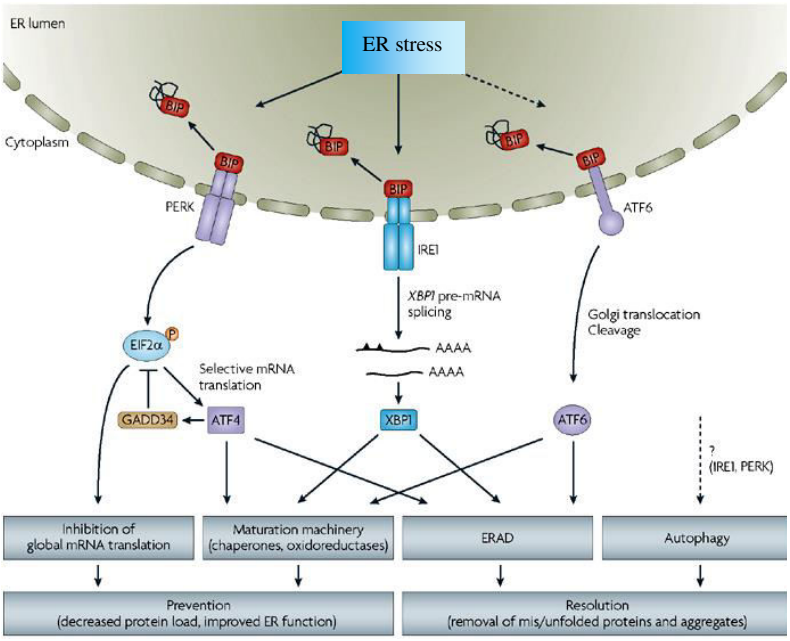


Figure 1.11. The unfolded protein response (UPR) pathway: Increased protein misfolding lead to the activation of 3 transducers of the UPR pathway: activation of IRE1 leads to spliced XBP-1 leading to ERAD and ATF6 which leads to transcriptional up-regulation of chaperone proteins. The third arm involves PERK, which oversees the attenuation of nascent protein translation via phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) (Wouters and Koritzinsky, 2008).

IRE1 is another transmembrane protein, which get activated and oligomerized by the unfolded proteins (Ron and Walter, 2007). IRE1 has a serine/threonine kinase domain and as well as an endoribonuclease domain (Calfon et al., 2002). The kinase domain activates JNK and NFkB, leading to the release of inflammatory cytokines (Xu et al., 2005). The endoribonuclease causes IRE1-mediated splicing of X box protein-1 (XBP1) mRNA (Calfon et al., 2002). The newly spliced XBP1 acts as a potent transcription factor, up-regulating the expression of UPR target genes (Yoshida et al., 2001).

ER stress causes the translocation of ATF6 to the Golgi apparatus, where it is proteolytically processed and the processed ATF6 is translocated to the nucleus and upregulates the expression of UPR target genes (Lai et al., 2007). ATF6 is also an inducer of XBP1 mRNA production (Yoshida et al., 2001).

The UPR sensors then activate the pathways that can ameliorate ER stress by increasing the production of molecular chaperons or by degrading the unfolded proteins by activating the apoptosis pathway. During ER stress, there is an up-regulation of genes involved in ER protein folding, including chaperones such as glucose-regulated protein 78 (BiP/GRP78), enzymes mediating folding such as protein disulfide isomerase (PDI), ER structural components, and components of the ER-associated degradation pathway (ERAD) (Oslowski and Urano, 2011). CHOP, which is also known as growth-arrest and DNA-damage- inducible gene 153 (GADD153) is the first identified protein that mediates ER stress-induced apoptosis. All the three UPR transducers can induce the transcription rate of CHOP. CHOP induces the expression of ER oxidoreductase enzyme, ER oxidoreductin-1alpha (ERO1α), which under normal conditions can regenerate PDI at the expense of molecular oxygen. The induction of CHOP is accompanied by an increased oxidative stress and apoptosis of the cell (Wang et al., 1996).

1.8. Perspectives in the management of insulin resistance

Understanding the mechanisms behind insulin signaling and IR will provide an insight into the development of therapeutic interventions for the prevention of IR and T2DM. Two main approaches for the management of IR include pharmacological interventions and non pharmacological means. The non-pharmacological means include lifestyle modifications which include reduction of excess body weight, modification of dietary habits, increased energy expenditure by physical activity etc. The pharmacological means to improve IR utilize drugs that are anti-hyperglycaemic agents which enhances insulin sensitivity by ameliorating the “glucose toxicity” (Matthaei et al., 2000). The currently available insulin sensitizers have different mode of actions and include biguanides, dipeptidylpeptidase-IV (DPP-IV) inhibitors, thiazolidinediones (agonists of PPARγ) etc.

1.8.1. Biguanides

Metformin is the important biguanide available and is the most important drug of choice in obese diabetic patients (Bailey, 1992; Bailey and Turner, 1996). Metformin through the activation of liver AMPK, reduces hepatic glucose output and thus hyperglycemia (Viollet et al., 2012). In T2DM patients, metformin is found to inhibit gluconeogenesis (Madiraju et al., 2014). Also, metformin was found to increase glucose uptake in skeletal muscles (Sarabia et al., 1992).

1.8.2. DPP-IV inhibitors

DPP-IV inhibitors are a class of promising medication for the treatment of diabetes. They are reported to control blood glucose level without causing severe hypoglycaemia (Richter et al., 2008). Inhibition of DPP-IV improves blood glucose level by the suppression of action of glucagon and enhancement of insulin secretion. DPP-IV is a cell surface peptidase and can cleave a large number of peptide hormones including glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP1), and inactivation or inhibition of DPP-IV is found to improve blood glucose level (Lamont and Drucker, 2008). Sitagliptin was the first gliptin approved by the Food and Drug Administration (FDA) of the United States in October 2006 and is now available worldwide and DPP-IV inhibitors are generally considered as well tolerated medicines. In addition to DPP-IV inhibitors, GLP-1 analogues are also used as antidiabetic agents. GLP-1 is secreted by the L cells of the small intestine and can enhance insulin biosynthesis and suppress glucagon release (Kim and Egan, 2008). The first GLP-1 agonist introduced in the market is exenatide, which is more resistant to digestion by DPP-IV and and has a longer plasma half life. Like DPP-IV inhibitors, GLP-1 analogues are also associated with less risk of hypoglycaemia (Nielsen et al., 2004).

1.8.3. Thiazolidinediones (TZDs)

TZDs or glitazones are a class of orally active drugs which are designed to enhance insulin sensitivity. These can increase insulin dependent glucose disposal and also reduce hepatic glucose output. In 1982, ciglitazone, a TZD was discovered, followed by a number of other compounds like pioglitazone, troglitazone, englitazone,

and others (Saltiel and Olefsky, 1996). TZDs mediate their action by binding with the transcription factor PPARγ. All TZDs share a thiazolidine-2-4-dione structure with some chemical modifications to improve their efficiency.

1.9. The PPAR family

PPARs are transcription factors that belong to the superfamily of nuclear receptors (Laudet et al., 1992). They are ligand activated transcription factors and are involved in cellular differentiation, energy metabolism, glucose homeostasis and fatty acid metabolism (Brown and Plutzky, 2007). The family of PPARs is represented by the following three members: PPAR alpha (PPARα), PPAR beta/delta (PPARβ/δ), and PPARγ, each isoform has its own specific tissue distribution. PPARα is highly expressed in tissues with a high oxidative capacity such as liver, heart and skeletal muscle, also present in kidney, intestinal mucosa and brown adipose tissue. PPARβ/δ is ubiquitously expressed in whole body, mainly found in skeletal muscle, kidney and intestine, PPARγ is mainly expressed in adipose tissue and it is also found in skeletal muscles, heart, kidney etc. (Grygiel-Górniak, 2014; Gilde et al., 2006). It is further subdivided into four isoforms (Evans et al., 2004).

- γ1 - expressed in virtually all tissues, including heart, muscle, colon, kidney, pancreas, and spleen.
- γ2 - expressed mainly in adipose tissue.
- γ3 - expressed in macrophages, large intestine, and white adipose tissue.
- γ4 - expressed in endothelial cells

1.9.1 Structural features of PPARs

All the three different isoforms of PPARs have similar structural features. The PPARs possess the canonical domain structure, including the amino-terminal activation function-1 (AF-1) transactivation domain, which is ligand independent domain. The ligand independent AF-1 domain offers constitutive activity of the receptor, which is negatively regulated by phosphorylation. The AF-1 region is followed by a DNA-binding domain (DBD), or C domain which is involved in the direct binding of the receptor to the PPAR response element (PPRE) in the promoter region of the target genes. The dimerization region or the D region or the hinge region act as the docking

site for various co-repressors (Pawlak et al., 2012). PPARs act on DNA response elements as heterodimers with the retinoid X receptor (RXR). Their natural activating ligands are lipid-derived substrates (Issemann et al., 1993; Shulman and Mangelsdorf, 2005). The ligand-dependent transactivation domain AF-2 is located at the carboxy-binding domain (Grygiel-Górniak, 2014; Tyagi et al., 2011). The ligand binding domain (LBD) or the E/F region is responsible for the specificity of various ligands. When a ligand gets bind with the LBD of the E/F domain, the receptor get stabilized, and causes the recruitment of various transcriptional co-activators like cAMP response element-binding protein and release of corepressors like silencing mediator of retinoid and thyroid hormone receptors (SMRT) resulting in the transcription of genes (Brown and Plutzky, 2007). The mechanism of action of PPARs is diagrammatically represented in Figure 1.12.

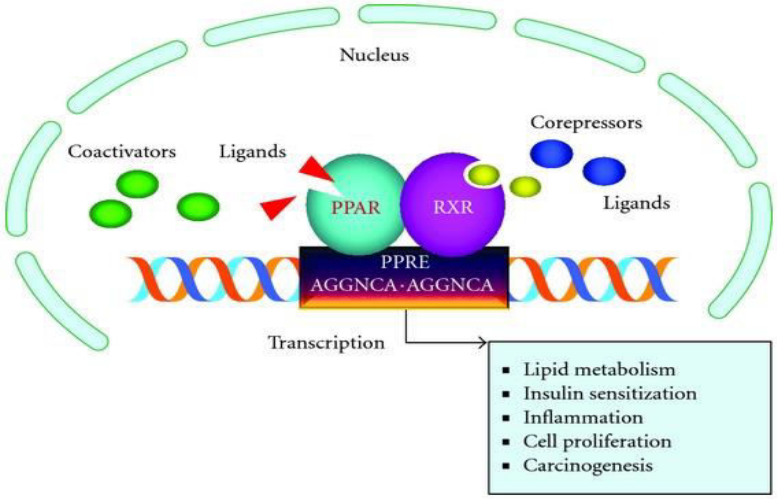


Figure 1.12. Mechanism of action of PPARs: Following ligand binding, PPAR forms a heterodimer with RXR, which binds to the PPRE of target genes and regulates the transcription of these genes (Lee et al., 2012). PPRE, PPAR response element; RXR, retinoid X receptor

1.9.2. PPARα

PPARα is mainly involved in lipid and lipoprotein metabolism, and is the first to be identified as a PPAR. It is mainly expressed in liver, kidney, heart muscle, digestive tract, vascular and immune cell types, endothelial cells, lymphocytes and microglia (Braissant et al., 1996; Chinetti et al., 1998; Diep et al., 2000; Inoue et al., 1998). PPARα acts as a master regulator of lipid metabolism by altering the expression of numerous genes involved in different aspects of lipid metabolism like fatty acid

uptake, oxidation, ketogenesis, lipoprotein and bile metabolism etc. Some of the genes which are activated by PPARα include peroxisomal acyl-coenzyme A (CoA) oxidase gene, fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36), lipoprotein lipase (LPL), apolipoprotein A-I, A-II etc. (Grygiel-Gorniak, 2014; Mandard et al., 2004). The endogenous agonists of PPAR-α include arachidonic acid, linoleic acid, leukotriene B_4 etc (Krey et al., 1997; Willson et al., 2000). The synthetic agonists of PPARα include various drugs used in the treatment of dyslipidemia like fenofibrate, bezafibrate, gemfibrozil etc. (Forman etal., 1996; Staels and Fruchart, 2005).

1.9.3. PPARβ/δ

PPARβ/δ is ubiquitously expressed in our body and it is reported to have anti-inflammatory activity by suppressing macrophage derived inflammation (Tenenbaum et al., 2005). It can promote fatty acid oxidation also. It can attenuate atherogenesis by reducing the expression of inflammatory mediators and adhesion molecules, thereby reducing atherogenesis (Graham et al., 2005). PPARδ ligands can inhibit NFκB activation, and thereby it can reduce cardiac hypertrophy (Tyagi et al., 2011). The endogenous ligands of PPARβ/δ include docosahexaenoic acid, linoleic acid, prostacyclins etc. The synthetic ligands include GW0742, GW501516 and L165041 etc.

1.9.4. PPARγ

PPARγ is one of the best characterized nuclear receptor and TZDs are the most widely studied and clinically relevant PPARγ ligands. PPARγ can control the expression of a large number of genes involved in glucose and lipid metabolism. In adipocytes, PPARγ can improve IR by ameliorating the effect of the proinflammatory cytokine TNF-α (Kubota et al., 2006). In adipocyte, PPARγ can modulate adipocyte differentiation, glucose metabolism and fatty acid storage through the activation of genes like FATP, ap2, CD36 etc. (Grygiel-Górniak, 2014). PPARγ also has an important role in the management of inflammation (Tyagi et al., 2011). The endogenous ligands of PPARγ include 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2), oxidized fatty acids such as 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoate or (5-oxo-ETE),

9-oxo-octadecadienoic acid (9-oxoODE), docahexanoid acid, arachidonic acid, prostaglandin PGJ_2 etc. (Itoh et al., 2008; Soares et al., 2005; Shiraki et al., 2005). The physiological importance of PPARγ agonists is represented in Figure 1.13

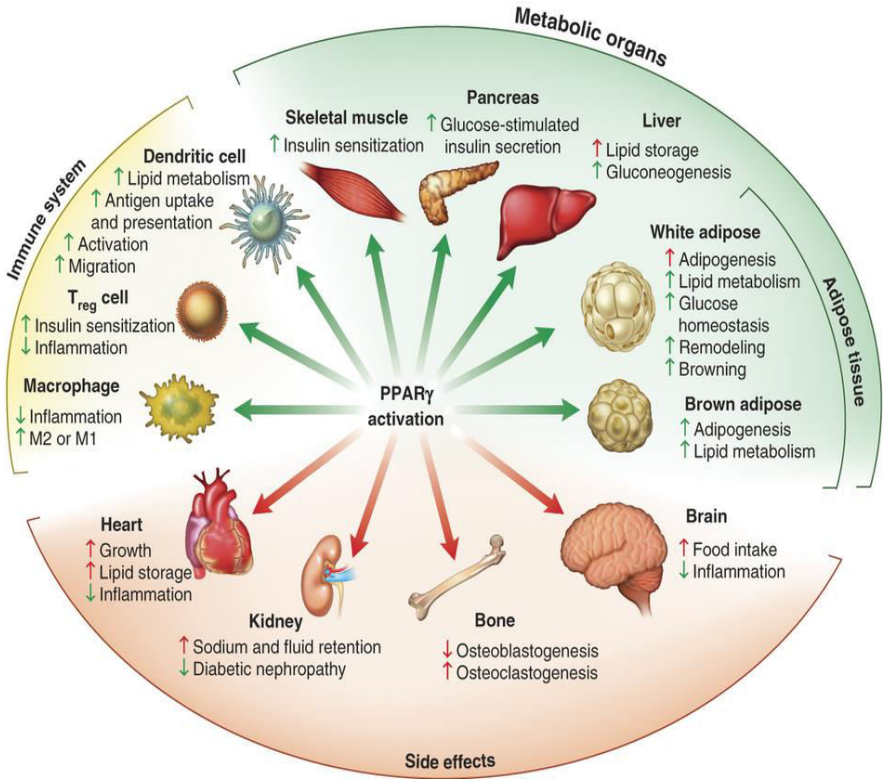


Figure 1.13. Physiologial importance of PPARγ: PPARγ can cause beneficial effects (indicated by green arrow) as well as adverse effects (indicated by red arrow) (Ahmadian et al., 2013).

The synthetic ligands include various antidiabetic drugs like troglitazone, RG, pioglitazone etc. Troglitazone was the first thiazolidinediones approved for IR and T2DM, which is followed by RG and pioglitazone. In 1997, troglitazone was withdrawn from the market due to its increased risk of drug induced hepatitis (Kohlroser et al., 2000; Watkins and Whitcomb, 1998). RG is another important insulin sensitizer which is marked in the name of Avanda; but its use is also restricted due to increased risk of myocardial infarction and fluid retention. The TZD, which is still available in the market is pioglitazone. It is also reported to have some side effects like occurrence of urinary bladder cancer (Wang et al., 2014).

1.9.5. Various strategies for the development of safer PPARγ agonists

Since PPARγ agonists are reported with several side effects, new strategies have been investigated for the development of PPARγ agonists with fewer side effects; like PPARα/γ dual agonists, selective PPARγ modulators (SPPARγMs), pan PPAR agonists etc. (Higgins and Depaoli, 2010; Wang et al., 2014). These combination agonist therapies utilize a combination of agonists, and can activate each receptor subtype involved. The combination concept can provide more beneficial effects and have less undesirable side effects (Cho et al., 2008). The partial or selective PPARγ agonist will have selective gene regulating properties, thereby decreasing the adverse effects (Gilde et al., 2006).

1.9.5.1. PPARα/γ dual agonists

PPARα/γ dual agonists can activate both PPARγ and PPARα; thus have positive influence on both lipid and glucose metabolism. They can be used for the treatment of T2DM with co-existing dyslipidemia (Munigoti and Harinarayan, 2014). Considering the benefits of these dual agonists, numerous compounds with dual action on PPARα and PPARγ have been developed, named as glitazars. Faglitazar was the first glitazar to be studied, and it was discontinued as it is reported to cause edema (Henke et al., 1998). Ragaglitazar was another glitazar and it was found to reduce fasting plasma glucose levels and triglyceride levels. Further plasma HDL was elevated and the total LDL and free fatty acids were decreased (Gilde et al., 2006). But it is reported with certain side effects like weight gain, anaemia and edema, and bladder cancer (Egerod et al., 2005; Gilde et al., 2006). Muraglitazar was proved to improve insulin sensitivity and diabetic dyslipidemia, but was withdrawn in 2006 due to adverse effects on cardiovascular system (Kendall et al., 2006). Tesaglitazar is reported with bone marrow and renal dysfunction (Fagerberg et al., 2005). Another important compound with dual PPARα/γ agonist activity is saroglitazar (trade name, Lipaglyn). It has antihyperglycemic and antidyslipidemic effects and it is not reported with any adverse effects as that of the other PPARα/γ dual agonists. But its long-term cardiovascular safety has yet to be established (Munigoti and Harinarayan, 2014).

1.9.5.2. Pan PPAR agonists

The compounds which can activate the three isoforms of PPARs are termed as pan PPAR modulators, so they can potentially exert their metabolic effects on IR, dyslipidemia and hypertension (Cho et al., 2008). The first clinically tested pan PPAR agonist is bezafibrate. Bezafibrate can reduce plasma glucose, TG and increase plasma HDL level and insulin sensitivity (Tenenbaum et al., 2005). Clinical studies with bezafibrate did not report any side effects like edema or weight grain and compounds with pan PPAR activity can be considered as promising strategies for the management of MS.

1.9.5.3. Selective PPARγ agonists

Another strategy to reduce the side effects of PPARγ agonists is the use of partial PPARγ agonists or selective PPARγ modulators (SPPARγMs). SPPARγMs can enhance insulin sensitivity but have lower adipogenic potential compared with the full PPARγ agonists (Cho et al., 2008). SPPARγMs bind with the ligand binding domain (LBD) of PPARγ in a manner which is different from that of the full PPARγ agonists, and causes differential displacement and recruitment of various co-factors, leading to differential gene expression (Zhang et al., 2007). SPPAR*γ*Ms like halofenate (HA) and PA-082 are recently reported. Halofenate can selectively regulate the expression of PPARγ responsive gene with the differential recruitment of co-factors (Allen et al., 2006). PA-082 is a non TZD group of compound and an isoquinoline derivative. It can decrease lipid accumulation compared with the full PPARγ agonist RG. At the same time it can increase the expression of insulin sensitive genes (Burgermeister et al., 2006). The detailed study of SPPARγMs will give more information about antidiabetic agents with PPAR*γ* agonist property with reduced side effects.

1.9.6. Impact of nutraceutical in the management of IR

Nutraceuticals are emerging as an alternative to modern medicines for the management of diabetes and MS. Though insulin and other pharmacological interventions can control some aspects of diabetes, they are inadequate to prevent secondary complications arising from diabetes (Bastaki, 2005; Dalle Grave et al., 2010). So the demand for nutraceutical compounds that might have potential efficacy

in the management of T2DM and MS is increasing these days (Davì et al., 2010). They have been shown to target the pathogenesis of diabetes, MS and their complications and to favourably modulate a number of biochemical and clinical endpoints (Ramaa et al., 2006).

1.9.7. PPARγ activation by natural products

Medicinal plants have been used for the treatment of various diseases including MS since ancient times. Since 19th century, many pure compounds isolated from medicinal plants are reported with significant biological activity. (Balunas and Kinghorn, 2005). Plant based compounds are attaining more attention these years because of their increased tolerance and less toxicity. Natural products serve as an important source for the discovery of new therapeutic options (Cragg and Newman, 2013). Significant research is going on to discover plant based PPARγ modulators which will have selective agonist property towards PPARγ. PPARγ ligands were reported from plants that are common food sources, including the tea plant (*Camellia sinensis*), soybeans (*Glycine max*), palm oil (*Elaeis guineensis*) and ginger (*Zingiber officinale*) (Table 1.1). Comparing with their synthetic counterparts, PPARγ agonists from natural sources are safer and can act at multiple targets, offering a synergistic effect (Bassaganya-Riera et al., 2011).

Table 1.1. Plant species with PPARγ agonist property, their traditional use and active compounds (Wang et al., 2014)

Plant species	Common name	Traditional use	Active compound with PPARγ agonist property
Amorpha fruticosa	Desert false indigo	Used to treat hypertension	Amorfrutins
Bixa orellana	Lipstick tree, Annatto	Used as diuretic, laxative, antibilious, antiemetic and astringent agent	Bixin, norbixin
Camellia sinensis	Tea plant	Used as stimulant, diuretic and astringent	(-)-Catechin
Cornus alternifolia	Green osier	Used as tonic, analgesic, and diuretic	Kaempferol-3-O β-glucopyranoside
Echinacea purpurea	Purple coneflower	Used for throat infection, cough etc.	Alkamides
Elaeis guineensis	Oil Palm	Used as laxative and diuretic, as a poison antidote, as a cure for gonorrhea and bronchitis etc.	Tocotrienols
Glycine max	Soybean	The beans of the plant are used as a food worldwide	Genistein
Momordica charantia	Bitter melon	Used to relieve diabetes, as stomachic,laxative, antibilious, emetic, and anthelmintic agent	Cucurbitane-type triterpene glycosides
Pueraria thomsonii Benth.	Kudzu	Traditionally used for the treatment of fever, diarrhea, diabetes, and cardiovascular diseases	Daidzein
Rosmarinus officinalis	Rosemary	The essential oil from flowers and leaves is used as anti-inflammatory and antiseptic agent	Carnosic acid and carnosol

Table 1.1. continues

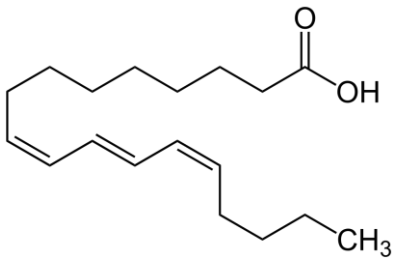
Plant species	Common name	Traditional use	Active compound with PPARγ agonist property
Sambucus nigra	Elderberry	Used for the treatmentof common cold, influenza, and sinusitis	a-Linolenic acid, linoleic acid, and
Terminalia bellerica	Bahera	Used to treat asthma, diarrhea, hypertension, inflammation etc.	Gallotannins
Thymus vulgaris	Thyme	Used in traditional medicine as antiseptic, antibacterial and antifungal agent	Carvacrol
Trifolium pratense	Red Clover	Used as antiinflammatory, expectorant, sedative, and anti-dermatosis agent	Isoflavones
Zingiber officinale	Ginger	Widely used as a spice worldwide. Fresh rhizomes are used to eliminate cold and arrest vomiting; dried rhizomes are used to dispel cold from the spleen and the stomach	6-Shogaol

1.9.7.1. Punicic acid

Punicic acid, also known as trichosanic acid, is an omega-5 long chain polyunsaturated fatty acid found in high concentrations in the seed of *Punica granatum* and *Trichosanthes kirilowii* (Joh et al., 1995; Sassano et al., 2009). About 64-83% of the pomegranate seed oil is constituted by PA (Ahlers et al., 1954; Kaufman and Wiesman, 2007). PA can activate PPARα and PPARγ *in vitro* and also can bind to PPARγ and δ ligand binding domain (Hontecillas et al., 2009). Also, oral administration of PA can ameliorate fasting plasma glucose concentration and can normalize glucose concentration in db/db mice, indicating that PA can ameliorate T2DM. PA can make increase in the expression of PPARα responsive genes in skeletal muscles and PPARα and PPARγ responsive genes in WAT (Hontecillas et al., 2009). PA can suppress the expression of inflammatory cytokines like TNF-α and also can decrease NF-κB activation. PA can also upregulate the expression of PPARδ in

pancreatic tissue (Bassaganya-Riera et al., 2011). The structure of PA is given in Figure 1.14.

A



B

Figure 1.14. Structure of Punicic acid: The compound in the present study-punicic acid, is mainly found in the seed oil of pomegranate fruit. A) structure of punicic acid B) Pomegranate fruit

1.10. Societal impact of the study

The prevalence of obesity and MS is increasing at an alarming rate worldwide. The high cost, dose and regular use of currently available drugs for the management of IR and T2DM is associated with financial burden and some adverse effects. PPARγ agonists like TZDs used in the treatment of IR are reported with many side effects and these adverse effects are a result of full PPARγ activation, compared with the weak agonistic property of endogeneous ligands like fatty acids. Therefore, there is a high demand of PPARγ modulators from natural sources with partial activity, which can improve glucose homeostasis and can be considered as a better alternative for the treatment for IR and T2DM. The present study suggested that PA, as a partial PPARγ agonist, can be considered as an alternative to TZDs for the treatment of IR. Since it is present in large amount in pomegranate seed oil, it can be considered as an ideal nutraceutical for the development of health care products for the management of IR and MS.

1.11. Aims and objectives of the study

A range of PPARγ activating natural compounds is recently described with immense potential in the management of IR and T2DM. Since the prevalence of MS and IR are increasing worldwide, the demand for pharmacological interventions that can improve the abnormalities associated with MS is also increasing. The adverse effects associated with the full PPARγ agonists like TZDs had focused the attention of current research more on the naturally occurring PPARγ agonists. In the present study, we have characterized PA as a partial PPARγ agonist and evaluated the potential of PA in improving IR and associated dysfunctions in 3T3-L1 adipocytes.

The main objectives of the study include

- To evaluate the activity of PA as a partial PPARγ agonist by employing *in vitro* and *in silico* methods
- To study the effect of PA on insulin signaling pathway in 3T3-L1 adipocytes
- To study the effect of PA on TNF-α induced IR in 3T3-L1 adipocytes
- To study the crosstalk between inflammation, mitochondrial dysfunction and ER stress in insulin resistant model of 3T3-L1 adipocytes and possible amelioration with PA

Chapter 2

Materials and methods

2.1. Materials

2.1.1. Chemicals

Punicic acid (PA) was purchased from Larodan fine chemicals (Sweden). 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), insulin, GW9662, 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA), ethylene diamine tetraacetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), JC-1 mitochondria staining kit, bovine serum albumin (BSA), 3,3' diaminobenzidine (DAB), sodium azide (NaN3), sodium citrate, Tween-20, oligomycin, phenyl methyl sulphonyl fluoride (PMSF), radioimmunoprecipitation (RIPA) buffer, TRIzol and protease inhibitor cocktail were purchased from Sigma Aldrich (St. Louis, Mo, USA). Trichloroacetic acid (TCA), dipotassium phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), potassium phosphate (K_2PO_4), sodium pyrophospahte, methanol, hydrogen peroxide (H_2O_2), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), 5,5'-dithio-bis-2-(nitrobenzoic acid) (DTNB-Ellman's reagent), glacial acetic acid, n-butanol, nicotinamide adenine dinucleotide phosphate (NADPH), sucrose, glycine, methanol, hydrogen peroxide (H_2O_2) and Triton-x100 were purchased from Merck specialities Pvt. Ltd. USA. Fetal bovine serum (FBS), penicillin-streptomycin antibiotics, Dulbecco's modified Eagle's medium (DMEM), Krebs' ringer phosphate buffer and trypsin-EDTA were purchased from Himedia Pvt Ltd (Himedia Pvt. Ltd, India). 2-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxy-D-glucose (2-NBDG) was from Invitrogen (Carlsbad, CA, USA). Tris hydroxymethyl aminomethane hydrochloride (Tris HCl) and paraformaldehyde was purchased from Sisco research laboratory, Mumbai, India. Rosiglitazone (RG) was from Cayman chemicals (Cayman Chemicals, MI, USA). Fura 2 AM, Mitotracker Deep Red FM and MitoSOX™ red were purchased from Molecular probes, Life technologies, USA. All other chemicals used were of analytical grade.

2.1.2. Assay kits and antibodies

Protein carbonyl, total antioxidant, reduced glutathione (GSH), aconitase, O_2 consumption, Ca^{2+} content, triglyceride (TG), nuclear extraction kit and nuclear factor-κβ (NF-κβ) expression cell based assay kits were used (Cayman Chemicals, MI, USA). JC-1 mitochondria staining kit was from Sigma-Aldrich, (St. Louis, MO, USA). 2-deoxy glucose (2-DG) uptake colorimetric assay and mitochondrial biogenesis assay kit were from Abcam (Abcam,Cambridge, MA, USA). Adiponectin enzyme immunoassay (EIA) kit was from SPI Bio (SPI Bio, Bertin Pharma, France). Leptin ELISA kit was from Merck (Merck Millipore, USA). BCA protein assay kit was from Pierce (Rockford, IL USA). Oil red EZStain adipocyte staining kit was from Himedia (Hi-media Pvt Ltd, Mumbai, India). ATP determination kit was from Molecular Probes Inc., Eugene, USA. Interleukin-6 (IL-6), interleukin-2 (IL-2), interleukin-10 (IL-10), MCP-1 and interferon-gamma (IFN-γ) assay kits were from BD Biosciences, USA. Time resolved-fluorescence resonance energy transfer (TR-FRET) assay kit was from Life Technologies (Life technologies, Invitrogen,USA). Primers for PCR were from Hysel India Pvt Ltd, India. Superscript III 1st strand synthesis system kit and SYBR Green master mix were from Life technologies, Bangalore, India. Primary antibodies for western blotting, horse radish peroxidase (HRP) conjugated secondary antibodies and fluorescein-isothiocyanate (FITC) conjugated secondary antibody were from Santacruz, USA.

2.2. Methods

2.2.1. Cell culture

3T3-L1 cells derived from mouse embryonic fibroblasts were obtained from American Type Culture Collection (ATCC), USA. Cells were cultured in DMEM containing 4.5 g/L glucose, 1.5 g/L sodium bicarbonate and 110 mg/L sodium pyruvate, supplemented with 10% FBS, and penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified incubator with 5% CO_2 at 37°C. Cells were passaged regularly and subcultured in 70% confluence before the experiments.

2.2.1.1. Differentiation of 3T3-L1 pre-adipocytes

3T3-L1 preadipocytes cells were seeded at a density of 4×10^4 cells/well in 24 well plate and maintained in DMEM supplemented with 10% FBS, antibiotic (100 U penicillin/ml and 100 μg streptomycin/ml) till confluency. To induce differentiation, 2-days post confluent 3T3-L1 preadipocytes were stimulated with 0.5 mM IBMX, 0.25 mM DEX, and 1 μg/ml insulin in DMEM with 10% FBS and antibiotics for 48 hours. The cells were then maintained in DMEM containing 1 μg/ml insulin (maintenance medium). The medium was replaced with fresh maintenance medium every 2 days. Adipocytes were used for experiments on 9^{th} day of initiation of differentiation.

2.2.2 MTT assay

Cytotoxicity of different concentration of PA (10, 20, 30, 50 and 75 μM) on 3T3-L1 preadipocytes and adipocytes was checked by MTT assay. It is based on the ability of viable cells to reduce the MTT to insoluble formazan crystals by mitochondrial dehydrogenase. The method was carried out as per previously described protocol (Wilson, 2000). PA was dissolved in DMSO, and the final concentration of DMSO was 0.1% (v/v) for every group and treatment. Cells treated with 0.1% DMSO in DMEM was selected as the control group. 3T3-L1 preadipocytes and adipocytes were treated with indicated concentrations of PA for 48 hours in 37°C, 5% CO_2 incubator. Then, media was removed and replaced with MTT (final concentration 0.5 mg/ml) in DMEM without FBS and incubated for 4 hours. After incubation, DMSO was added to dissolve insoluble crystals and the absorbance was read at 570 nm using a multimode reader (Biotek Synergy 4, USA). The absorbance is directly proportional to the number of viable cells.

2.2.3. Adipogenic studies of PA

For evaluating the adipogenic potential of PA, cells were treated with differentiation medium along with indicated concentrations of PA (5, 10 and 30 μM) throughout the process of adipocyte differentiation. To study the effect of GW9662, an antagonist of PPARγ, cells were treated with GW9662 (10 μM) along with the higher concentration of PA (30 μM) throughout the process of differentiation. Rosiglitazone (RG; 100 nM) was used as the positive control for adipogenesis studies.

2.2.4. Oil red O staining

Oil red staining was done with Himedia EZStain adipocyte staining kit (Himedia Pvt Ltd, Mumbai, India). The cells were treated as described in section 2.2.3. After treatment, the cells were washed with wash buffer and fixed for 1 hour at room temperature with the standard fixing solution provided with the kit. After fixing, the solution was aspirated off and cells were washed with distilled water, and appropriate volume of permeabilization solution was added and incubated for 5 minutes. Working solution of oil red O was prepared by mixing 3 parts of oil red O staining solution with 2 parts of distilled water. The working solution was incubated for 10 minutes at room temperature and filtered through Whatmann No.1 filter paper. The permeabilization solution was removed and appropriate volume of filtered staining solution was added and incubated at room temperature for 5 minutes. After staining, the cells were washed with distilled water and photographed under a microscope. Oil red was extracted using 100% isopropanol and absorbance was measured at 500 nm using isopropyl alcohol as blank on multimode reader.

2.2.5. Triglyceride (TG) accumulation

Cells treated as above with the peak stage of adipogenesis with maximum clusters of lipid droplets were washed with PBS and collected by centrifugation. Triglyceride was estimated by Cayman triglyceride estimation kit (Cayman chemicals, MI, USA). For this cells were lysed by sonication in the diluted standard diluent provided in the kit and 10 µl of cell lysate was mixed with 150 µl of enzyme buffer solution, incubated at room temperature for 15 minutes and read at 540 nm. The kit utilizes the action of the enzyme, lipases, which cause the enzymatic hydrolysis of triglyceride to glycerol and fatty acid. The glycerol formed is phosphorylated by glycerol kinase to form glycerol-3-phosphate, which is then oxidized to dihydroxy acetone phosphate and hydrogen peroxide by glycerol phosphate oxidase. The H_2O_2 formed is coupled with 4-aminoantipyrine and N-ethyl-N-(3-sulfopropyl)-m-anisidine (ESPA), in a redox coupled reaction by peroxidase, producing a brilliant purple colour, whose intensity is proportional to the amount of triglyceride in the sample. The intensity of the colour formed was measured at an optical density of 540 nm on multimode reader.

2.2.6. Glycerol-3-phosphate dehydrogenase (GPDH) estimation

GPDH estimation was carried out in cells treated as described above according to the method of Wise & Green, 1978. Cells treated as described in section 2.2.2 were washed and lysed in standard lysis buffer (RIPA buffer). The standard reaction mixture was prepared which contain 100 mM tris-HCl (pH 7.5), 2.5 mM EDTA, 0.12 mM NADH, 0.2 mM dihydroxyacetone phosphate (DHAP), 0.1 mM β-mercaptoethanol and cell extract containing defined protein concentration all in a final volume of 1 ml. The change in absorbance at 340 nm was measured by multiplate reader and one unit of enzyme activity corresponds to the oxidation of 1 nM of NADH per minute.

2.2.7. Adiponectin estimation

Adiponectin was estimated in the conditioned medium using a mouse adiponectin enzyme immunoassay (EIA) kit (SPI Bio, Bertin Pharma, France). Fully differentiated 3T3-L1 adipocytes were treated with different concentrations of PA (5, 10 and 30 μM) or RG for 24 hours. After respective treatments, conditioned media were collected and added to wells coated with a monoclonal antibody specific of mouse adiponectin. After one-hour incubation, the wells were washed and polyclonal anti-mouse adiponectin antibody conjugated to biotin was added and incubated for one hour. After washing, streptavidin-horseradish peroxidase (HRP) tracer was added and incubated for 30 minutes. The concentrations of the adiponectin were then determined by measuring the enzymatic activity of the HRP using the hydrogen peroxide/3 ,3' -5, 5' tetramethylbenzidine (TMB) solution by measuring the OD at a wavelength of 450 nm using a multimode reader .

2.2.8. 2-deoxy glucose uptake assay

2-deoxy glucose (2-DG) uptake assay was carried with Abcam glucose uptake colorimetric assay kit (Abcam,Cambridge, MA, USA) according to the manufacturer's instructions using RG (100 nM) as the positive control. Briefly, cells were seeded in a 96 well plate and differentiated to mature adipocytes. Differentiated 3T3-L1 adipocytes were treated with indicated concentrations of PA (5, 10 and 30 μM) or RG for 24 hours. Then the cells were starved for glucose by preincubating in Krebs-Ringer-Phosphate-Hepes (KRPH) buffer containing 2 % BSA for 40 minutes. Cells were

stimulated with insulin (10 µg/ml) for 20 minutes and treated with 2-DG, mixed and incubated for 20 minutes. In principle 2-DG can be taken up by glucose transporters and metabolized to 2-DG-6-phosphate (2-DG6P) and cannot be further metabolized, and thus accumulates in the cells. The NADPH generated from oxidised 2-DG6P can be determined by an enzymatic recycling amplification reaction, which was measured at 412 nm and is directly proportional to 2-DG uptake by the cells.

2.2.9. Glucose uptake activity using 2-NBDG

Glucose uptake in 3T3-L1 adipocytes was also measured using fluorescent glucose analogue 2-NBDG using RG (100 nM) as the positive control. Differentiated 3T3-L1 adipocytes were treated with indicated concentrations of PA (5, 10 and 30 µM) or RG for 24 hours. Cells were then incubated in low glucose medium again in the presence of different concentrations of PA and RG for 3 hours. Cells were stimulated with insulin (10 µg/ml) for 10 minutes and then treated with 100µM NBDG for 1 hour. The fluorescence intensity of 2-NBDG in the cells were recorded using a FACS Aria II flow cytometer (BD Biosciences, USA) at FITC range (excitation 490 nm, emission 525 nm band pass filter). The mean fluorescence intensities of different groups were analyzed by BD FACS Diva software and corrected for auto fluorescence from unlabelled cells. For each measurement, data from 5000 single cell events were collected using the flow cytometer.

2.2.10. Immunofluorescence imaging of GLUT4

Immunofluoresence imaging of GLUT4 was done according to the method of Emoto et al., 2001 with some modifications. Fully differentiated 3T3-L1 cells treated with indicated concentrations of PA (5, 10 and 30 µM) or RG (100 nM) for 24 hours were sensitized with insulin (10 µg/ml) for 10 minutes, fixed in 3.7% formaldehyde and permeabilized in 0.5% triton X-100. The permeabilized cells were incubated at room temperature for 1 hour with monoclonal GLUT4 primary antibody followed by incubation for 1 hour with FITC-conjugated secondary IgG antibody. Then the cells were washed and imaged using spinning disk fluorescent microscope (BD Biosciences, USA).

2.2.11. Molecular docking

Docking experiment of PA and RG into the PPAR γ was done using the software Autodock 4.2 and iGEMDOCK v2.1 using default docking parameters. These docking softwares were used to find the appropriate binding and conformations of the ligand to the receptor. The 3D model of PPAR γ was retrieved from the Brookhaven Protein Data Bank (PDB) (http:// www.rcsb.org/pdb/) (PDB ID 2Q5S). PA (ChemSpider ID**:** 4444570) and RG (ChemSpider ID: 70383) structures were downloaded from ChemSpider (http://www.chemspider.com) and converted to PDB file using Chem3D Pro10. The docking fitness of the ligand molecules to PPARγ and the amino acids of receptor (PPARγ) involved in interaction were predicted using iGEMDOCK. The interaction table and the binding energy of compounds to the LBD of PPARγ were analysed by using iGEMDOCK.

2.2.12. Lanthascreen TR-FRET PPARγ competitive binding assay

The binding of PA on PPARγ ligand binding domain was studied using Lanthascreen TR-FRET PPARγ competitive binding assay kit from Invitrogen according to the kit protocol. A mixture of 5 nM of GST (glutathione S-transferase) tagged PPARγ ligand binding domain (GST–PPARγ–LBD), 5 nM Tb ant-GST-antibody, 5 nM Fluormone Pan-PPAR Green, and serial dilutions of PA, GW9662 or RG were mixed and incubated in dark for 2 hours. The TR-FRET signals were measured by excitation at 340 nm and emission at 520 nm for fluorescein and excitation at 340 nm and emission at 490 nm for terbium in Tecan multiplate reader (Tecan Infinite 200PRO, Mannedorf, Switzerland). The ratiometric emission 520/490 was plotted against the concentrations of the compound to determine the IC_{50} value of the compounds for the displacement of pan PPAR-green. DMSO was used as the no ligand control.

2.2.13. Induction of insulin resistance and treatment

Insulin resistance was induced in 3T3-L1 adipocytes as previously described with some modifications (Houstis et al., 2006). Fully differentiated 3T3-L1 adipocytes were treated with 10ng/ml recombinant recombinant human TNF-α (Gold biotechnology, USA) for 24 hours. Cultured 3T3-L1 adipocytes exposed to TNF-α

become insulin resistant as assessed by the ability of insulin to stimulate glucose uptake. To see the effect of PA and RG on TNF-α induced insulin resistance, 3T3-L1 adipocytes were treated with indicated concentrations of PA or RG for 24 hours along with TNF-α.

2.2.14. Quantification of leptin secretion

The secretion of leptin was quantified in conditioned media using leptin ELISA kit (Merck Millipore, USA). This assay is a sandwich ELISA based method. Conditioned media after respective treatment (as described in section 2.2.13) were collected and leptin in the conditioned media was bound by a pre-titered antiserum and resulting complexes were immobilized in the wells of the microtiter plate. After washing, purified biotinylated detection antibody was added to the immobilized leptin. Then HRP enzyme solution was added to the immobilized biotinylated antibodies. The concentration of leptin was determined by measuring the enzymatic activity of the HRP using the H_2O_2/TMB solution. The enzyme activity was measured spectrophotometrically at 450 nm and 590 nm within 5 minutes.

2.2.15. Estimation of inflammatory cytokines

Inflammatory cytokines (IL-6, IL-1β, MCP-1 and IFN-γ) were estimated in conditioned media, using ELISA kits (BD Bioscience, USA). For performing these assays, 100 µl diluted capture antibody were added to the wells and incubated overnight at 4°C. The solution was then discarded and the wells were washed 3 times. 200 µl blocking buffer was added to each wells and incubated for 1 hour at room temperature. After washing, 100 µl of conditioned media from respective treatment (as described in section 2.2.13), were added to the wells and incubated for 2 hours at room temperature. The wells were then washed and 100 µl working detector were added and incubated for 1 hour at room temperature. After washing, 100 µl of substrate solution was added and incubated for 30 minutes in dark. The reaction was then stopped by addition of stop solution and the absorbance was read at 450 nm.

2.2.16. NF-κB p65 translocation assay

NF-κB p65 transcription factor activity was determined using Cayman NF-κB p65 translocation assay kit Chemicals (Cayman Chemical Company, USA) as per manufacturer's instructions. The method is based on ELISA technique and a specific double stranded DNA sequence containing NF-κB response element is immobilized on the bottom of microplate wells. NF-κB contained in the cytoplasmic and nuclear extract binds specifically to the NF-κB response element and NF-κB p65 is detected by the addition of specific primary antibody.

The cells are treated as described in section 2.2.13 and are collected by centrifugation. The cytoplasmic and nuclear fractions were isolated using nuclear extraction kit (Cayman Chemical Company, USA). 10 µl samples containing NF-κB was added to the wells and incubated overnight at 4 °C. After that all the wells were washed with 200 µl 1x wash buffer. Then to the all wells except blank, 100 µl NF-κB (p65) primary antibody was added and incubated for 1 h at room temperature. The wells were washed with wash buffer and to all the wells except blank, 100 µl diluted HRP conjugated goat anti-rabbit secondary antibody was added and incubated for 1 h at room temperature. The wells were again washed with wash buffer. Then, to all wells 100 µl developing solution was added and incubated for 30 minutes with gentle agitation. After that 100 µl of stop solution was added to all the wells and the absorbance was read at 450 nm in a multiplate reader. The absorbances obtained is an indication of NF-κB p65 concentration in the nuclear and cytoplasmic extracts.

2.2.17. Indirect enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was carried out by the method of Engvall and Perlman, 1971, with slight modification. Cell lysate precoated to ELISA plates (96 well, NUNC) served as antigen. After blocking (5% non-fat dry milk) and washing (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween-20), the plates were incubated with specific primary antibody (1:500 dilution) for 2 hours at room temperature. The plates were washed and then treated with HRP-conjugated secondary antibody (1:1000 dilution). The colour developed using TMB substrate solution (BD bioscience) was measured spectrophotometrically at 450 nm in a multimode reader.

2.2.18. Inhibition of reactive oxygen species (ROS)

Intracellular ROS content was determined by oxidative conversion of cell-permeable DCFH-DA to fluorescent 2', 7'dichlorofluorescein (DCF). For this, 3T3-L1 adipocytes were seeded in 96-well plate and subjected to differentiation. The cells were treated as described above. After respective treatments DCFHDA stain in serum-free medium was added to the cells and incubated at 37^0C for 20 minutes. After three washes, images were collected using high-content spinning disk facility (BD Pathway 855; BD Biosciences, USA) and fluorescence intensity was measured at excitation and emission wavelengths of 490 nm and 525 nm, respectively in a multiplate reader .

2.2.19. Detection of mitochondrial superoxide radical production

Mitochondrial superoxide productions was assayed using a fluorescent dye, mitoSOX . Briefly after respective treatments, cells were washed with PBS (pH 7.4) and loaded with mitoSOX (5 μM) in the medium and incubated for 20 minutes at 37°C in CO_2 incubator. Images of the cells were collected using spinning disk microscope (BD PathwayTM Bioimager System, BD Biosciences) and fluorescence intensity was measured using multimode reader (Biotek Synergy 4 USA) at excitation and emission wavelengths of 510 nm and 580 nm, respectively.

2.2.20. Mitochondrial content measurement

Mitochondrial content was determined using mitotracker red (Mitotracker Deep Red FM, Invitrogen). Briefly, the cells after treatment as described above were incubated with 5 μM stain in serum free media for 30 minutes at 37^0C. The stain was washed off with PBS and examined under spinning disk microscope and images were collected (BD PathwayTM Bioimager System, BD Biosciences). Fluorescence intensity was measured at excitation and emission wavelengths of 644 nm and 665 nm, respectively in a multimode reader.

2.2.21. Mitochondrial biogenesis assay

Mitochondrial biogenesis was analysed with MitoBiogenesis™ In-Cell ELISA Kit (Abcam, MA, USA) in control and treated groups according to manufacturer's instruction. In brief, the cells after treatment were fixed with 4% paraformaldehyde.

Cells were then washed with PBS followed by the addition of 100 µl of freshly prepared 0.5% acetic acid for 5 minutess to block endogenous alkaline phosphatise (AP) activity. Cells were washed again with PBS and permeabilized with 0.1% Triton X-100 for 30 mins and followed by the addition of 200 µl of 1X Blocking Solution for 2 ours. These cells were then incubated with primary antibodies specifically against mtDNA encoded COX-I (subunit I of Complex IV, cytochrome c oxidase-1), and nuclear-DNA encoded SDH-A (a subunit of complex II, succinate dehydrogenase) proteins overnight at 4ºC. Cells were washed with a washing buffer and incubated with AP for SDH-A and HRP for COX-I secondary antibodies for 1 hour. After thorough washing, AP Substrate was added and colour development was measured at 405 nm for SDH-A. Then the wells were emptied and added HRP Substrate. The colour developed was measured at 600 nm in a multimode reader for COX-I. COX-I signal and SDH-A signal were then plotted independently for analysing the data.

2.2.22. Assay for mitochondrial membrane potential

Mitochondrial membrane potential was measured using mitochondrial staining kit, JC1. The experiment was done as per the protocol provided with the kit (JC1 kit, Sigma). The kit uses the cationic, lipophilic dye, JC-1 . In normal cells, due to the electrochemical potential gradient, the dye concentrates in the mitochondrial matrix, where it forms red fluorescent aggregates (JC-1 aggregates). Any event that dissipates the mitochondrial membrane potential prevents the accumulation of the JC-1 dye in the mitochondria and thus, the dye is dispersed throughout the entire cell leading to a shift from red (JC-1 aggregates) to green fluorescence (JC-1 monomers). The cells after respective treatments as described above were incubated with JC-1 staining solution for 20 minutes at 37^0C. The stain was washed off with PBS and examined under spinning disk microscope and images were collected and fluorescence intensity was also measured. For JC-1 monomers, the fluorescence was measured at 490 nm excitation and 530 nm emission wavelengths, and for JC-1 aggregates, the fluorescence was measured at 525 nm excitation and 590 nm emission wavelengths. Valinomycin (1 µg/ml) was used as positive control for the measurement of dissipation of mitochondrial membrane potential.

2.2.23. O_2 consumption assay

O_2 consumption in the cells was measured using Cayman's O_2 consumption assay kit (Cayman Chemicals, MI, USA). Cayman's oxygen consumption rate assay kit utilizes phosphorescent oxygen probe, MitoXpress®-Xtra to measure oxygen consumption rate. In brief, cells were seeded in 96 well plate and were allowed to differentiate. After respective treatments as described above, the culture medium was removed and replaced with fresh medium. Blank wells were added with culture medium alone. MitoXpress®-Xtra solution was added to all wells except blank wells and then the wells were covered with HS mineral oil. The fluorescence was read at excitation; 380 nm and emission; 650 nm kinetically for 150 minutes in a multiplate reader (Tecan Infinite 200PRO, Tecan, Austria). In principle the fluorescent signal of MitoXpress is quenched by molecular oxygen and the signal is inversely proportional to the amount of oxygen present in the media. Antimycin A, which is an inhibitor of mitochondrial electron transport chain, is used as the positive control for the measurement of impaired oxygen consumption.

2.2.24. ATP production assay

ATP production was measured by using ATP determination kit (Molecular Probes, USA). It is a bioluminescence assay for quantitative determination of ATP using recombinant firefly luciferase and its substrate D-luciferin. Cells after respective treatments were rinsed with phosphate-buffered saline (PBS) and lysed in ATP-releasing buffer containing 100 mM potassium phosphate buffer at pH 7.8, 2 mM EDTA, 1 mM dithiothreitol (DTT) and 1% Triton X-100 (Wang et al., 2001). 20 µl of cell lysate were mixed with 180 µl of standard reaction mixture, which contains 8.9 ml distilled water, 0.5 ml 20X reaction buffer, 0.1 ml 0.1 M DTT, 0.5 ml 10 mM D-luciferin, 2.5 µl firefly luciferase (5 mg/ml stock solution) and luminescence was measured in Tecan multiplate reader. ATP concentrations in the samples were calculated from standard ATP curve and normalised to the protein content. Cells treated with oligomycin (8 µM for 8 hours) was used as the positive control for measuring depletion in ATP content due to mitochondrial dysfunction (Hafizi and Bakar, 2015).

2.2.25. Determination of aconitase activity

Aconitase activity was measured by using Cayman's assay kit. The kit measures the absorbance of NADPH at 340 nm which is generated in a coupled reaction of aconitase with isocitrate dehydrogenase. The rate at which NADPH is generated is proportional to the activity of aconitase. Briefly, after respective treatments, cells were lysed in the homogenization buffer and cell lysate was mixed with assay buffer, NADP+, isocitric dehydrogenase and sodium citrate (substrate for aconitase). The reaction mixture was incubated at 37^{o}C for 15 minutes and the absorbance was read once every 10 minutes at 340 nm for 30 minutes.

2.2.26. Estimation of lipid peroxidation

The levels of lipid peroxidation in cell lysates were estimated by TBARS assay according to the method of Niehius and Samuelsson (1968). Decomposition of the unstable peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde (MDA), which was quantified. In brief, cells after treatment were washed twice in cold PBS, and lysed in PBS supplemented with 150 µM butylated hydroxytoluene (BHT). 100 µl of the cell lysate was combined with freshly made 50 µl 30% trichloroacetic acid, 0.75% thiobarbituric acid, and 0.5 N HCl, and incubated for 15 minutes at 100°C. The reaction mixture was then centrifuged for 8 minutes at 13,500 g. The absorbance of the supernatant was measured at 532 nm.

2.2.27. Estimation of protein carbonyl content

The protein carbonyl content in cell lysate was estimated using assay kit from Cayman Chemical Company as per the manufacturer's instructions. The kit utilizes the reaction between 2, 4-dinitrophenylhydrazine (DNPH) and protein carbonyls in the cell lysate. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically. Briefly 200µL of cell lysate were mixed with 800 µL of DNPH, mixed and kept at room temperature in dark for one hour; vortexing the tube every 15 minutes. Then, 1 ml of 20% TCA was added to each tube vortexed and incubated in ice for 5 minutes. The tubes are centrifuged at 10,000 x g for 10 minutes at 4^{0}C, the supernatant was discarded and the pellet was washed two times in 1 ml of 1:1 mixture of ethanol/ethyl

acetate mixture. The tubes were centrifuged at 10,000 x g for 10 minutes at 4^0C. After final wash, the pellet was dissolved in 500 µL of guanidine hydrochloride by vortexing. The tubes were again centrifuged and the absorbance of the supernatant was read at 385 nm.

2.2.28. Preparation of cell lysate for antioxidant enzyme activities

After respective treatments, cells were washed in ice cold PBS and harvested in PBS. The harvested cells were homogenized with 20 mM Tris/HCl buffer (pH 7.5) containing 0.2% Triton x-100 and 0.5 mM PMSF and sonicated for 30 seconds on ice. Total cell lysates were then centrifuged at 3000 rpm at 4°C for 15 minutes. The supernatant was collected and aliquoted and utilized for different enzymatic assays.

2.2.29. Activities of antioxidant enzymes.

Activity of the antioxidant enzyme superoxide dismutase (SOD) was estimated according to the method of Kakkar et al., 1984. The assay of SOD is based on the inhibition of the formation of NADH-PMS-NBT formazan. In brief, the assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, 0.2 ml of the cell extract and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2 ml of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0 ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a multimode reader. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration, which gives 50% inhibition of NBT reduction in one minute under assay conditions. SOD activity was expressed as U/mg of protein.

The assay of glutathione reductase was done according to the procedure of David and Richard (1983). To 0.1 ml of sample, 1 ml of potassium phosphate buffer (0.12M pH 7.2), 0.1ml of EDTA, 0.1ml of sodium azide and 0.1 ml of oxidized glutathione were added and the volume was made up to 2 ml with water. The reaction mixture was kept at room temperature for three minutes and 0.1ml of NADPH was

added. The absorbance at 340nm was recorded at intervals of 15 seconds for three minutes. One unit of GR is expressed as μM of NADPH consumed per minute.

Catalase activity in the cell lysate was measured according to the method of Aebi (1984). The method measuresd the disappearance of H_2O_2 at 240 nm. Briefly, 0.1 ml of cell lysate was added to a cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). The reaction was started by addition of 1 ml of freshly prepared 30 mM H_2O_2. Catalase activity was expressed as amount of enzyme that catalyzed the conversion of one μM of H_2O_2 in a minute/mg protein.

GPx activity was quantified according to the method of Rotruck et al., 1973 with some modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10 mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of water and 0.5 ml of cell lysate was incubated at 25^0C for 90 seconds. The reaction was terminated with 0.5 ml of 10% TCA. The reaction mixture was then centrifuged and 1ml of the supernatant was added to 1.5 ml of phosphate buffer and 0.5 ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the enzyme activity was expressed in terms of μg of glutathione utilized/minute/mg protein.

2.2.30. Estimation of reduced glutathione (GSH)

GSH content was determined using Cayman's GSH estimation kit (Cayman chemicals, USA). The kit utilizes glutathione reductase for the quantification of GSH. The sulfhydryl group of GSH reacts with 5,5'-dithio-bis-2-(nitrobenzoic acid) (DTNB-Ellman's reagent) and produces a yellow coloured 5-thio-2-nitrobenzoic acid (TNB), which can be read at 405 nm. The mixed disulfide, GSTNB (produced between GSH and TNB) which is concomitantly produced is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is proportional to this recycling reaction which in turn is proportional to the concentration of GSH in the sample.

2.2.31. Intracellular calcium (Ca^{2+}) overload

After respective treatments, cells were treated with 5μM of Fura-2AM and incubated at 20 minutes at 37^0C. After incubation, cells were washed three times with

PBS and the images were visualized using BD PathwayTM Bioimager System (BD Biosciences). The intensity of the colour is directly proportional to the concentration of calcium in the sample. The intensity of the colour is fluorimetrically read at excitation of 335 nm and emission of 505 nm by multiplate reader.

2.2.32. Quantitative real time polymerase chain reaction (qRT-PCR)

TotalRNA from 3T3-L1 adipocytes was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was reverse transcribed using Super Script III reverse transcriptase and random hexamers (Life technologies, Invitrogen,USA). The expression levels of different genes were analysed by quantitative real-time PCR (qRT-PCR), conducted using the CFX96 Real Time PCR system (Bio-rad, USA) using the following conditions: an initial denaturation for 10 minutes at 95ºC, followed by 39 cycles of 15 seconds denaturation at 95ºC, 30 seconds annealing at the optimal primer temperature and 10 seconds extension at 72ºC. Each sample was assayed in triplicate in a 20µl reaction volume containing 1µl cDNA, 10µl SYBR Green master mix (Life technologies, Invitrogen,USA), 5.81µl DEPC water and 1.6 µl of each primer. Negative controls (no template) were run as well to ensure the absence of contamination. Analysis was performed using comparative critical threshold (Ct) method using β-actin as the housekeeping gene. The primers for various genes were designed using the Primer 3 and synthesised by Hysel India Pvt. Ltd. Based on the amplification results, the comparative CT method (ΔΔCT) was used to calculate the relative multiple of the starting copy number that existed in the template from each experimental group.

Fold change = 2^(-Δ(ΔCT)where ΔCT = CT (target) - CT (β-actin) and

ΔΔCT = ΔCT (stimulated) - ΔCT (control)

CT (threshold cycle) is the intersection between an amplification curve and a threshold line.

Table 2.1 primer sequences for qRT-PCR

mRNA		Primer sequence
Irs2	Forward	5'-GGCCTCTGTGGAAAATGTCTC-3'
	Reverse	5'-CTGTGGCTTCCTTCAAGTGAT-3'
C/ebpa	Forward	5'-AGACATCAGCGCCTACAT-3'
	Reverse	5'-TGTAGGTGCATGGTGGTCTG-3'
Pparg	Forward	5'-GCTGTTATGGGTGAAACTCTG-3'
	Reverse	5'-ATAAGGTGGAGATGCAGGTTC-3'
Cd36	Forward	5'-GAGAACTGTTATGGGACTAT-3'
	Reverse	5'-TTAAACTCGAGAGGCAAAGG-3'
Glut4	Forward	5'-CAACGTGGCTGGGTAGGC-3'
	Reverse	5'-ACACATCAGCCCAGCCGGT-3'
Socs3	Forward	5'-ATTCACCCAGGTGGCTACAG-3'
	Reverse	5'-AACACAGGACCAGTTCCAGG-3'
Tfam	Forward	5'-GGAATGTGGAGCGTCCTAAAA-3'
	Reverse	5'-TGCTGGAAAAACACTTCGGAATA-3'
Pgc1a	Forward	5'-CGGAAATCATATCCAACCAG-3'
	Reverse	5'-TGAGGACCGCTAGCAAGTTTG-3'
Nrf1	Forward	5'-TGGTCCAGAGAGTGCTTGTG-3'
	Reverse	5'-TTCCTGGGAAGGGAGAAGAT-3'
MtDNA	Forward	5'-CCACTTCATCTTACCATTTA-3'
	Reverse	5'-ATCTGCATCTGAGTTTAATC-3'
Grp78	Forward	5'-CCACTTCATCTTACCATTTA-3'
	Reverse	5'-ATCTGCATCTGAGTTTAATC-3'
Chop	Forward	5'-GTCCAGCTGGGAGCTGGAAG-3'
	Reverse	3'-GTCCAGCTGGGAGCTGGAAG-3'
β-Actin	Forward	5'-AGTACCCCATTGAACGC-3'
	Reverse	5'-TGTCAGCAATGCCTGGGTAC-3'

2.2.33. Western blotting

Fully differentiated 3T3-L1 cells after respective treatments were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma Aldrich, St Louis, MO, USA). After incubation, the cell suspensions were centrifuged at 12000 rpm for 15 minutes at 4^0C, and the supernatants were used for immunoblot analyses. Protein content in the supernatant was measured using a bicinchoninic acid kit (Pierce, Rockford, IL, USA). Briefly, 40 µg of total protein was separated by 10% sodium dodecyl sulphate

polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred onto poly(vinylidene difluoride) (PVDF) membranes. The membrane was blocked with BSA in Tris buffered saline-Tween 20 (TBST) for 1 hour at room temperature, washed incubated with the specific primary antibodies (1:500), and β-actin (1:500) in 1% BSA in TBST with gentle agitation at 4 °C overnight. The membrane was washed three times with TBST for 10 minutes in a shaker, followed by addition of HRP-conjugated secondary antibodies (1:1000) in 0.25% BSA in TBST, and was incubated for 60 min at room temperature with shaking. After three washes with TBST, the membranes were developed using DAB tablets and the relative intensity of bands were quantified using Bio-Rad Quantity One version 4.5 software in a Bio-Rad gel documentation system.

2.34. Statistical data analysis

Values are given as mean ± standard deviation (n=6). Data were analyzed by one-way ANOVA and the significances of differences between means were calculated by Duncan's multiple range tests using SPSS for Windows standard version 7.5.1 (SPSS, Inc.). $p \leq 0.05$ was considered to be significant.

Chapter 3

Characterization of punicic acid as a partial PPARγ agonist

3.1. Introduction

The demand for nutraceutical compounds in the management of T2DM and MS is increasing these days. They have been shown to target the pathogenesis of diabetes, heart diseases and their complications, and to favourably modulate a number of biochemical and clinical endpoints (Ramaa et al., 2006). These compounds include antioxidants, vitamins (vitamins C, E and D), conjugated linoleic acid, omega-3 fatty acids, minerals such as chromium and magnesium, α-lipoic acid, phytoestrogens, dietary fibers, organic acids including abscisic acid etc. (Bassaganya-Riera et al., 2010; Davi et al., 2010; Ramaa et al., 2006; Viladomiu et al., 2013). Although plant extracts have been used for the treatment of T2DM for hundreds of years worldwide, more research is needed for the identification of their active compounds and their mode of action for wider acceptability (Rizvi and Mishra 2013). Numerous mechanisms of antidiabetic action have been proposed for several plant extracts, like inhibition of alpha glucosidase enzyme and the increase of insulin-stimulated glucose uptake (Patel et al., 2012; Mentreddy, 2007; Kalekar et al., 2013).

One of the targets of interest for antidiabetic drugs is PPARγ. Activation of PPARs has been shown to play an important role in the regulation of energy homeostasis by modulating glucose and lipid metabolism (Glide et al., 2006). Adipose tissue is the major mediator of PPARγ action in insulin sensitivity, and so, adipose tissue is now a days emerging as a major drug target for obesity and IR (Leonardini et al., 2009). PPARγ agonists can induce insulin sensitivity by activating the expression of IRS2 and also by increasing tyrosine phosphorylation of IRS1 leading to the activation of Akt/PKB (Jiang et al., 2002; Smith et al., 2001). TZD family of antidiabetics which include the widely applied drug RG (Avandia), strongly activate PPARγ and enhances adipocyte differentiation (Cho and Momose, 2008). Recently, these PPARγ activators have come under scrutiny because of certain undesirable clinical side effects (Ahmadian et al., 2013; Rubenstrunk et al., 2007). So there is a demand for naturally occurring PPARγ modulators from edible items due to minimum adverse effect and high tolerance to human body (Penumetcha and Santanam, 2012).

PPARγ modulators with partial agonism exhibit differential binding property as compared with the full agonists. The partial PPARγ agonists are expected to uncouple insulin sensitization from lipid accumulation in diabetic patients and thus can avoid many of the side effects of full PPARγ agonists (Guasch et al., 2011).

Pomegranate extracts have been widely used as antidiabetic agents and they contain a large number of bioactive substances (Viladomiu et al., 2013). PA, one among them is a polyunsaturated fatty acid mainly found in its seed oil and is reported to have PPARγ agonist activity by different groups (Bassaganya-Riera et al., 2011; Hontecillas et al., 2009). Hontecillas et al had conducted studies on PPARγ agonist property of PA and found up regulation of PPARγ target genes in intra abdominal white adipose tissue of obese mouse model on treatment with PA (Hontecillas et al., 2009).

In this chapter, we checked the effect of PA on insulin signaling pathway in 3T3-L1 adipocytes. Effect of PA on secretion of adiponectin, which is an important adipokine for its role in MS, is also evaluated. Also the effect of PA on adipocyte differentiation markers like CD36 and C/EBPα were studied. We also used *in silico* method and *in vitro* ligand binding assay along with cell-based *in vitro* models to characterize its activity as a partial PPARγ ligand. The adipogenic potential, PPARγ ligand binding activity and insulin sensitizing activity of PA in 3T3-L1 adipocytes were compared with that of RG, the full PPARγ agonist.

3.2. Methods

Experiments were conducted to see the adipogenic potential and insulin sensitizing activity of PA compared to RG in 3T3-L1 adipocytes. Also, the characterization of PA as a partial PPARγ agonist was done using *in silico* method as well as by TR-FRET assay. The detailed procedures of all the methods are given in chapter 2.

The methodologies in the chapter include

- Cytotoxicity evaluation of PA
- Preadipocyte differentiation and oil red O staining assay
- Estimation of TG content
- Determination of GPDH activity

- Estimation of adiponectin
- Effect of PA on insulin signaling pathway in 3T3-L1 adipocytes
- GLUT4 translocation assay
- TR-FRET assay for PPARγ ligand binding efficiency
- Docking studies of PA with PPARγ LBD

3.3. Results

3.3.1. Cytotoxicity evaluation of PA

Cytotoxicity of different concentrations of PA (10, 20, 30, 50 and 75 µM) was conducted in 3T3-L1 preadipocytes and adipocytes. None of the used concentrations of PA were found to be cytotoxic (Figure 3.1).

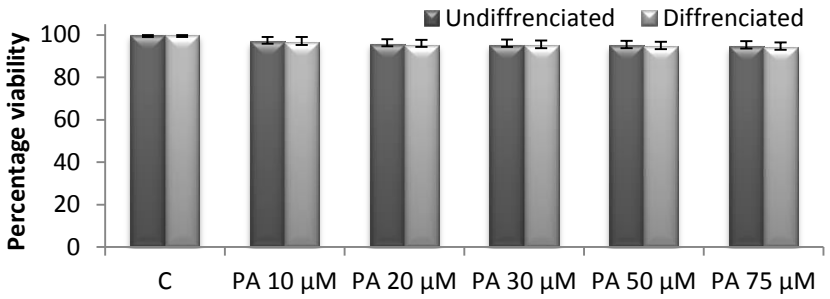


Figure 3.1. Cytotoxicity evaluation of different concentrations of punicic acid in undifferentiated and differentiated adipocytes.

3.3.2. PA moderately enhanced adipocyte differentiation in 3T3-L1 preadipocytes compared to the full agonist RG

For adipogenesis studies, 3T3-L1 preadipocytes were treated with indicated concentrations (5, 10 and 30 µM) of PA or RG (100 nM) throughout the process of adipocyte differentiation for 8 days. To confirm the role of PA as a modulator of PPARγ, GW9662 (an antagonist of PPARγ) at a concentration of 10 µM was used along with the higher concentration of PA (30 µM) during the process of differentiation (Jiang et al., 2002; Liao et al., 2012; Shimojo et al., 2007). Lipid accumulation was found to be increased in a dose dependent manner on treatment with PA (Figure 3.2 A). Adipocyte differentiation was confirmed by oil red O staining of lipid droplets (Figure 3.2 B). The optical density of eluted oil red O concentration was significantly increased by 1.12 and 1.15 fold compared to the control group with 10 and 30 µM concentrations of PA respectively ($p \leq 0.05$), but it was significantly less compared

with the RG treated cells ($p \leq 0.05$). In RG treated cells, eluted oil red O concentration was increased significantly ($p \leq 0.05$) to 1.35 fold compared to the control (Figure 3.2 C). These results revealed that PA stimulated adipocyte differentiation in a moderate level compared with RG. Further it also indicated that in presence of GW9662, a potent inhibitor of PPARγ, the adipogenic potential of PA was decreased and the optical density of eluted oil red O concentration was reduced significantly to 1.21 fold compared to the 30 µM PA alone treated cells, indicating that PA acts through PPARγ. Overall results of adipocyte differentiation showed that PA only moderately enhanced differentiation of preadipocytes as compared with the full PPARγ agonist RG.

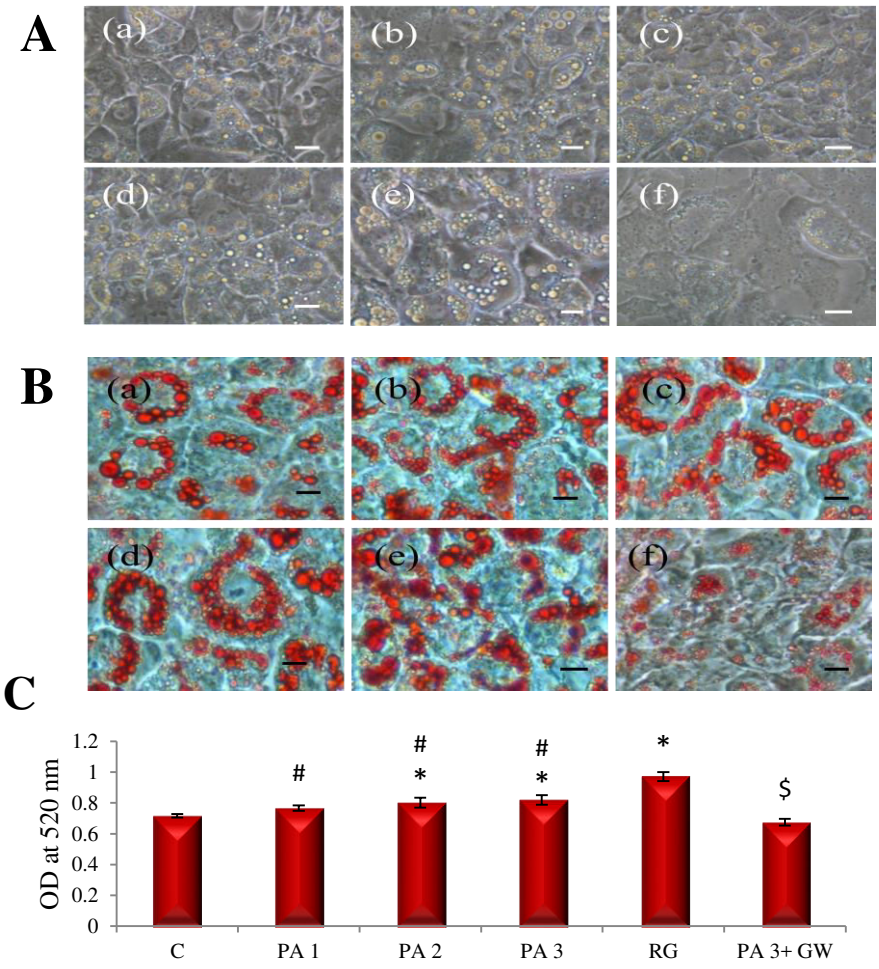


Figure 3.2. Adipocyte differentiation assay and Oil red O staining: (A) Phase contrast micrograph of lipid accumulation in adipocytes treated with various concentrations of punicic acid, rosiglitazone and punicic acid + GW9662. **(B)** Phase contrast micrograph of oil red O staining in differentiated adipocytes. (a) control (b) punicic acid 5 µM (c) punicic acid 10 µM (d) punicic acid 30 µM (e) rosiglitazone 100 nM (f) punicic acid 30 µM+ GW9662 10 µM**.** Scale bars correspond to 100 µm**. (C)** Quantification of lipid droplet in differentiated adipocytes by oil red O staining. C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG rosiglitazone 100 nM, PA3 + GW-punicic acid 30 µM+ GW9662 10 µM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant difference from control group ($p \leq 0.05$). #indicates significant difference from rosiglitazone treated group ($p \leq 0.05$). $ indicates significant difference from 30 µM PA treated group.

3.3.3. PA enhanced GPDH expression and TG accumulation in 3T3-L1 adipocytes

During the conversion of preadipocytes into adipocytes there is an increase in the accumulation of lipid and also the activity of the cytosolic enzyme GPDH (Wise and Green, 1979). TG accumulation and GPDH activity were also determined in the cells treated with PA. PA was found to increases TG accumulation and GPDH activity in moderate level but significantly less ($p \leq 0.05$) than RG. In details, PA caused a significant increase ($p \leq 0.05$) in TG content by 1.17 and 1.27 fold with 10 and 30 µM doses of PA respectively (Figure 3.3 A). PA also caused a significant increase ($p \leq 0.05$) in GPDH activity with 10 (1.12 fold compared to the control group) and 30 µM (1.16 fold increase compared to the control group) concentrations (Figure 3.3 B). RG significantly increased ($p \leq 0.05$) TG and GPDH activity by 1.93 and 1.50 fold compared to the control group (Figure 3.3 A, B). The effect of GW9662 on GPDH activity and TG accumulation in PA treated cells were also analyzed. The presence of GW9662 significantly reduced ($p \leq 0.05$) the TG accumulation and GPDH activity in PA (30 µM) treated cells (reduced to 1.45 and 1.30 fold respectively with respect to the 30 µM of PA alone treated group; Figure 3.3 A, B). These results again confirmed the activity of PA as a partial PPARγ agonist.

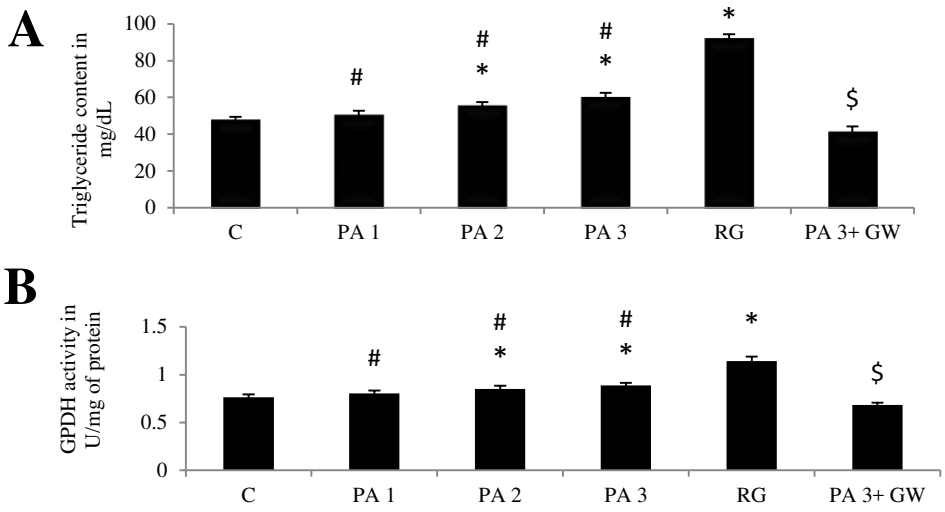


Figure 3.3. Triglyceride content and glycerol-3-phosphate dehydrogenase (GPDH) activity in 3T3-L1 adipocytes: A) Triglyceride content in various groups **B)** GPDH activity in various groups. C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG- rosiglitazone 100 nM, PA3 + GW-punicic acid 30 µM+GW9662 10 µM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant difference from control group ($p \leq 0.05$). #indicates significant difference from rosiglitazone treated group ($p \leq 0.05$). $ indicates significant difference from 30 µM of PA treated group.

3.3.4. PA enhanced the expression of adipogenic genes C/EBPα and CD36

Effect of PA on the expression of adipogenic markers like C/EBPα and CD36 was analyzed by qRT-PCR. The expression of both C/EBPα and CD36 were up-regulated in a dose dependent manner indicating an increased adipogenic potential on treatment with PA. CD36 expression was increased significantly ($p \leq 0.05$) by 1.20, 1.47 and 1.52 fold with the three concentrations (5, 10, and 30 µM) of PA compared to that of control group, and in RG treated cells, CD36 expression was increased significantly ($p \leq 0.05$) by 1.67 fold compared to the control group (Figure 3.4). C/EBPα expression was increased significantly ($p \leq 0.05$) by 1.22 and 1.96 fold with 10 and 30 µM of PA compared to that of control group, and in RG treated cells, C/EBPα expression was increased significantly ($p \leq 0.05$) by 2.46 fold compared to the control group (Figure 3.4). These results again confirm a less adipogenic potential of PA compared to the full agonist RG.

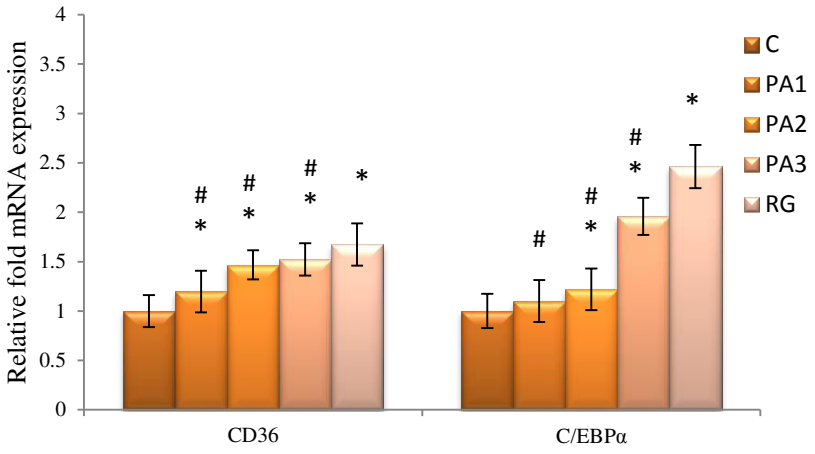


Figure 3.4. mRNA expressions of CD36 and C/EBPα in different experimental groups: C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG- rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant difference from control group ($p \leq 0.05$). #indicates significant difference from rosiglitazone treated group ($p \leq 0.05$).

3.3.5. PA enhanced the expression of PPARγ in 3T3-L1 adipocytes

Next we studied the effect of PA on the expression of PPARγ by qRT-PCR and western blot. PA and RG up-regulated the mRNA level expression of PPARγ significantly in a dose dependent manner (increased by 1.20, 1.33, 1.46 and 2.33 fold compared to the control group for 5, 10 and 30 µM of PA and 100 nM of RG respectively; $p \leq 0.05$; Figure 3.5 A). The protein level expression of PPARγ was also

increased in a dose dependent manner on treatment with PA, which was evident from western blot result. The expression of PPARγ protein level was increased significantly ($p \leq 0.05$) by 1.19, 1.30 and 1.37 fold for 5, 10 and 30 µM of PA, and for RG, the protein was increased significantly ($p \leq 0.05$) by 1.59 fold compared to the control group (Figure 3.5 B, C). But the increase in the mRNA and protein expression levels were significantly ($p \leq 0.05$) lower compared with RG treated cells, indicating the quality of PA as a partial PPARγ agonist.

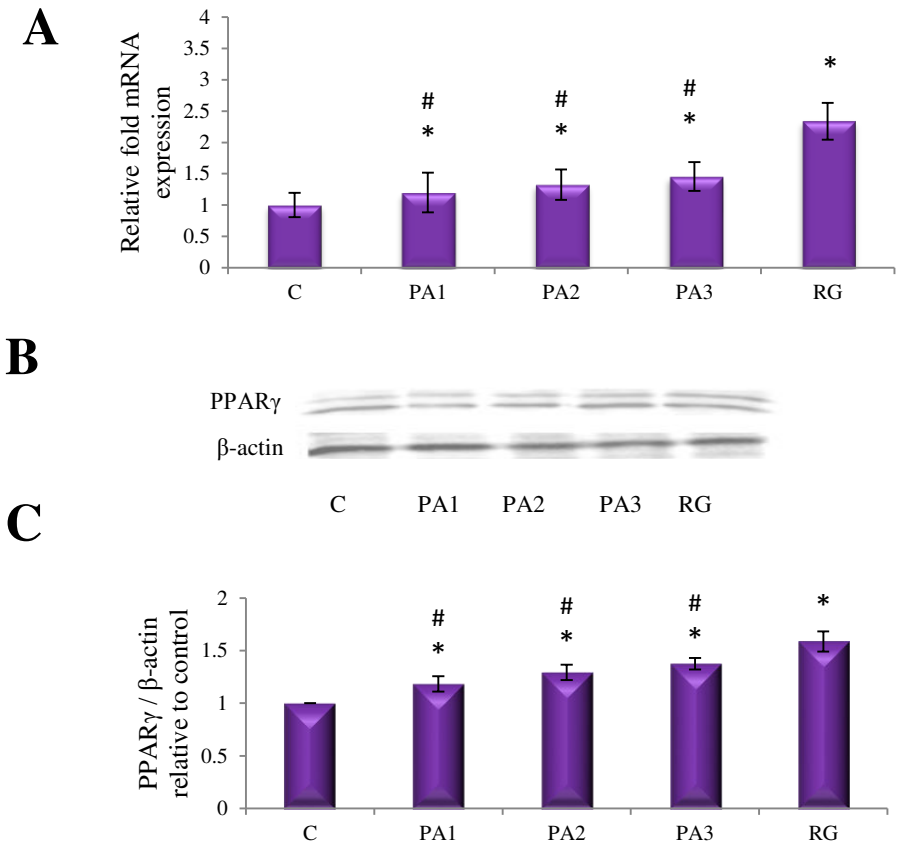


Figure 3.5. mRNA and protein expression of PPARγ in different experimental groups: A) mRNA expression of PPARγ **B)** Immunoblot analysis of PPARγ **C)** Densitometric analysis of protein expression of PPARγ with respect to β-actin. C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG- rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant difference from control group ($p \leq 0.05$). #indicates significant difference from rosiglitazone treated group ($p \leq 0.05$).

3.3.6. PA enhanced adiponectin secretion in 3T3-L1 adipocytes

Fully differentiated 3T3-L1 cells were treated with different concentrations of PA or RG for 24 hours and the release of adiponectin from the cells into the medium was determined by using mouse adiponectin ELISA kit. The result showed that PA significantly increased ($p \leq 0.05$) adiponectin secretion in a dose dependent manner

Figure 3.6). Adiponectin secretion was increased by 1.11, 1.16 and 1.20 fold with 5, 10, and 30 µM of PA respectively compared to the control group and with RG (100 nM) it was increased significantly ($p \leq 0.05$) by 1.34 fold. This result supports the beneficial property of PA as an insulin sensitizing agent.

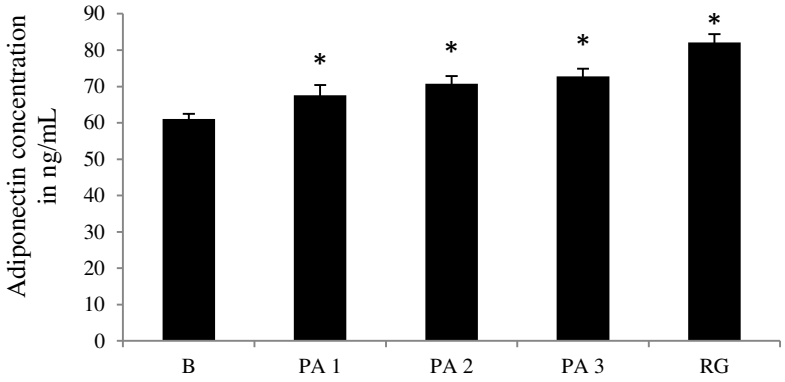


Figure 3.6. Secretion of adiponectin in various groups: Fully differentiated 3T3-L1 adipocytes were treated with different concentrations of punicic acid and rosiglitazone and adiponectin was estimated in the conditioned media. C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG- rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant difference from control group ($p \leq 0.05$).

3.3.7. Competitive binding affinity of PA to PPAR γ

In vitro binding of PA to PPARγ was analyzed by TR-FRET PPARγ competitive binding assay (Raman et al., 2011), using RG and GW9662 as the positive controls. The main reaction of this assay includes the binding of the test compounds (RG, PA and GW9662) with PPARγ ligand binding domain (LBD) by displacing the Fluromone Pan PPAR green. This will cause a reduction in the ratiometric emission at 520 nm/490 nm. The FRET signals were measured in Tecan multiplate reader and are plotted against various concentrations of the ligands.The IC_{50} value of the compounds for the 50% displacement of Pan PPAR green was calculated from the response curve and was found to be 4.82 µM for PA, whereas for RG and GW9662 it was 53.09 nM and 12.88 nM respectively (Figure 3.7).

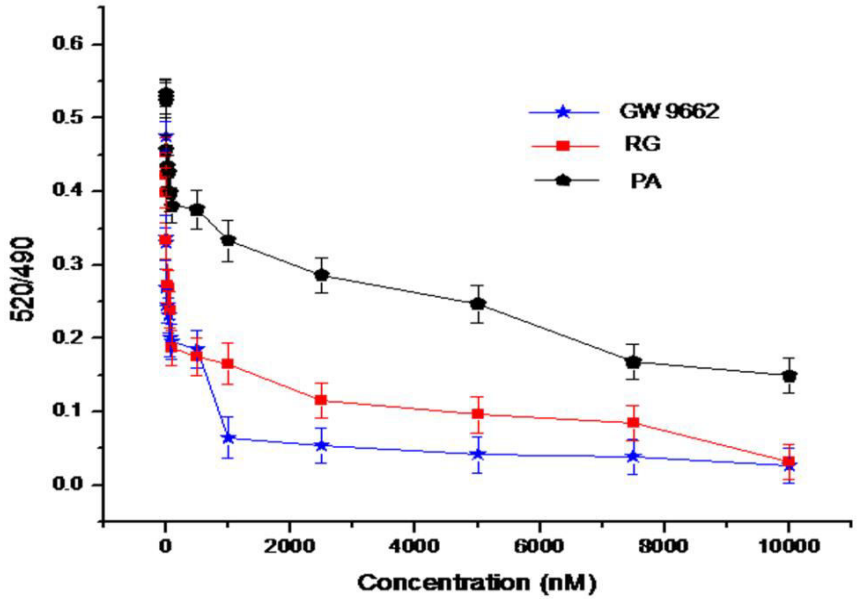


Figure 3.7. Lanthascreen TR-FRET PPARγ competitive binding assay of punicic acid, rosiglitazone and GW9662: The reaction mixture containing 0.5 nM PPAR-LBD (GST), 5 nM Terbium-tagged anti-GST antibody, 5 nM Fluormone Pan-PPAR Green, 5 mM dithiothreitol, and varying concentrations of punicic acid, rosiglitazone or GW9662 was incubated in dark for 2 hours and TR-FRET signals were measured. The values are means, with standard deviations represented by vertical bars (n=6).

3.3.8. Docking analysis of PPARγ with PA

By our molecular docking experiment using Autodock 4.2 and iGEMDOCK v2.1, appropriate binding and conformation of PA and RG to the LBD of PPARγ was estimated (Hsu et al., 2011; Mahindroo et al., 2006; Morris et al., 2009**).** Autodock has estimated the free binding energy of PA to be -5.91 kcal/mol showing the high potential binding affinity into the binding site. The docking fitness obtained for PA using iGEMDOCK is 614.678. For RG, the free energy of binding was found to be -6.99 kcal/mol and fitness value was found to be 2116.08. So by molecular docking study it was predicted that PA could bind to the LBD of PPARγ though slightly weak as compared with RG (Figure 3.8). The binding energy of the ligands with PPARγ was analysed by using the software iGEMDOCK (Hsu et al., 2011) and it is represented in Table 3.1. Other groups had also predicted the more or less similar level of binding affinity of PA to the PPARγ LBD by molecular docking (Lewis et al., 2011).

Thus molecular docking study and competitive binding assay confirmed proper binding of PA to the LBD of PPARγ predicting agonist property of PA. But the binding affinity was less compared to RG.

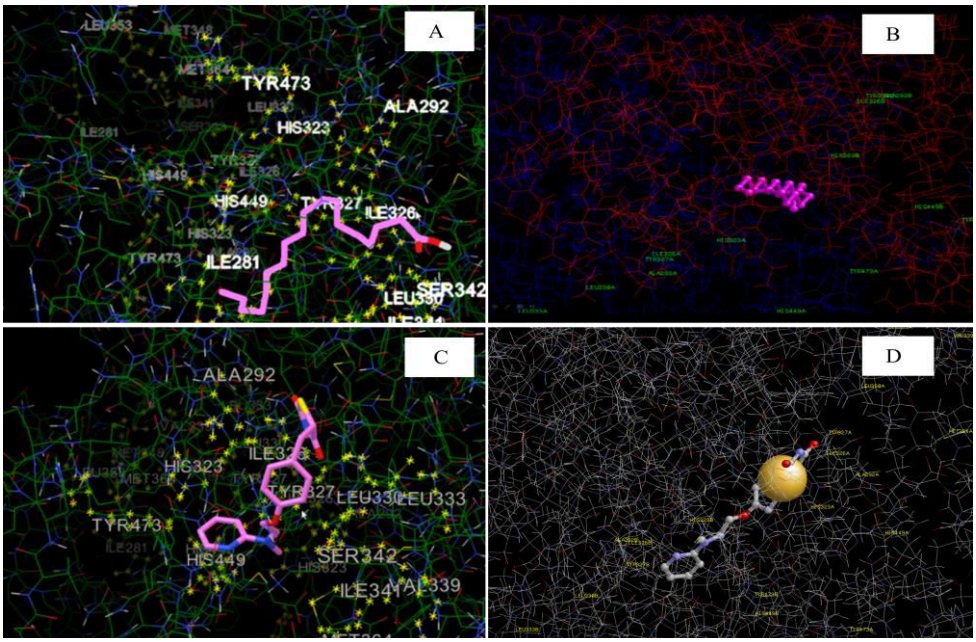


Figure 3.8. Docking of punicic acid and rosiglitazone to the LBD of PPARγ (A) Autodock 4.2 representing the binding of PA with PPARγ LBD. **(B)** iGEMDOCK v2.1 representing the best docking pose of PA to PPARγ LBD. **(C)** Autodock 4.2 representing the binding of RG with PPARγ LBD. **(D)** iGEMDOCK v2.1 representing the best docking pose of RG to PPARγ LBD

Compund	Energy (kcal/mol)	VDV	H bond	Elect
PA	-85.0884	-85.0884	0	0
RG	-100.512	-100.512	0	0

Table 3.1. Binding energy of PA and RG to the LBD of PPARγ obtained by iGEMDOCK

3.3.9. PA up-regulated the expression of IRS2 in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocytes with different concentrations of PA significantly up-regulated ($p \leq 0.05$) the mRNA expression of IRS2 (the expression was increased to 1.24, 1.90 and 2.14 fold compared to the control group). RG treatment also significantly increased ($p \leq 0.05$) IRS2 gene expression to 3.35 fold compared to the control group ($p \leq 0.05$; Figure 3.9 A). Also, western blot analysis showed that PA treatment increased the protein level expression of IRS2 ($p \leq 0.05$; Figure 3.8 B, C) in a dose dependent manner which was comparable to that of RG treated group. IRS2 protein level was increased significantly ($p \leq 0.05$) to 1.20, 1.57, 1.62 and 1.71 fold for 5, 10 and 30 μM of PA and 100 nM of RG respectively, compared to the control group.

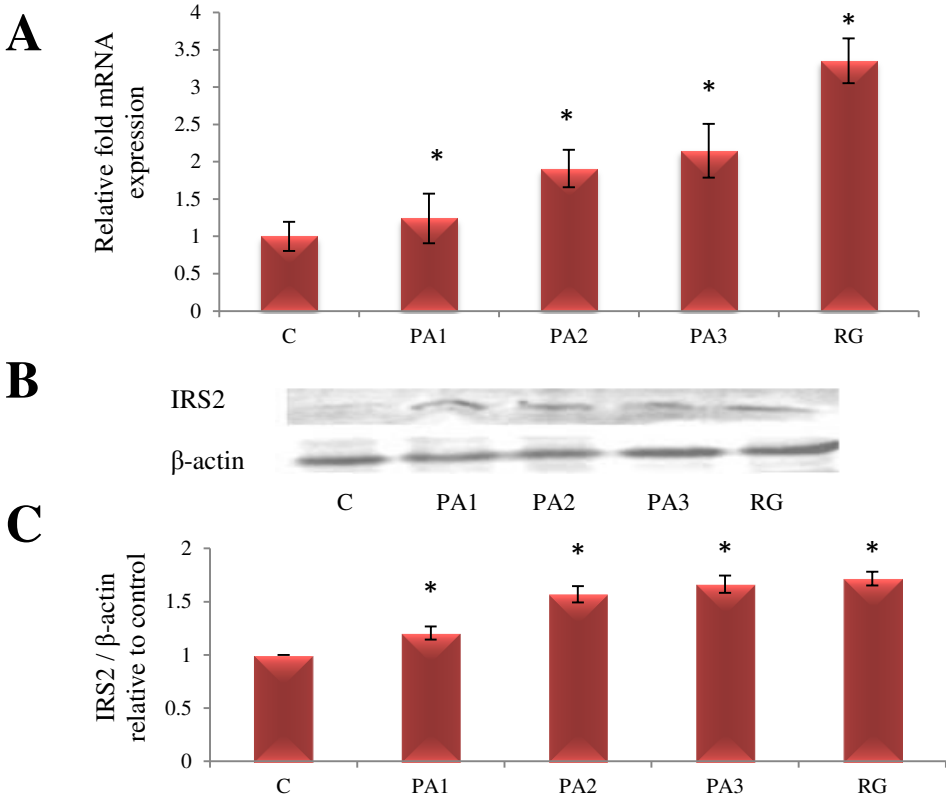


Figure 3.9. mRNA and protein expression of IRS2 in different experimental groups: A) mRNA expression of IRS2 **B)** Immunoblot analysis of IRS2 **C)** Densitometric analysis of protein expression of IRS2 with respect to β-actin. C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG- rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant difference from control group ($p \leq 0.05$).

3.3.10. PA up-regulated PI3K expresion and Akt phosphorylation in 3T3-L1 adipocytes

Western blot analysis showed that PA treatment increased the protein level expression of pAkt and PI3K significantly ($p \leq 0.05$). On treatment with different concentrations of PA and RG, PI3K level was increased by 1.45, 1.67, 1.79 and 1.87 fold for 5, 10 and 30 µM of PA and 100 nM of RG respectively (Figure 3.10 A, B). PA or RG treatment increased the expression of pAkt/Akt ratio to 1.46, 1.73, 1.81 and 1.64 fold compared to the control group for 5, 10 and 30 µM of PA and 100 nM of RG respectively (Figure 3.10 C, D).

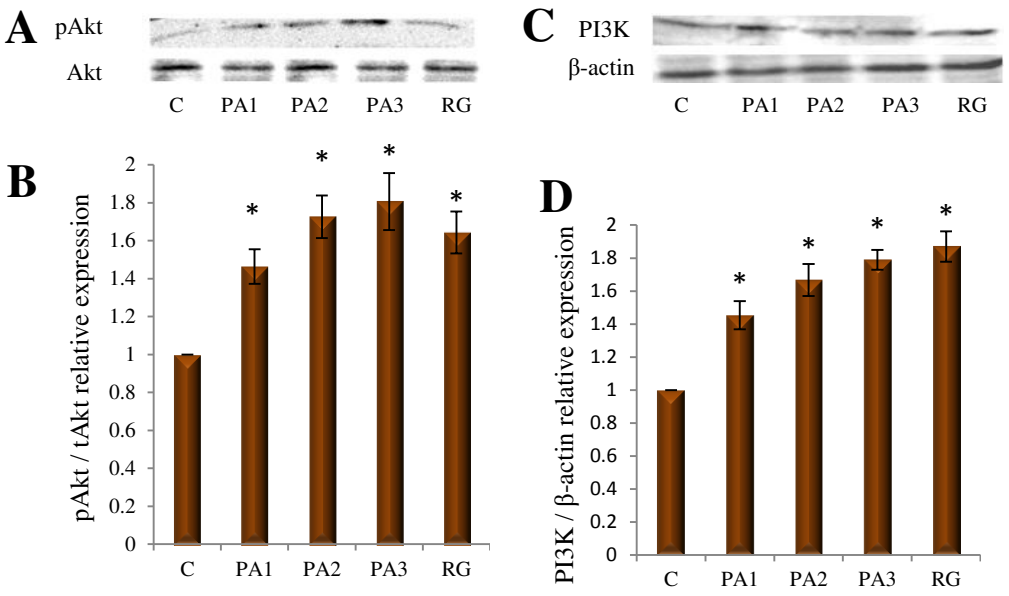


Figure 3.10. Protein expression of pAkt and PI3K in different experimental groups: A) Immunoblot analysis of pAkt **B)** Densitometric analysis of expression of pAkt with respect to total Akt **C)** Immunoblot analysis of PI3K **D)** Densitometric analysis of expression of PI3K with respect to β-actin. C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG- rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant difference from control group ($p \leq 0.05$).

3.3.11. PA up-regulated the expression of GLUT4 in 3T3-L1 adipocytes

PA treatment also increased the mRNA level expression of GLUT4 significantly in a dose dependent manner (increased to 1.30, 1.53 and 1.94 fold compared to the control group on treatment with 5, 10 and 30 µM of PA respectively; $p \leq 0.05$; Figure 3.11 A) and RG also increased the expression of GLUT4 mRNA significantly (increased to 1.86 fold compared to the control group, $p \leq 0.05$; Figure 3.11 A). Also, the protein level expression of GLUT4 was increased significantly on treatment with PA and RG (increased to 1.27, 1.42, 1.50 and 1.60 fold increase for 5, 10 and 30 µM of PA and 100 nM of RG respectively ($p \leq 0.05$; Figure 3.11 B, C).

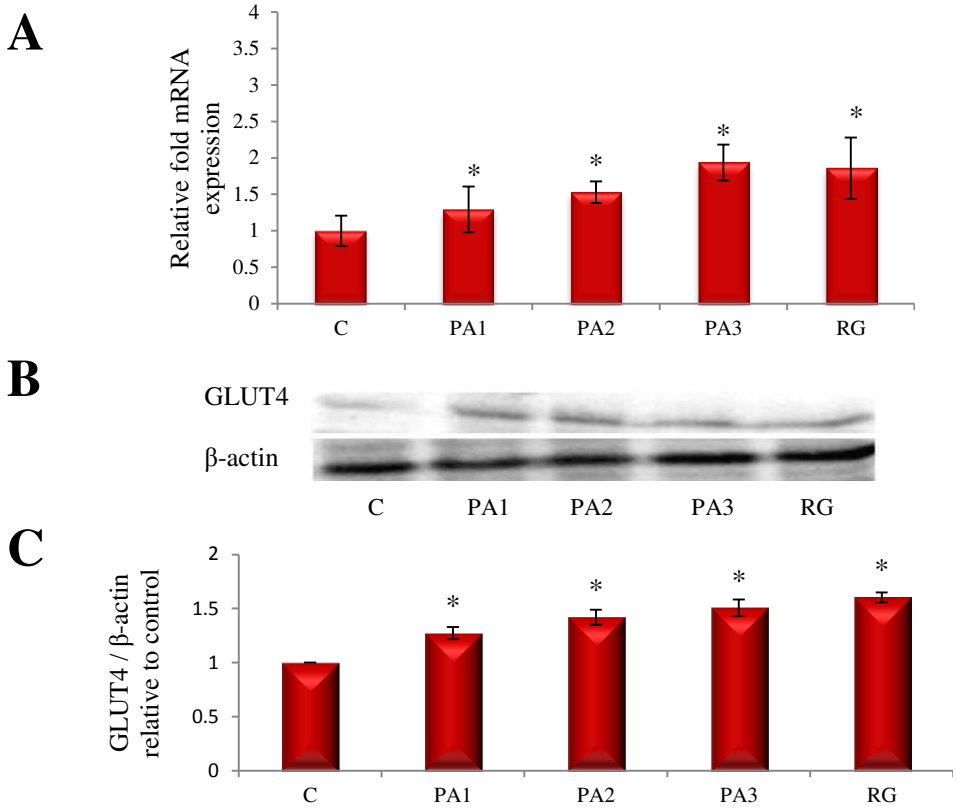


Figure 3.11. mRNA and protein expression of GLUT4 in different experimental groups: A) mRNA expression of GLUT4 **B)** Immunoblot analysis of GLUT4 **C)** Densitometric analysis of protein expression of GLUT4 with respect to β-actin. C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG- rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant difference from control group ($p \leq 0.05$). #indicates significant difference from rosiglitazone treated group ($p \leq 0.05$).

3.3.12. PA enhanced GLUT4 translocation to the plasma membrane

Next we studied the effect of PA on the translocation of GLUT4 into the plasma membrane by immunofluorescence analysis (Figure 3.12). PA treatment increased the translocation of GLUT4 into the plasma membrane in a dose dependent manner. RG treatment also increased the translocation of GLUT4 into the plasma membrane.

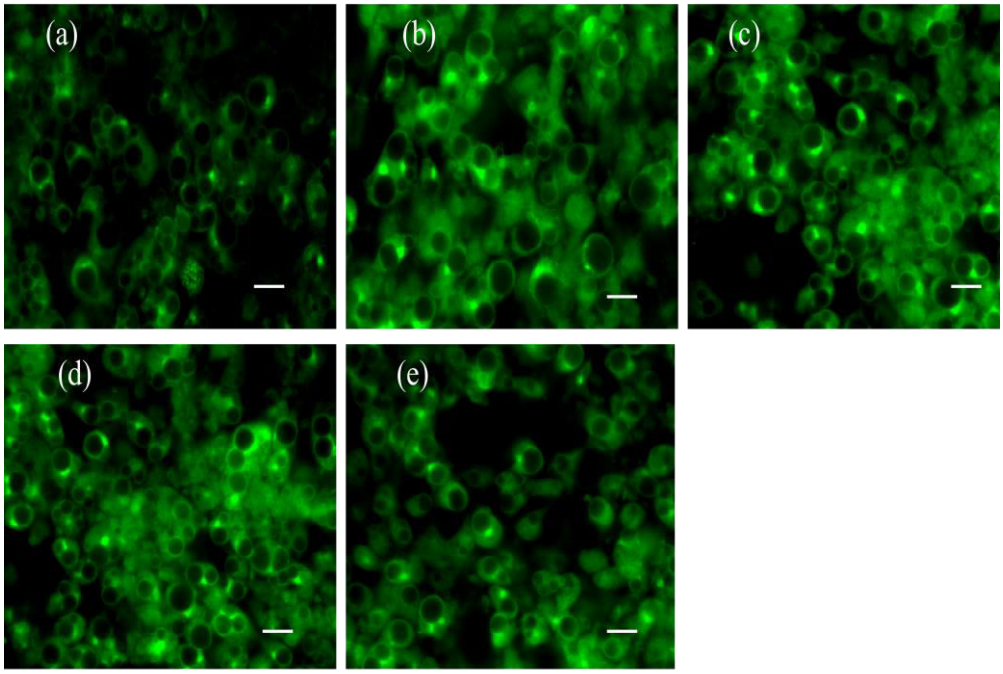


Figure 3.12. Immunofluorescence imaging of GLUT4 translocation in control and treated cells: (a) control (b) punicic acid 5 µM (c) punicic acid 10 µM (d) punicic acid 30 µM (e) rosiglitazone 100 nM (f) punicic acid 30 µM+ GW9662 10 µM**.** Scale bars correspond to 100 µm**.**

3.3.13. PA enhanced glucose uptake in 3T3-L1 adipocytes

Next, insulin stimulated glucose uptake activity in PA treated cells was measured by using commercially available kit (colorimetry) and by using 2-NBDG, a fluorescent analogue of glucose (cytometry) using RG (100 nM) as the positive control. The results indicated that PA and RG significantly increased ($p \leq 0.05$) insulin stimulated glucose uptake in adipocyte in a dose dependent manner (Figure 3.13). In 2-deoxy glucose uptake assay, glucose uptake was found to be increased to 1.20, 1.65 and 2.25 fold with the three concentrations (5, 10, and 30 µM) of PA whereas RG caused an increase of 1.80 fold compared to the control group (Figure 3.13 A). Results obtained by flow cytometry using 2-NBDG also showed the same trend as that of 2-deoxy glucose uptake assay (Figure 3.13 B).

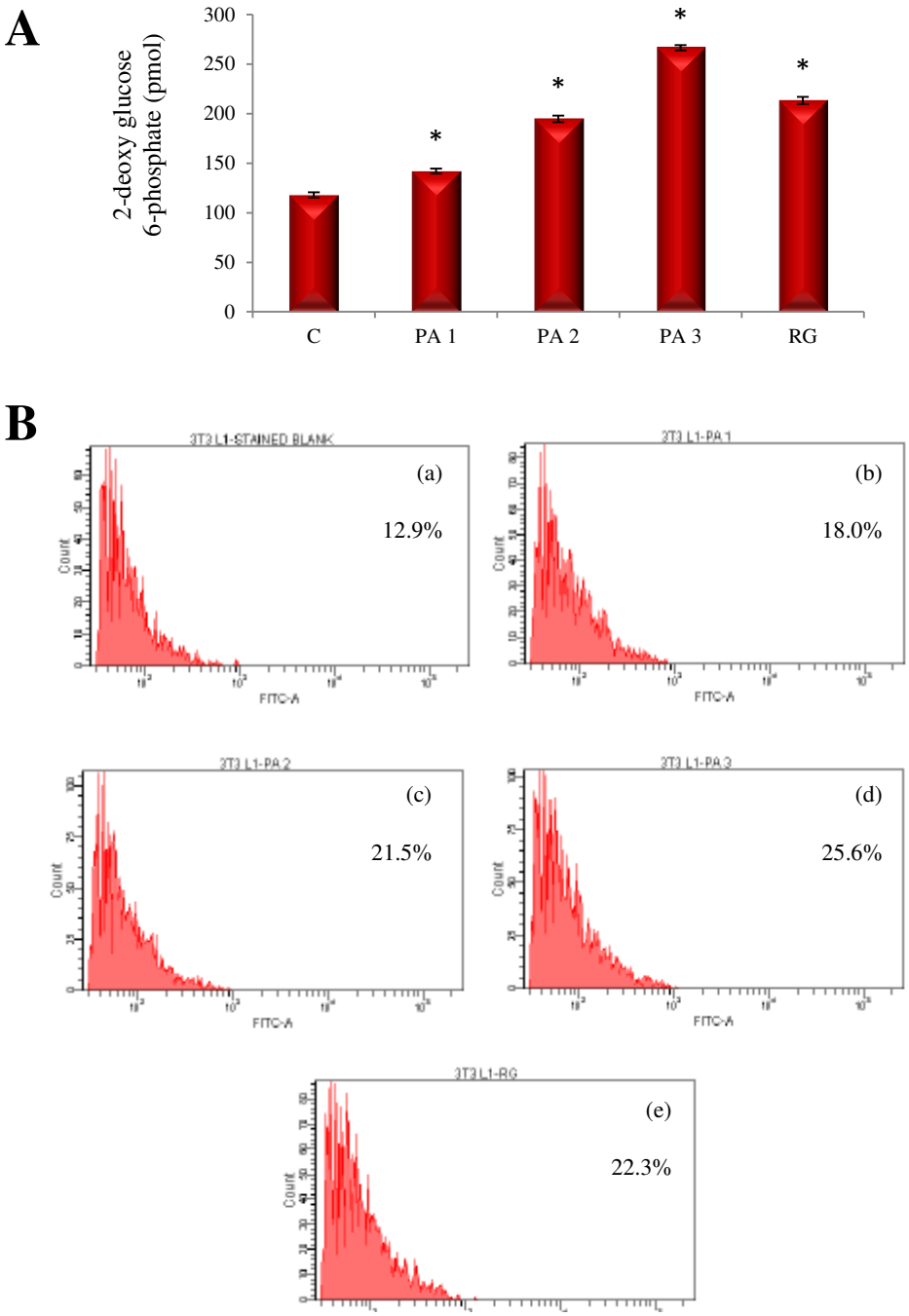


Figure 3.13. Glucose uptake activity in 3T3-L1 adipocytes: (A) 2-deoxy glucose uptake activity in various groups. C-control, PA1- punicic acid 5 µM, PA2- punicic acid 10 µM, PA3- punicic acid 30 µM, RG- rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *indicates significant different from control group ($p \leq 0.05$). **(B)** 2-NBDG uptake activity in various groups**.** Representative histogram shows percentage cells with fluorescent intensity which indicates glucose uptake activity. (a) control group, (b) punicic acid 5 µM, (c) punicic acid 10 µM, (d) punicic acid 30 µM, (e) rosiglitazone 100 nM.

3.4. Discussion

PPARγ agonists of natural origin are now gaining more importance than the synthetic ones because of their fewer side effects. New lead PPARγ agonists from natural resources are showing much potential in the treatment of diabetes and associated disorders, suggesting the relevance of nutraceuticals as PPARγ agonists for the management of MS (Rau et al., 2006; Salam et al., 2008). In the present chapter, we characterized PA as a partial PPARγ agonist and evaluated the potential of PA as a nutraceutical by conducting detailed study on its insulin sensitizing property in 3T3-L1 adipocytes. We studied the effect of PA on the expression of PPARγ, IRS2, PI3K and pAkt in 3T3-L1 adipocytes. Effect of PA on insulin mediated glucose uptake, adiponectin secretion, expression and translocation of GLUT4 were also studied. Various biochemical and molecular studies were conducted on adipogenesis and associated signaling pathways to elucidate its PPARγ agonist property in comparison with RG. The extent of adipocyte differentiation was studied by morphological examination of the cells for lipid accumulation, quantification of lipid droplets by oil red O staining, measurement of TG accumulation by colorimetric method, measurement of the activity of the enzyme GPDH and also by studying the expression of adipocyte differentiation markers like C/EBPα and CD36.

Adipocyte differentiation is characterized by highly coordinated signaling pathways and it includes morphological and biochemical changes in adipocytes. Synthetic PPARγ agonists enhance adipocyte differentiation and they can act as insulin sensitizers and are used in the treatment of T2DM. But these are full agonists of PPARγ and are associated with various side effects like weight gain, edema, fluid retention and congestive heart failure (Guasch et al., 2013). Nutritional items that can induce adipocyte differentiation in a moderate level with better insulin sensitizing property can be considered as better options for the management of MS and associated disorders.

PPARγ is a master regulator of adipocyte differentiation (Choi et al., 2009). During adipocyte differentiation, PPARγ is up regulated and PPARγ agonists are reported to enhance adipocyte differentiation (Gregoire et al., 1998). Adipocyte differentiation is accompanied by increased lipid accumulation and there is an increase in the activity of the lipogenic enzyme, GPDH (Wise and Green, 1979). PPARγ is

involved in the terminal differentiation process along with c/EBPα by activating the expression of aP2 and LPL and there by facilitating the cytoplasmic storage of TG in adipocytes (Gregoire, 1998). Also, C/EBPα is involved in PPARγ dependent expression and maintenance of insulin sensitivity (Farmer, 2006). CD36 is another important marker of differentiation of pre-adipocytes into adipocytes and is an integral membrane glycosylated protein. CD36 is a fatty acid transporter found in many mammalian cell types such as adipocyte macrophages, hepatocytes, endothelial cells etc. (Silverstein and Febbraio, 2009). In CD36 null mice, fatty acid uptake in adipocytes was found to be reduced to around 60% (Coburn et al., 2000). Our results indicated that PA moderately enhanced adipocyte differentiation in 3T3-L1 pre-adipocytes compared with the full agonist RG, which is evident from the results of TG estimation and oil red O staining. Also, PA induced pre-adipocyte differentiation was inhibited on treatment with GW9662, an antagonist of PPARγ, indicating that PA enhanced adipocyte differentiation by activating PPARγ. PA induced increase in adipocyte differentiation is also accompanied by moderate increase in the activity of the enzyme GPDH and moderate increase in the expression of C/EBPα, PPARγ and CD36, suggesting the property of PA as a partial PPARγ agonist.

Our molecular docking study and TR-FRET assay proved that PA can bind with the ligand binding domain of PPARγ but with less affinity compared with the full PPARγ agonist RG, again confirming that PA can be considered as a partial PPARγ agonist. Molecular docking study revealed that the binding energy of PA to the PPARγ LBD was less compared to the full agonist RG. Full PPARγ agonists like RG interacts with PPARγ LBD through hydrogen bonds with the amino acid residues Ser289, His323, His449 and Tyr473. The interaction profile for RG to the PPARγ LBD obtained using Autodock was consistent with the previous reports (Guasch et al., 2013; Lima et al., 2014)**.** The key interactions between partial agonists and the LBD of PPARγ are different than those of the full agonists, which affects the recruitment of various coactivators causing differential transcriptional activity of PPARγ (Lu et al., 2006; Pochetti et al., 2007). Our docking study revealed that PA did not share the common binding site as that of RG, indicating that it behaves more like a partial agonist.

Adiponectin is an important adipokine secreted by small adipocytes, which enhance insulin sensitivity (Rau et al., 2006; Yamauchi et al. 2001). Adiponectin is a PPARγ target gene which is involved in adipocyte differentiation (Choi et al., 2009). It is reported that adiponectin increases insulin sensitivity in adipocytes by increasing the number of insulin sensitive adipocytes and the level of adiponectin is increased during adipocyte differentiation and decreased during conditions like T2DM (Fu et al., 2005; Naowaboot et al., 2012). Our result indicates that treatment with PA dose dependently enhanced adiponectin secretion into the medium. This result supports the beneficial property of PA as an insulin sensitizing agent.

PPARγ agonists like TZDs are reported to enhance IRS2 gene expression (Smith et al., 2001). Tyrosine phosphorylation of IRS can activate PI3K. PI3K phosphorylates phosphatidyl inositol lipids, in the plasma membrane. Thus generated phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) recruits the serine/threonine protein kinases PDK1 and PKB/Akt to the plasma membrane (James et al., 1996). The phosphorylation of PKB/Akt at threonine 308 causes its partial activation and phosphorylation at serine 403 causes maximal activation (Alessi et al., 1996; Alessi et al., 1997; Stokoe et al., 1997). Phosphorylated PKB/Akt causes GLUT4 translocation to the plasma membrane and increases glucose uptake (Summers et al., 1998). PI3K is involved in the insulin stimulated glucose uptake activity in adipocytes (Yang et al., 2007) and the activation of PPARγ is reported to up-regulate PI3 kinase activity (Naowaboot et al., 2012). Insulin stimulated GLUT4 translocation is an important event in glucose uptake in adipocytes (Zhiyong et al., 2012). PPARγ ligands are known to increase glucose transport in insulin sensitive tissues. The synthetic ligands of PPARγ like TZDs enhance glucose uptake by increasing the expression of GLUT1 or GLUT4 or by increasing the translocation of GLUT4 (Kuhn et al., 2011). The present study showed that treatment of adipocytes with PA resulted in increased expression of IRS2, PI3K, pAkt, PPARγ and GLUT4 and translocation of GLUT4. Thus PA enhanced insulin stimulated glucose uptake and insulin sensitivity in 3T3-L1 adipocytes.

Effect of PA on insulin stimulated glucose uptake activity in 3T3-L1 adipocytes was comparatively higher than that of RG treated cells. At the same time, adipogenic potential of PA was moderate compared to RG. The results of both glucose uptake

activity and adipogenic potential of PA gave an idea that PA is a relevant compound in nutraceutical perspectives compared with the existing full agonist like RG. These results revealed that PA is a partial PPARγ agonist with potent insulin stimulated glucose transport capacity and moderate adipogenic potential. In addition, PA improves adiponectin secretion. Because of high tolerance to humans due to its edible nature, the prospects of PA as a nutraceutical is very attractive compared to RG. For maximum utilization of its potential as nutraceuticals, detailed investigations are needed with respect to its mode of action on various other relevant biochemical targets of insulin signaling pathway. Keeping this in mind detailed investigation had been conducted on the effect of PA on inflammation induced insulin resistance in 3T3-L1 adipocytes. The details of this study are given in the next chapter.

Chapter 4

Punicic acid improves insulin resistance induced via inflammation by TNF-α in 3T3- L1 adipocytes

4.1. Introduction

Insulin resistance is defined as a condition in which there is a reduced ability of cells or tissue to respond to the physiological levels of insulin and is a characteristic condition in the pathogenesis of T2DM (Zhang et al., 2009). IR is a defining factor in the development of MS (de Luca and Olefsky 2008). Inflammation in adipose tissue is strongly associated with the development of IR in humans and rodents (Kwon and Pessin, 2013). Chronic low grade inflammation in adipocytes is a stimulus to initiate IR and T2DM (Hotamisligil, 2006; Shoelson et al., 2006). Inflammation in adipocytes can lead to oxidative stress, mitochondrial dysfunction, ER stress etc. and can contribute to the activation of stress responsive genes including JNK, IKKβ, NF-kB etc. which in turn augment the expression of inflammatory genes thus contributing to systemic inflammation (Samuel and Shulman, 2012).

Among the different types of proinflammatory cytokines TNF-α is the first one to be identified to play an important role between obesity, inflammation and IR (Fujishiro et al., 2003). TNF-α is produced by adipocytes and macrophages infiltrating adipocytes, and the amount of TNF-α is increased in obese and diabetic subjects (Weisberg et al., 2003). Treatment with TNF-α causes an inhibition in insulin signaling and results in a state of IR in several isolated cell system including 3T3-L1 adipocytes, human primary adipocytes and animal models (Hotamisligil et al., 1996; Plomgaard et al., 2005; Shibasaki et al., 2003; Shoelson et al., 2006). It also causes a reduction in the expression of various proteins involved in insulin signaling pathway like IRS1, GLUT4, LPL, C/EBP and PPARγ, leading to a reduction in glucose uptake (Ruan et al., 2002; Stephens et al., 1997).

Obese patients having T2DM are reported to have increased levels of TNF-α in circulation and adipose tissue (Hotamisligil et al., 1993; Kern et al., 1995; Kwon and Pessin, 2013). TNF-α is found to increase the phosphorylation of several inhibitory kinases including JNK and IKKβ, which can further cause the activation and transportation of NF-κB p65 into the nucleus (Boura-Halfon and Zick, 2009; Gual et

al., 2005; Tanti and Jager, 2009). The activated NF-κB can further induce the production of inflammatory markers and also activate SOCS3 and PTP1B, which can cause a decline in insulin sensitivity. The increased PTP1B expression can cause dephosphorylation of tyrosine residue of IRS1 leading to a decline in insulin sensitivity (Chuang et al., 2010). Activation of NF-κB is reported to inhibit the transcriptional activation of PPARγ, leading to a state of IR (Remels et al., 2009).

Activation of PPARγ by thiazolidinediones is reported to partially ameliorate IR (Olefsky, 2000). Besides, PPARγ has also been demonstrated as a key repressor in obesity linked IR and inflammation (Moller and Berger, 2003). PPARγ agonists like TZDs have been shown to block the effect of TNF-α in important insulin signaling events including the tyrosine phosphorylation IRS1 (Peraldi et al., 1997). PPARγ agonists can interfere with the signaling cascade of NF-κB thus inhibiting the expression of proinflammatory cytokine, ameliorating inflammation and IR.

The preliminary work related to adipogenic potential and insulin sensitizing property of PA was found to be very attractive and promising for management and control of MS. Keeping this in mind, studies related to the effect of PA on inflammation mediated IR was conducted and details of the same are given in this chapter.

4.2. Methods

Experiments were conducted to see the effect of PA and RG on TNF-α induced insulin resistant model of 3T3-L1 adipocytes. In order to see the effect of PA and molecular mechanisms behind its activity detailed investigations were conducted on insulin signaling pathway. For this the following experiments were conducted. The detailed procedures of all the methods are given in chapter 2.

- Induction of IR in 3T3-L1 adipocytes by TNF-α treatment (10 ng/ml, 24 hour)
- 2-deoxy glucose uptake assay
- Estimation of adiponectin and leptin
- NF-κB translocation assay
- Estimation of inflammatory cytokine release
- Analysis of PTP1B expression by indirect ELISA
- Analysis of SOCS3 expression by qRT-PCR

- Analysis of JNK1/2 and pJNK½ expression by western blot
- Analysis of pIRS1 (serine 307) expression by western blot
- Expression of PPARγ and GLUT4 by qRT-PCR and western blot

4.3. Results

4.3.1. PA improved TNF-α induced reduction in 2-deoxy glucose uptake

TNF-α treatment (10 ng/ml for 24 hours) caused a significant reduction in insulin stimulated glucose uptake (1.7 fold reduction with respect to control group; $p \leq 0.05$). Treatment with various concentrations of PA or RG along with TNF-α significantly restored ($p \leq 0.05$) the glucose uptake capacity (1.23, 1.45, 1.5 and 1.51 fold increase than the TNF-α treated group for 5, 10 and 30 µM of PA and for 100 nM of RG respectively) (Figure 4.1).

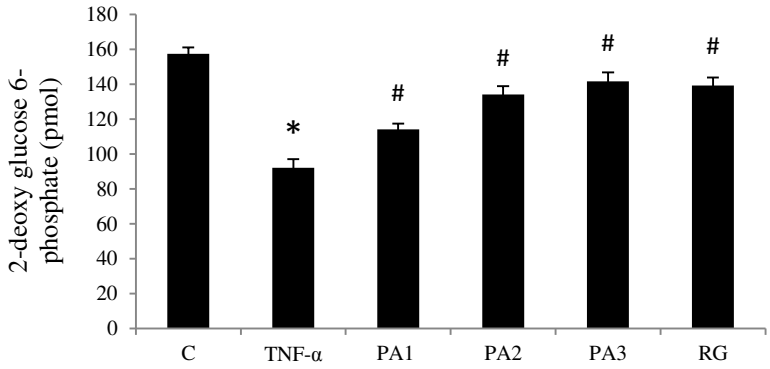


Figure 4.1. Punicic acid reversed TNF-α induced decrease in insulin stimulated glucose uptake in 3T3-L1 adipocytes: Fully differentiated 3T3-L1 adipocytes were treated with 10ng/mL of TNF-α for 24 hours in the presence or absence of various concentration of punicic acid or rosiglitazone and 2-deoxyglucose uptake assay was performed. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *$p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

4.3.2. PA increased adiponectin and decreased leptin secretion in TNF-α induced insulin resistant model of 3T3-L1 adipocytes

We quantified the level of adiponectin and leptin by ELISA methods in the conditioned media after respective treatments. TNF-α treatment significantly reduced ($p \leq 0.05$) the secretion of adiponectin in the medium (3.75 fold decrease compared to the control group; Figure 4.2 A). PA and RG treatment significantly restored ($p \leq 0.05$)

the adiponectin secretion (1.31, 1.60, 2.21 and 3.36 fold increase compared with the TNF-α treated group for 5, 10 and 30 μM of PA and for 100 nM of RG respectively).

TNF-α treatment significantly increased ($p \leq 0.05$) the secretion of leptin in to the media (1.60 fold increase compared to the control group). In PA and RG treated groups, leptin levels were reduced significantly ($p \leq 0.05$) compared to the TNF-α treated group (1.13, 1.23, 1.40 and 1.65 fold decrease for 5, 10 and 30 μM of PA and for 100 nM of RG respectively; Figure 4.2 B).

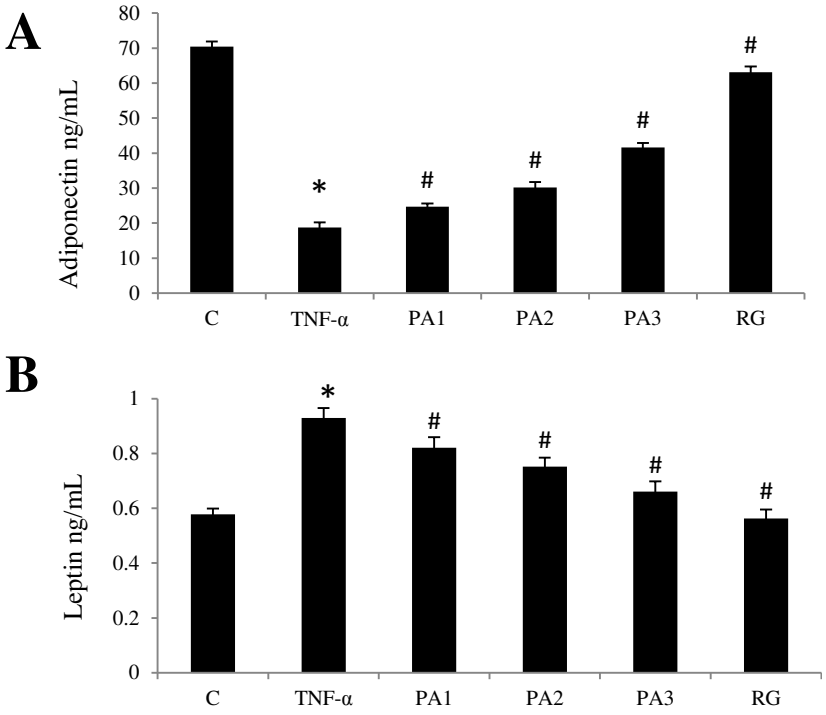


Figure 4.2. Adiponectin and leptin secretion in various experimental groups: **A)** Estimation of adiponectin in conditioned media by ELISA **B)** Estimation of leptin in conditioned media by ELISA. C- control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 μM, PA2- TNF-α + punicic acid 10 μM, PA3- TNF-α + punicic acid 30 μM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *$p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

4.3.3. PA decreased TNF-α induced NF-κB p65 translocation in adipocytes

NF-κB p65 translocation into the nucleus was studied by ELISA of nuclear and cytosolic fractions of different experimental groups (Figure 4.3). In TNF-α treated group there was a significant increase ($p \leq 0.05$) in the translocation of NF-κB p65 to the nucleus (2.69 fold increase compared to the control group). In PA treated groups, the translocation was decreased significantly ($p \leq 0.05$) in a dose dependent manner

(1.25, 1.58 and 2.01 fold reduction compared to the TNF-α treated group). In RG treated group also NF-κB p65 translocation was decreased significantly ($p \leq 0.05$) to 1.65 fold compared to the TNF-α treated group.

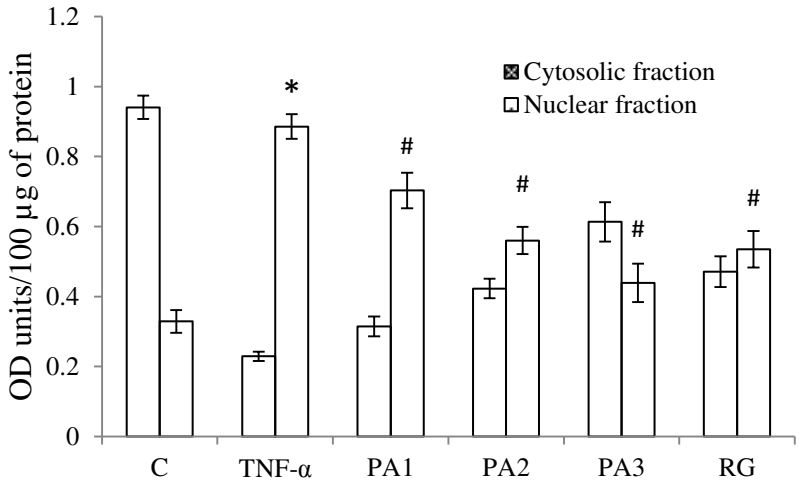


Figure 4.3. NF-κB p65 translocation in various experimental groups: C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5µM, PA2- TNF-α + punicic acid 10µM, PA3- TNF-α + punicic acid 30µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *$p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

4.3.4. PA decreased TNF-α induced JNK phosphorylation in 3T3-L1 adipocytes

Immunoblot analysis showed that TNF-α treatment significantly increased JNK phosphorylation in 3T3-L1 adipocytes compared with control group. Treatment with PA and RG lowered the levels of phosphorylated JNK in a dose dependent manner (Figure 4.4 A, B). In TNF-α treated group, pJNK was increased significantly ($p \leq 0.05$) with respect to total JNK level to 1.86 fold compared with the control group. 5, 10 and 30 µM of PA and 100 nM of RG significantly decreased ($p \leq 0.05$) the level of pJNK by 1.20, 1.29, 1.40 and 1.38 fold respectively compared to the TNF-α treated group.

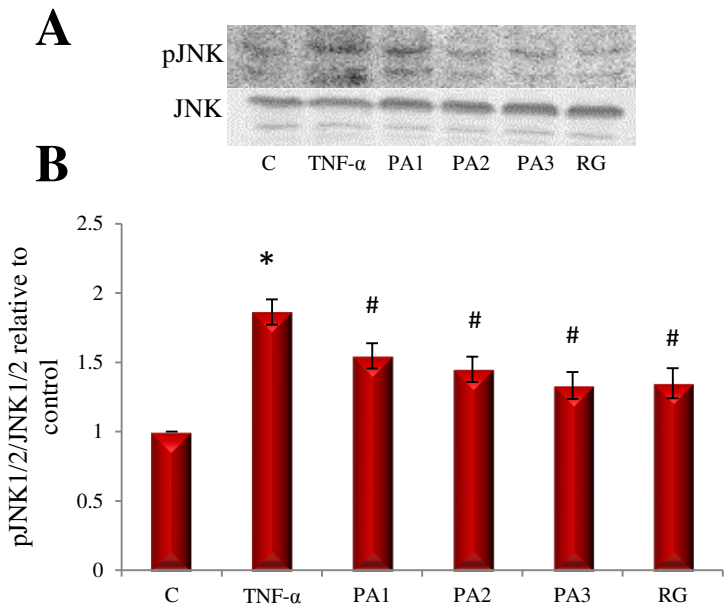


Figure 4.4. Levels of pJNK in various experimental groups: A) Immunoblot analysis of pJNK **B)** Densitometic analysis of pJNK with respect of total JNK. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5μM, PA2- TNF-α + punicic acid 10μM, PA3- TNF-α + punicic acid 30μM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *$p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

4.3.5. PA decreased TNF-α induced serine phosphorylation of IRS1 in 3T3-L1 adipocytes

Immunoblot analysis showed that TNF-α treatment significantly increased ($p \leq 0.05$) the level of serine phosphorylated IRS1 with respect to the total IRS1 level in 3T3-L1 adipocytes (increased by 1.80 fold compared with control group). Treatment with PA and RG significantly lowered ($p \leq 0.05$) serine phosphorylation of IRS1 by 1.13, 1.21, 1.28 and 1.31 fold for 5, 10 and 30 μM of PA and for 100 nM of RG respectively compared to TNF-α treated group, indicating protection from TNF-α induced IR (Figure 4.5 A, B).

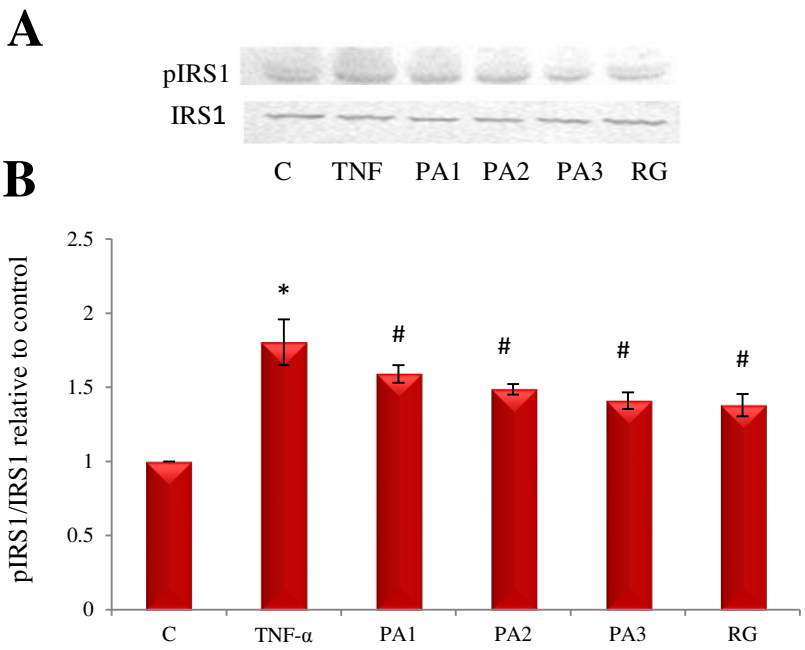


Figure 4.5. Levels of pIRS1(ser 307) in various experimental groups: A) Immunoblot analysis of pIRS1 **B)** Densitometic analysis of pIRS1 with respect of total IRS1. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *p ≤ 0.05 relative to control group; # p ≤ 0.05 relative to TNF-α treated group.

4.3.6. PA decreased TNF-α induced SOCS3 expression in 3T3-L1 adipocytes

SOCS3 is an important marker of IR. In TNF-α induced insulin resistant model, the mRNA level of SOCS3 was increased significantly ($p \leq 0.05$) by 2.21 fold compared to the control group and treatment with PA and RG significantly decreased ($p \leq 0.05$) the expression of SOCS3 in a dose dependent manner. Expression was decreased by 1.19, 1.32 and 1.23 fold for 10 and 30 µM of PA and for 100 nM of RG respectively compared to TNF-α treated group (Figure 4.6).

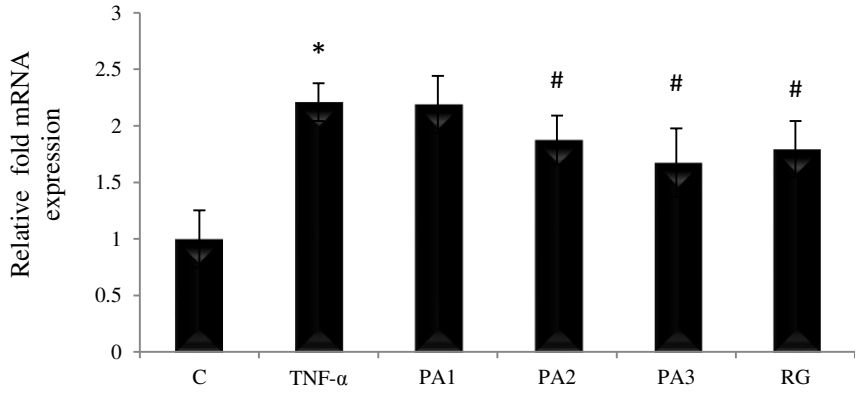


Figure 4.6. Levels of SOCS3 mRNA expression in various experimental groups: C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5μM, PA2- TNF-α + punicic acid 10μM, PA3- TNF-α + punicic acid 30μM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *p ≤ 0.05 relative to control group; # p ≤ 0.05 relative to TNF-α treated group.

4.3.7. PA decreased TNF-α induced PTP1B expression in 3T3-L1 adipocytes

The level of PTP1B was determined using indirect ELISA method. In TNF-α induced insulin resistant model, the level of PTP1B was increased significantly ($p \leq 0.05$) by 1.30 fold compared to the control group; indicating the induction of IR. On treatment with PA and RG the PTP1B levels were decreased significantly ($p \leq 0.05$) by 1.21, 1.35, 1.39 and 1.45 fold for 5, 10 and 30 μM of PA and for 100 nM of RG respectively compared to the TNF-α treated group, indicating an enhancement of insulin sensitivity (Figure 4.7).

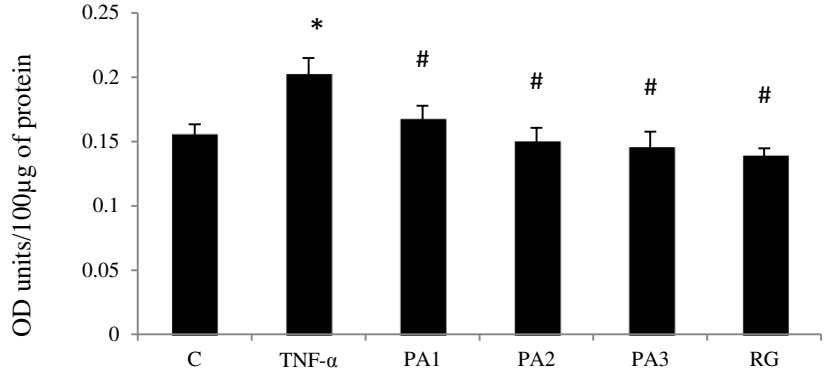


Figure 4.7. PTP1B protein expression in various experimental groups: C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 μM, PA2- TNF-α + punicic acid 10 μM, PA3- TNF-α + punicic acid 30 μM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *p ≤ 0.05 relative to control group; # p ≤ 0.05 relative to TNF-α treated group.

4.3.8. PA improved TNF-α induced alterations in secretion of inflammatory markers in 3T3-L1 adipocytes

TNF-α treatment induced the secretion of pro-inflammatory cytokines such as IL-6, MCP-1, IFN-γ and IL-1β significantly and treatment with PA and RG decreased TNF-α induced secretion of cytokines (Table 4.1). IL-6 level was increased significantly ($p \leq 0.05$) in TNF-α treated group compared to the control group (2.84 fold increase) and on treatment with PA, IL-6 levels were decreased significantly (1.15, 1.24, 1.55 and 1.23 for 5, 10 and 30 μM of PA and 100 nM of RG respectively compared with the TNF-α treated group; $p \leq 0.05$). In TNF-α treated group the levels of the inflammatory cytokine MCP-1 was increased significantly ($p \leq 0.05$; 2.18 fold increase compared with the control group). On treatment with PA the levels of MCP-1 was reduced significantly ($p \leq 0.05$) by 1.57, 1.94 and 2.41 fold respectively for 5, 10 and 30 μM of PA. In RG treated group also MCP-1 level was reduced by 1.53 fold compared to TNF-α treated group. The level of IFN-γ was increased significantly by 3.82 fold compared with the control group ($p \leq 0.05$) and on treatment with PA it was reduced in a dose dependent manner (IFN-γ levels were reduced significantly by 1.14, 1.28, 1.89 and 1.19 fold compared to the TNF-α treated group for 5, 10 and 30 μM of PA and 100 nM of RG respectively; $p \leq 0.05$). In TNF-α treated group, the level of IL-1β was increased significantly ($p \leq 0.05$; 3.87 fold increase compared to the control group). On treatment with 5, 10 and 30 μM of PA and 100 nM of RG the level of IL-1β was reduced significantly ($p \leq 0.05$) by 2.13, 2.58, 3.45 and 3.07 fold compared to the TNF-α treated group.

	IL-6 (pg/mL)	MCP-1 (pg/mL)	IFN-γ (pg/mL)	IL-1β (pg/mL)
Control Mean± SD	66.83 ± 3.25	69.41 ± 2.78	27.70 ± 2.30	33.33 ± 2.08
TNF-α Mean± SD	190.16 ± 3.29*	151.87 ± 3.45*	105.89 ± 3.96*	129.0 ± 2.64*
PA1 Mean± SD	164.66 ± 3.40#	95.65 ± 3.83#	92.45 ± 2.75#	60.33 ± 3.51#
PA2 Mean± SD	153.0 ± 3.27#	78.07 ± 3.04#	82.37 ± 2.05#	50.0 ± 3.6#
PA3 Mean± SD	122.50 ± 2.78#	62.78 ± 3.0 #	55.86 ± 2.87#	37.33 ± 2.88#
RG Mean± SD	153.00 ± 2.29#	98.62 ± 3.39#	37.33 ± 2.88#	42.0 ± 2.64#

Table 4.1. Concentration of inflammatory cytokines in various experimental groups: C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 μM, PA2- TNF-α + punicic acid 10 μM, PA3- TNF-α + punicic acid 30 μM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *$p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

4.3.9. PA improved PPARγ expression in TNF-α induced insulin resistant model of 3T3-L1 adipocytes

Western blot and qRT-PCR analysis showed that TNF-α induced IR caused a reduction in the expression of PPARγ in 3T3-L1 adipocytes. The mRNA expression of PPARγ was reduced significantly ($p \leq 0.05$) by 1.76 fold in TNF-α treated group compared to the control group and in PA and RG treated group, PPARγ mRNA expression was increased significantly ($p \leq 0.05$) by 1.31, 1.42 and 1.52 fold for 10 and 30 μM of PA and 100 nM of RG respectively compared to TNF-α treated group (Figure 4.8 A). Western blot analysis showed that the protein level expression of PPARγ was reduced significantly ($p \leq 0.05$) by 1.82 fold in TNF-α treated group compared to the control group and on treatment with PA and RG, the expression was increased significantly ($p \leq 0.05$) by 1.17, 1.33, 1.55 and 1.63 fold for 5, 10 and 30 μM of PA and 100 nM of RG respectively compared to TNF-α treated group (Figure 4.8 B, C).

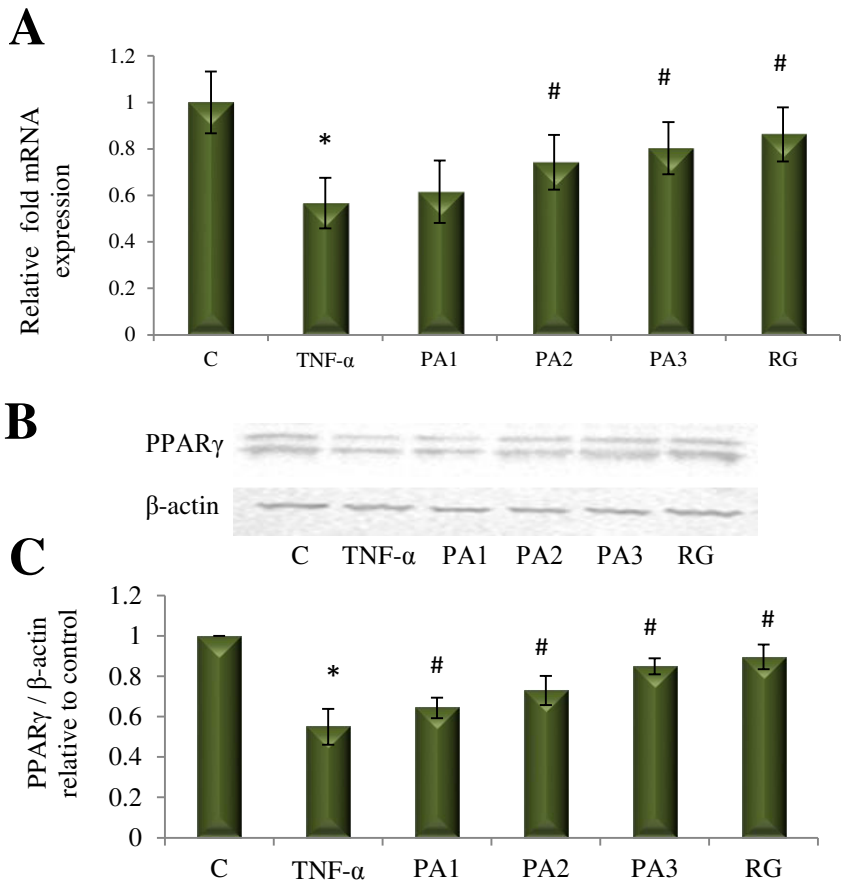


Figure 4.8. mRNA and protein levels of PPARγ in various experimental groups: A) mRNA expression of PPARγ **B)** Immunoblot analysis of PPARγ **C)** Densitometric analysis of protein expression of PPARγ with respect to β-actin. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *$p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

4.3.10. PA improved GLUT4 expression in TNF-α induced insulin resistant model of 3T3-L1 adipocytes

Western blot and qRT-PCR analysis showed that TNF-α induced IR caused a down regulation in the expression of GLUT4 in 3T3-L1 adipocytes. The mRNA expression of GLUT4 was reduced significantly ($p \leq 0.05$) to 2.07 fold in TNF-α treated group compared to the control group and on treatment with PA and RG, GLUT4 mRNA expression was increased significantly ($p \leq 0.05$) by 1.31, 1.47, 1.60 and 1.66 fold for 5, 10 and 30 µM of PA and 100 nM of RG respectively compared to TNF-α treated group (Figure 4.9 A). Western blot analysis showed that the protein level expression of GLUT4 was reduced significantly by 1.94 fold in TNF-α treated group compared to the control group and on treatment with PA and RG, the expression

was increased significantly ($p \leq 0.05$) by 1.22, 1.40, 1.63 and 1.69 fold for 5, 10 and 30 µM of PA and 100 nM of RG respectively compared to TNF-α treated group (Figure 4.9 B, C).

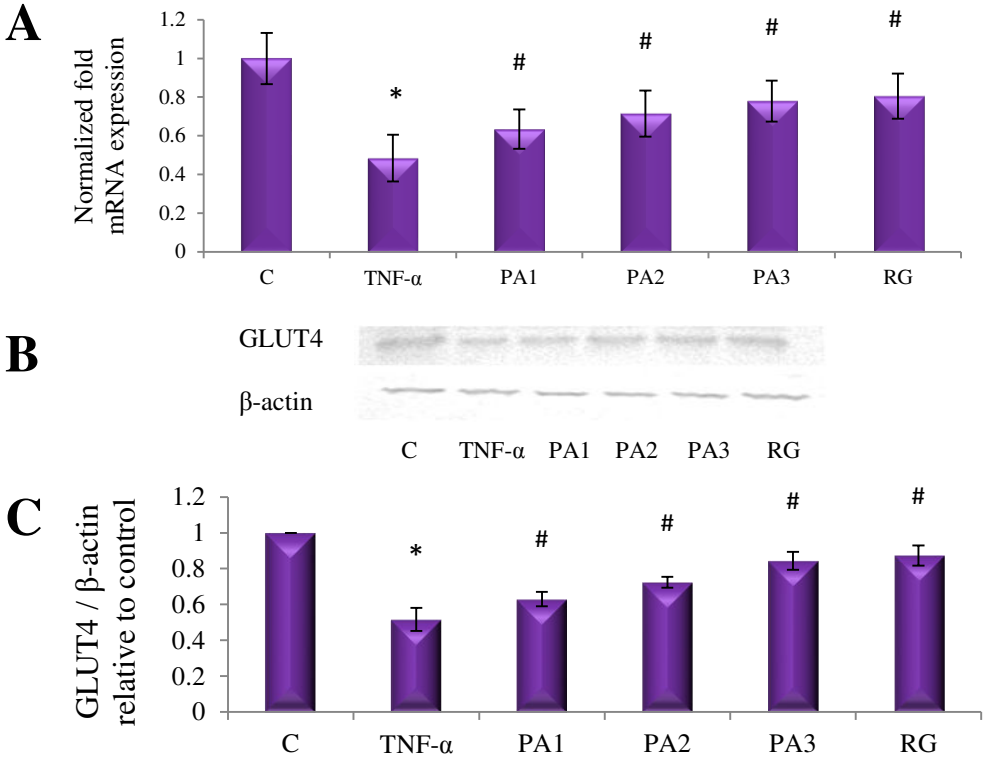


Figure 4.9. mRNA and protein levels of GLUT4 in various experimental groups: A) mRNA expression of GLUT4 **B)** Immunoblot analysis of GLUT4 **C)** Densitometric analysis of protein expression of GLUT4 with respect to β-actin. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5µM, PA2- TNF-α + punicic acid 10µM, PA3- TNF-α + punicic acid 30µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). *$p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group.

4.4. Discussion

Among the different kinds of adipokines secreted by adipocytes, TNF-α is the first one to connect obesity, inflammation and IR (Chen et al., 2010). This is also highly expressed in the adipose tissue of obese subjects having T2DM and mice lacking the expression of TNF-α or its receptor displayed a protection against the development of IR (Hotamisligil et al., 1995; Uysal et al., 1997). Administration or over expression of TNF-α in cultured cells or animal models resulted in a decreased insulin sensitivity (Hotamisligil et al., 1996; Shibasaki et al., 2003). These suggest that TNF-α over expression in adipocytes significantly contribute to the progression of IR. In this regard, we used TNF-α to induce IR and inflammation in 3T3-L1 adipocytes.

The present chapter demonstrated the protective effect of PA on various inflammatory genes and negative regulators of insulin sensitivity in TNF-α induced insulin resistant model of 3T3-L1 adipocytes.

TNF-α induced IR is associated with the activation of several signaling pathways involved in inflammation, like mitogen-activated protein kinase (MAPK), activator protein 1 (AP1) (Lee et al., 2011). TNF-α causes a translocation of NF-κB to the nucleus under the influence of JNK and MAPK, which may cause an increase in the secretion of various pro-inflammatory cytokines (Chuang et al., 2011). The secreted cytokines has a negative regulation in insulin signaling through an endocrine or paracrine fashion (Greenberg and Obin, 2006; Trayhurn and Wood, 2004). The expression of the proinflammatory cytokin IL-6 is found to be increased in the fat cells of human insulin resistant subjects, and causes an inhibition in the expression of IRS1, GLUT4 and PPARγ (Rotter et al., 2003). MCP-1 is another important pro-inflammatory cytokine and the expression of MCP-1 is found to be elevated in *in vitro* insulin resistant models in 3T3-L1 adipocytes and also in *in vivo* obese models and inhibit several adipogenic genes like LPL, ap2, GLUT4, PPARγ etc. (Sartipy and Loskutoff, 2003). Also, the level of other pro-inflammatory cytokines IL-1β and IFN-γ is reported to be increased in IR (Lagathu et al., 2006). PPARγ has a key role in the regulation of systemic insulin action and is recently emerging as a major regulator in obesity associated inflammation (Sakamoto et al., 2014). PPARγ agonists can directly inhibit the phosphorylation of JNK and it is relevant to the hypoglycaemic properties of PPARγ agonists (Diaz-Delfin et al., 2007). Also, the synthetic PPARγ agonist rosiglitazone is reported to inhibit JNK phosphorylation in cerebral ischemia (Okami et al., 2013).

Our results showed that in TNF-α induced insulin resistant model of 3T3-L1 adipocytes, there is increased pJNK level, NF-κB translocation to the nucleus and increased secretion of various proinflammatory cytokines, indicating the activation of inflammatory pathway. The levels of these inflammatory mediators are found to be inhibited significantly on co-treatment with the partial PPARγ agonist PA, indicating an improvement in inflammatory state, which can contribute to the enhancement of insulin sensitivity.

Phosphorylation of IRS1 at certain serine residues, S307 and S612 has been reported to inhibit insulin signaling (Jiang et al., 2004). The inhibitory phosphorylation at serine residues of IRS1 will inhibit the interaction between IR and IRS1 and promotes the degradation of IRS1 (Tanti and Jager, 2009). In consistent with these data, our results showed that TNF-α treatment caused increased phosphorylation of JNK and S307 of IRS1, leading to an impairement in insulin signaling. TZDs like RG is reported to inhibit the serine phosphorylation of IRS1 *in vitro* and *in vivo* and potentiate insulin signaling (Jiang et al., 2004). Our result showed that PA dose dependently decreased the serine phosphorylation of IRS1 in TNF-α treated groups and thereby increased insulin signaling and sensitivity.

SOCS3 plays an important role in the pathogenesis of IR and may be considered as a good target for the therapeutic interventions in IR. The expression of SOCS3 is found to be augmented in IR. In 3T3-L1 cells, over expression of SOCS3 is found to downregulate glucose uptake and in the muscles and lungs of db/db and LPS injected mice, SOCS3 expression is found elevated. Also, the knockdown of SOCS3 effectively ameliorated IFNγ mediated degradation of IRS1 in 3T3-L1 adipocytes (Wada et al., 2011). Overexpression of SOCS3 reduces AMPK activation, tyrosine phosphorylation of IRS1, PI3K activity, and AKT phosphorylation (Yang et al., 2012).

A PPARγ agonist pioglitazone reduced the expression of SOCS3 mRNA in adipose tissue of db/db and high fat fed mice which is associated with increased expression of PPARγ, increased signal transducer and activator of transcription 3 (STAT3) phosphorylation and adiponectin production, thus leading to improvement in whole body insulin sensitivity (Kanatani et al., 2007).

PTP1B is an intracellular enzyme involved in the negative regulation of insulin signaling (Ahmad et al., 1995; Elchebly et al., 1999). PTP1B causes a decline in insulin signaling by dephosphorylating tyrosine residue on IRS1 (Fernández-Veledo et al., 2006; Zabolotny et al., 2008). PTP-1B null mice are reported to have increased sensitivity to insulin and resistance to diet induced obesity (Elchebly et al., 1999). Reduction of PTP1B expression using PTP1B antisense oligonucleotide caused increased insulin sensitivity in ob/ob mice (Gum et al., 2003). In consistent with these data our results showed that TNF-α treatment caused a significant increase in the protein level expression of pJNK, serine phosphorylated (307) IRS1, PTP1B and

SOCS3 mRNA, leading to decreased glucose uptake and IR. Co-treatment with PA or RG showed significant decrease in the expression of all these markers of IR in a dose dependent manner, improving insulin sensitivity. Also, there is a decrease in the expression of mRNA and protein levels of GLUT4 and PPARγ in TNF-α treated group, causing impairment in insulin signaling leading to decreased glucose uptake. PA and RG treatment increased the mRNA and protein level of both GLUT4 and PPARγ significantly, improving insulin sensitivity.

Based on the result of the various studies related to partial PPARγ activity, insulin sensitizing property of PA and amelioration of IR induced by TNF-α in adipocytes, it is scientifically possible to consider PA as a potential nutraceutical against IR and associated complications. So, it was our interest to see how does it behave with oxidative stress, mitochondrial dysfunction and ER stress in adipocyte which are playing significant role in the genesis of IR and MS. The details of the studies related to these areas are given in the next chapter (chapter 5).

Chapter 5

Punicic acid safeguards mitochondria and ER function during insulin resistance by TNF-α in 3T3-L1 adipocytes

5.1. Introduction

Mitochondria in recent years have been gaining more importance in obesity and IR since IR and T2DM are associated with impaired mitochondrial function (Kim et al., 2008). Mitochondria has been considered as a global regulator of energy homeostasis and factors like nutrient excess, inflammation, oxidative stress, ER stress etc. lead to mitochondrial dysfunctions and accelerate development of IR (Kusminski and Scherer, 2012). Impairment in mitochondrial ATP production, respiration rate, membrane potential and increased mitochondrial ROS production are the hallmarks of mitochondrial dysfunctions (Brand and Nicholls, 2011). The key regulatory molecules modulating mitochondrial biogenesis like PGC-1α and NRFs are decreased in IR (Hao et al., 2010). In addition, TNF-α induced IR is associated with altered mitochondrial dynamics in cultured adipocytes as well as in muscle cells (Hahn et al., 2014; Chen et al., 2010; Jheng et al., 2012).

Obesity related IR has been linked to the development of oxidative stress. Also in obesity and IR, there are organelle dysfunctions including ER stress (Ozcan and Tabas, 2012). Inflammation and oxidative stress are two major contributors in the pathogenesis of IR. TNF-α induced IR is associated with oxidative stress in adipocytes and TNF-α can induce oxidative stress and the same can lead to the development of ER stress and unfolded protein response (Xue et al., 2005). Thus oxidative stress and ER stress can be considered as closely interlinked pathological events in IR. Oxidative protein folding taking place in the ER lumen is a source of ROS production and accumulated ROS in ER will deplete the reduced glutathione level in ER, further aggravating the oxidative stress (Riemer et al., 2009). It was reported that UPR, which is the indicative of ER stress was activated in IR and obesity in experimental animals (Hotamisligil, 2008). The development of ER stress during obesity and IR was more prominent in adipose tissue which further contributes to its dysfunction.

There is an innate antioxidant system in our body to cope up with the development of reactive oxygen species. These act as the first line of antioxidant

defence in our body. These include enzymatic as well as non enzymatic antioxidant defence systems. The antioxidant enzymes include SOD, CAT, GPx etc. (Matough et al., 2012). SOD exists in three isoforms and is involved in the conversion of superoxide radicals into oxygen and hydrogen peroxides (H_2O_2). GPx helps in the removal of H_2O_2 at the expense of GSH and oxidize it to oxidized glutathione (GSSG). GSH is the most important non enzymatic antioxidant in the living cell. It is a tripeptide and is mainly found in the cytoplasm of the cell and directly scavenges free radicals and it acts as a cofactor for many antioxidant enzymes including glutathione-s-transferase. GR helps in the regeneration of GSH from GSSG using NADPH as the reducing agent. CAT is mainly located in peroxisomes and also found in cytosol and helps in the conversion of H_2O_2 to H_2O and molecular oxygen (Gomes et al., 2012; Ursini et al., 1995; Weydert and Cullen, 2010). The levels of theses enzymes and the non-enzymatic antioxidant GSH are found reduced in oxidative stress as well as in MS (Tangvarasittichai, 2015).

In oxidative stress the levels of oxidized proteins, lipids and DNA are increased. Protein carbonyls are an important indicator of oxidized protein content in the cell. Protein carbonyl levels are found to have a positive correlation with IR and is found to be increased in obesity (Ruskovska and Bernlohr, 2013). Thiobarbituric acid reacting species (TBARs) generated as a result of oxidative stress to lipid moieties in the cell, is also an indication of oxidative damage to the cells.

Since mitochondrial function also appears as a critical point in the management of insulin sensitivity, improving mitochondrial function by various naturally occurring partial PPARγ agonists could be used as a strategy for improving IR. In this scenario the present study is aimed to analyze the effect of PA on oxidative stress, mitochondrial dysfunction and associated ER stress in TNF-α induced insulin resistant model of 3T3-L1 adipocytes to extrapolate the role of PPARγ in improving mitochondrial and ER function. Based on the results of this chapter it appears that PA alleviates oxidative stress, ER stress and mitochondrial dysfunction in 3T3-L1 adipocytes, making it a preferably new bioactive for the management of MS.

5.2. Methods

Experiments were conducted to see the effect of PA on mitochondrial and ER dysfunctions in TNF-α induced insulin resistant model of 3T3-L1 adipocytes. The detailed procedures of all the methods are given in chapter 2.

The methodologies in the chapter include,

- Detection of intracellular reactive oxygen species (ROS)
- Mitochondrial content measurement and mitochondrial biogenesis assay
- Activity of mitochondrial biogenesis markers by qRT-PCR
- Analysis of mitochondrial membrane potential by JC-1 staining
- O_2 consumption and ATP production assay
- Determination of aconitase activity
- Analysis of expression of mitochondrial fission-fusion proteins by western blotting
- Determination of intracellular Ca^{2+} homeostasis.
- Analysis of expression of ER stress markers

5.3. Results

5.3.1. PA ameliorated intra cellular ROS generation in TNF-α induced insulin resistant model

TNF-α treatment significantly increased the ROS production in the cells (2.18 fold increase than the control group; $p \leq 0.05$) and it was significantly reduced in PA treated groups (1.47, 1.64 and 2.12 fold reduction compared with the TNF-α treated group for 5, 10 and 30 μM of PA; $p \leq 0.05$). On treatment with RG also ROS accumulation was reduced significantly (1.33 fold reduction compared with TNF-α treated group; $p \leq 0.05$) (Figure 5.1 A, B).

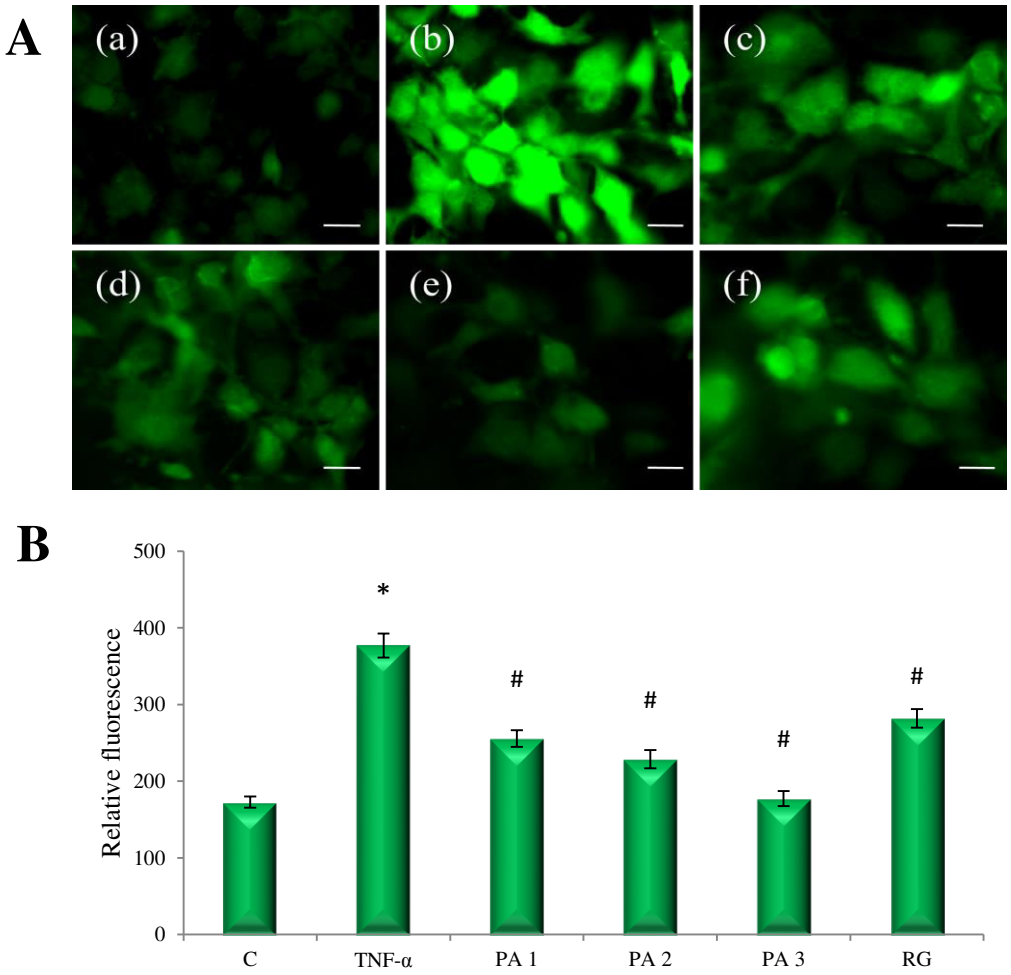


Figure 5.1. Intracellular ROS generation in various experimental groups: A) ROS accumulation in various groups determined by DCFH-DA staining; the representative images of ROS-induced fluorescence. (a) control, (b)TNF-α treated group, (c) TNF-α + punicic acid 5 µM, (d) TNF-α + punicic acid 10 µM, (e) TNF-α + punicic acid 30 µM, (f) TNF-α + rosiglitazone 100 nM. Scale bar corresponds to 100 µm. **B)** Relative fluorescent intensity of the fluorescent images**.** C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

5.3.2. PA ameliorated mitochondrial superoxide production

There was an increased generation of mitochondrial superoxide radicals in TNF-α induced insulin resistant model (significantly increased; $p \leq 0.05$) of 3T3-L1 cells compared to control cells (3.01 fold increase) while PA and RG treatment significantly reduced ($p \leq 0.05$) the generation of superoxide radicals by 1.13, 1.35, 1.57 and 1.43 fold for 5, 10 and 30 µM of PA and for 100 nM RG respectively compared to the TNF-α treated group (Figure 5.2 A, B).

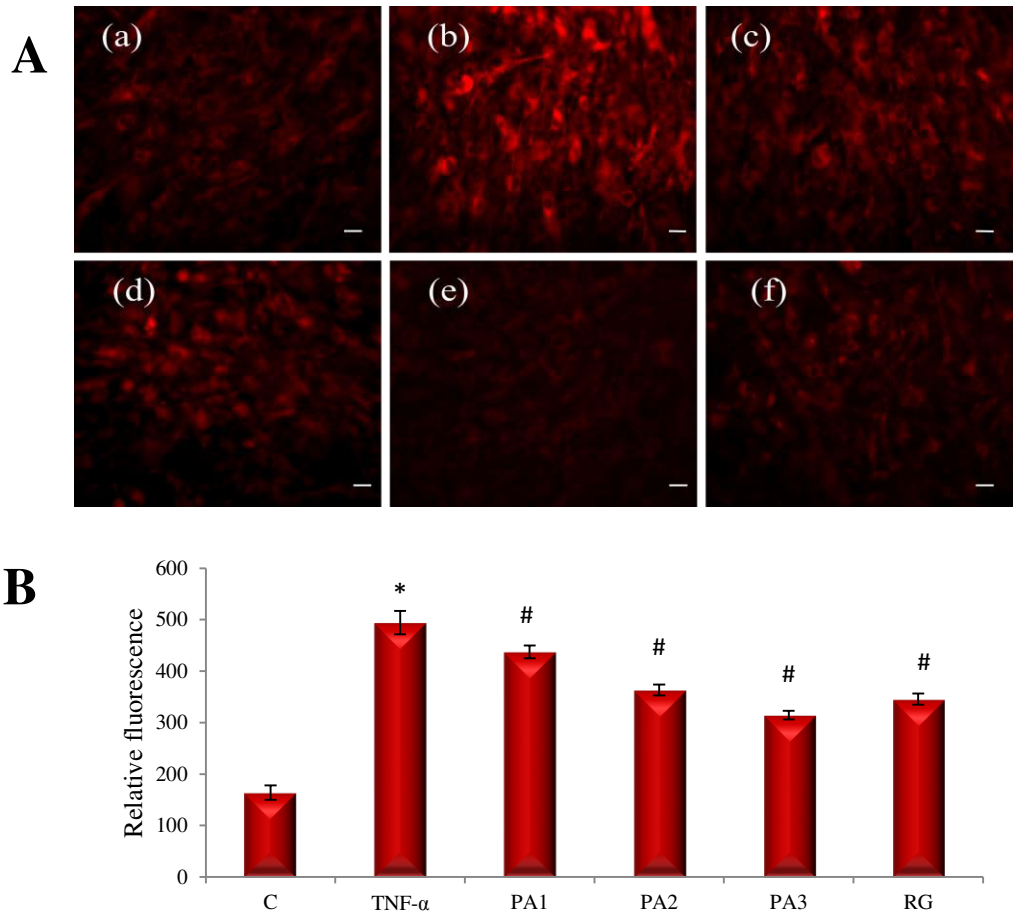


Figure 5.2. Mitochondrial superoxide generation in various experimental groups determined using mitoSOX: A) Representative images of mitochondrial superoxide generation in control and treated cells. (a) control, (b)TNF-α treated group, (c) TNF-α + punicic acid 5 µM, (d) TNF-α + punicic acid 10 µM, (e) TNF-α + punicic acid 30 µM, (f) TNF-α + rosiglitazone 100 nM. Scale bar corresponds to 100 µm. **B)** Relative fluorescent intensity of the fluorescent images**.** C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

5.3.3. PA improved aconitase activity in 3T3-L1 adipocytes

TNF-α treatment caused a significant reduction in aconitase activity (decreased by 1.88 fold compared with control group; $p \leq 0.05$) which was restored significantly on PA treatment (increased to 1.40, 1.55 and 1.60 fold compared with TNF-α treated group; $p \leq 0.05$). RG treatment also significantly restored the aconitase activity (increased by 1.76 fold compared with TNF-α treated group; $p \leq 0.05$; Figure 5.3).

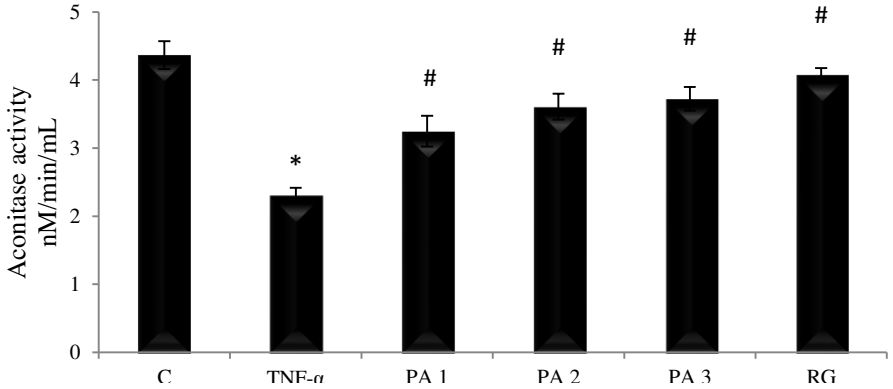


Figure 5.3. Aconitase activity in various groups: C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5μM, PA2- TNF-α + punicic acid 10 μM, PA3- TNF-α + punicic acid 30 μM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group.

5.3.4. PA improved endogeneous antioxidant status in different experimental groups

Evaluation of antioxidant status of the cells by studying the levels of various parameters like TBARS, antioxidant enzymes, GSH, protein carbonyls and trolox equivalent total antioxidant content of the cells provide an indication of the oxidative damage in TNF-α induced insulin resistant model of 3T3-L1 cells (Table 5.1). TNF-α treatment increased lipid peroxidation (MDA level) by 2.01 fold compared to the control group ($p \leq 0.05$). Treatment with PA significantly decreased the MDA levels by 1.24, 1.65 and 2.01 fold for 5, 10 and 30μM of PA respectively compared to the TNF-α treated group, indicating a protection from oxidative stress ($p \leq 0.05$; Table 5.1). TNF-α treatment also significantly increased protein carbonyl levels (increased by 2.43 fold compared to the control cells; $p \leq 0.05$). PA treatment significantly decreased the protein carbonyl levels significantly (decreased by 1.54, 1.77 and 1.97 fold for 5, 10 and 30μM of PA respectively compared to the TNF-α alone treated group; $p \leq 0.05$). RG treatment also decreased the MDA and protein carbonyl level significantly ($p \leq 0.05$; 1.59 and 1.46 fold reduction respectively compared to TNF-α treated group; Table 5.1).

TNF-α treatment also decreased the GSH content significantly by 1.88 fold compared to the control group ($p \leq 0.05$). On treatment with PA, the GSH levels were increased significantly by 1.18, 1.28 and 1.49 fold respectively for 5, 10 and 30μM of PA compared to the TNF-α treated group ($p \leq 0.05$). On treatment with RG also GSH

levels were increased significantly ($p \leq 0.05$) by 1.29 fold compared to control (Table 5.1).

TNF-α treatment decreased the levels of various antioxidant enzymes. The levels of the antioxidant enzymes SOD, CAT, GR and GPx were reduced significantly ($p \leq 0.05$) by 1.72, 2.12, 2.28 and 2.14 fold respectively compared with the control group (Table 5.1). PA treatment significantly improved the levels of the antioxidant enzymes in a dose dependent manner ($p \leq 0.05$). Level of SOD was increased by 1.17, 1.40, 1.57 and 1.48 fold with 5, 10 and 30μM of PA and 100 nM of RG respectively. Catalase activity was increased by 1.28, 1.64, 1.70 and 1.52 fold compared to TNF-α treated group with 5, 10 and 30 μM of PA and 100 nM of RG respectively. On treatment with 10 and 30μM of PA and 100nM of RG, GR activity was increased by 1.41, 2.03 and 1.63 fold compared to the TNF-α treated group. GPx activity was increased by 1.14, 1.18 and 1.44 fold compared to the TNF-α treated group for 10 and 30μM of PA and 100nM of RG respectively. Also, there was a significant reduction ($p \leq 0.05$) in total antioxidant content of the cell in TNF-α treated group (reduced by 1.83 fold compared to the control). PA and RG treatment improved the total antioxidant level significantly ($p \leq 0.05$) by 1.13, 1.35, 1.5 and 1.09 fold for 5, 10 and 30 μM of PA and for 100 nM RG respectively compared to TNF-α treated group (Table 5.1). The analysis of the antioxidant status of the cell on treatment with TNF-α and PA or RG indicates that TNF-α induced IR in 3T3-L1 adipocytes is associated with a reduction in endogeneous antioxidant status of the cell and PA and RG can improve the antioxidant status and thus can ameliorate oxidative damage to the cell.

Parameters	Control Mean ±SD	TNF-α Mean ±SD	PA1 Mean ±SD	PA2 Mean ±SD	PA3 Mean ±SD	RG Mean ±SD
TBARS (nM MDA/mg protein)	31.13 ± 0.72	62.59 ± 0.82*	50.35 ± 1.01#	37.92 ±.038#	31.08 ± 0.64#	39.20 ± 0.95#
Proteincarbonyls (nmol/ml)	2.59 ± 0.11	6.29 ± 0.25*	4.06 ± 0.34#	3.54 ± 0.23#	3.18 ± 0.23#	4.29 ± 0.22#
SOD (Unit/mg protein)	4.75 ± 0.12	2.76 ± 0.14*	3.21 ± 0.09#	3.86 ± 0.09#	4.34 ±0.09#	4.07 ± 0.09#
Catalase (µmoles of H_2O_2 decomposed/min/mg protein)	5.63 ± 0.28	2.66 ± 0.28*	3.56 ± 0.26#	4.42 ± 0.19#	4.58 ± 0.28#	4.11 ± 0.19#
GPx (Unit/mg protein)	0.22 ± 0.007	0.10 ± 0.005*	0.11 ± 0.006	0.12 ± 0.008#	0.19 ± 0.009#	0.05 ± 0.009#
GR (Unit/mg protein)	3.04 ± 0.22	1.33 ± 0.30*	1.41 ± 0.12	1.88 ± 0.28#	2.70 ± 0.27#	2.17 ± 0.12#
GSH (nM/mg protein)	6.72 ± 0.16	3.57 ± 0.10*	4.22 ± 0.11#	4.57 ± 0.11#	5.35 ± 0.25#	4.59 ± 0.16#
Total antioxidant activity (mM)	0.118 ±.002	0.064 ± .002*	0.073 ±.004#	0.087 ± .003#	0.096 ±.002#	0.070 ± .006#

Table 5.1. Endogenous antioxidant status in various experimental groups. TBARS, Thiobarbituric acid-reactive substances; MDA, Malondialdehyde; SOD, Superoxide dismutase; GPx, Glutathione peroxidase; GSH, Reduced glutathione; GR, Glutathione reductase. Each value represents mean ± SD (n=6). * indicates values are significantly different from control cells ($P \leq 0.05$) and # indicates values are significantly different from TNF-α treated cells ($P \leq 0.05$).

5.3.5. Effect of PA on TNF-α induced alterations in mitochondrial transmembrane potential (Δψm) in 3T3-L1 adipocytes

Alterations in mitochondrial transmembrane potential were assessed using JC-1. In TNF-α treated cells, JC-1 green monomers were significantly increased ($p \leq 0.05$) compared to the control (3.71 fold increase in green monomers) indicating a decrease in Δψm. Treatment with PA reduced the alterations in Δψm and the green fluorescence was decreased significantly ($p \leq 0.05$) by 2.32, 3.23, 3.55 and 2.84 fold with 5, 10 and 30 µM of PA and 100nM of RG respectively compared with the TNF-α treated group (Figure 5.4 A, B). In valinomycin treated group, the green monomers were increased significantly (4.49 fold increase compared to the control group; $p \leq 0.05$).

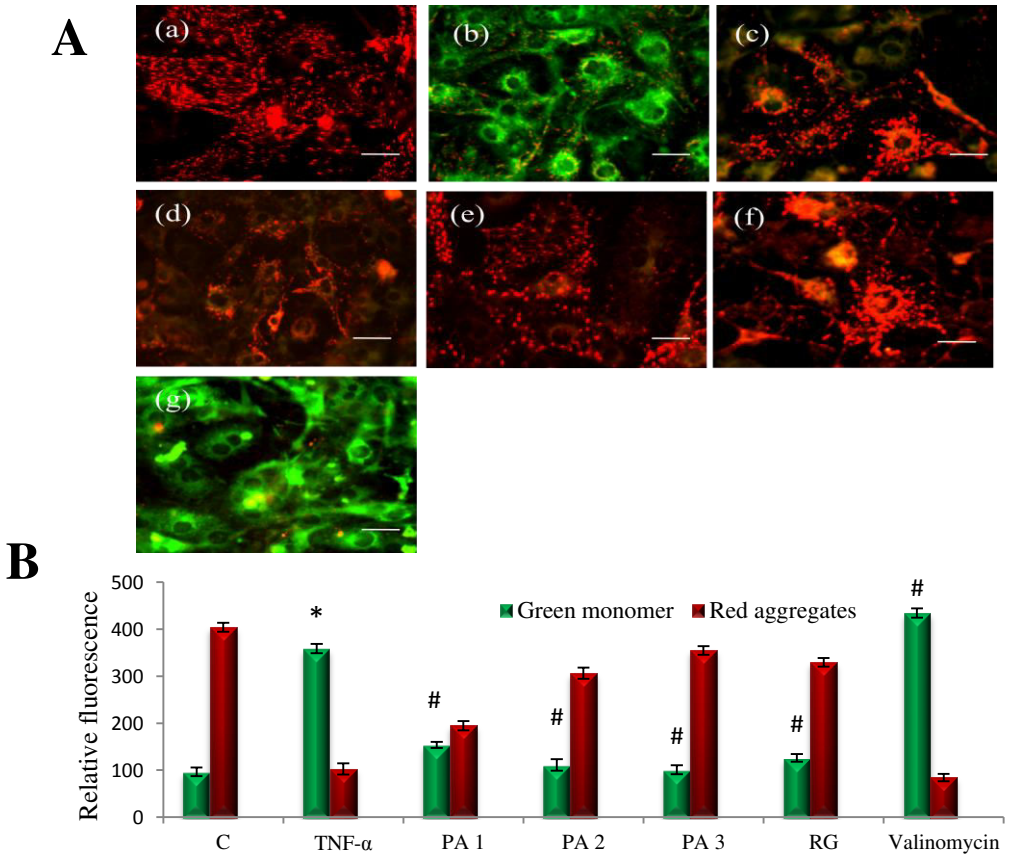


Figure 5.4. Mitochondrial transmembrane potential changes in control and TNF-α treated groups determined by JC-1 staining: A) Fluorescent microscopic merged images of 3T3-L1 cells; (a) control, (b) TNF-α treated group, (c) TNF-α + punicic acid 5µM, (d) TNF-α + punicic acid 10µM, (e) TNF-α + punicic acid 30µM, (f) TNF-α + rosiglitazone 100nM, (g) Valinomycin (1 µg/mL). Scale bar corresponds to 100 µm. **B)** Relative intensities of green and red fluorescence. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5µM, PA2- TNF-α + punicic acid 10µM, PA3- TNF-α + punicic acid 30µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

5.3.6 Effect of PA on TNF-α induced alteration in mitochondrial content in TNF-α treated 3T3-L1 adipocytes

Treatment of TNF-α caused a reduction in mitochondrial content and mitochondrial biogenesis. Mitochondrial content was assessed using mitotracker red. TNF-α caused a significant reduction in mitochondrial content as compared with the control (2.47 fold reduction; $p \leq 0.05$). In PA treated cells, mitochondrial density was restored significantly in a dose dependent manner compared with the TNF-α group (1.21, 1.87 and 2.08 fold increase with 5, 10 and 30 µM of PA respectively than the TNF-α treated group; $p \leq 0.05$). RG treatment also restored mitochondrial content significantly (2.21 fold increase compared with TNF-α alone treated group; $p \leq 0.05$; Figure 5.5 A,B).

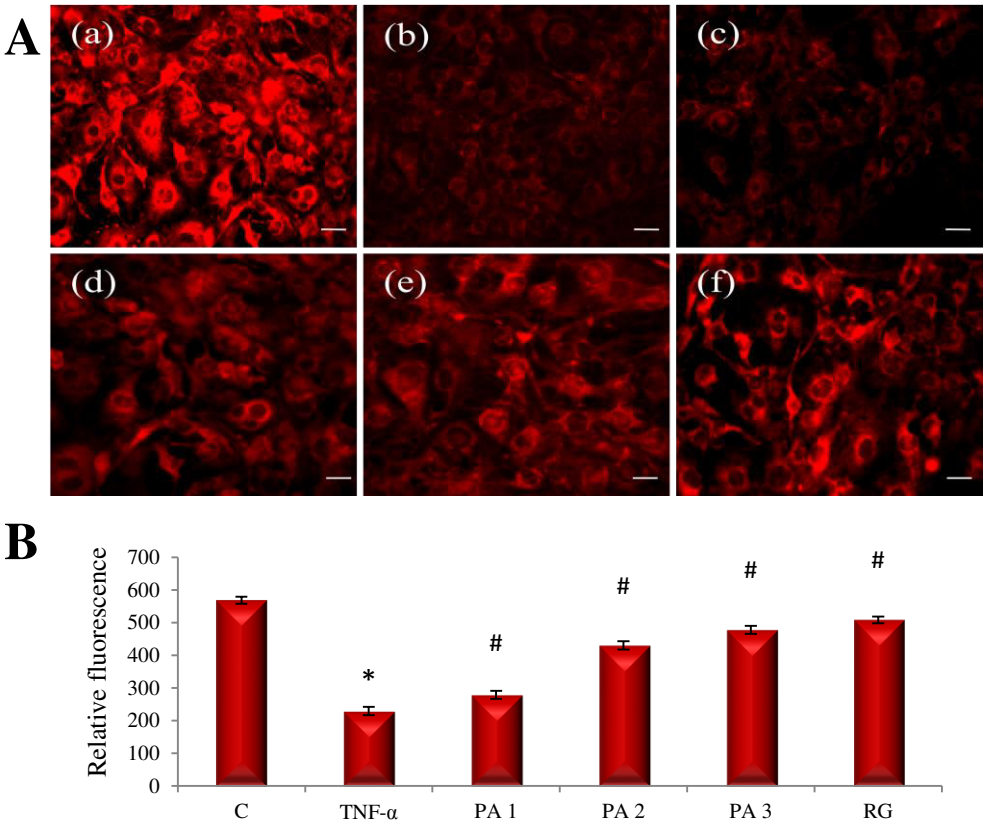


Figure 5.5. Mitochondrial content in control and treated groups: A) Fluorescent microscopic images of 3T3-L1 cells stained with mitotracker red. (a) control, (b) TNF-α treated group, (c) TNF-α + punicic acid 5 µM, (d) TNF-α + punicic acid 10 µM, (e) TNF-α + punicic acid 30 µM, (f) TNF-α+ rosiglitazone 100 nM. Scale bar corresponds to 100 µm. **(b)** Relative fluorescence intensity of mitochondrial content. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group.

5.3.7 Effect of PA on TNF-α induced alterations in O_2 consumption and ATP production in TNF-α treated 3T3-L1 adipocytes

ATP content in the cells was analyzed using commercially available kit (Molecular probes, USA). In insulin resistant model the ATP content was reduced significantly (2.48 fold compared with the control group; $p \leq 0.05$). Treatment with different doses of PA or RG increased the ATP content significantly (1.08, 1.23, 1.60 and 1.83 fold increase for 5, 10 and 30 μM of PA and 100 nM RG respectively; $p \leq 0.05$) as compared with the insulin resistant group (Figure 5.6 A). In oligomycin treated group ATP content was reduced significantly (5.34 fold compared with the control group; $p \leq 0.05$).

O_2 consumption was analyzed by using Cayman's O_2 consumption assay kit. The kit utilizes a phosphorescent probe mitoExpress whose fluorescence decreases with increase in O_2 content. The oxygen consumption rate was calculated from the change in mitoExpress probe signal over time. In TNF-α treated cells, the oxygen consumption rate was decreased significantly with respect to the control (3.35 fold decrease; $p \leq 0.05$). Antimycin A, which was used as a positive control of mitochondrial dysfunction also significantly reduced O_2 consumption in the cells ($p \leq 0.05$). PA treatment significantly increased ($p \leq 0.05$) O_2 consumption rate in TNF-α treated cells in a dose dependent manner (1.35, 1.82 and 2.26 fold increase compared with TNF-α group for 5, 10 and 30 μM for PA) and RG caused 2.41 fold increase compared with TNF-α group (Figure 5.6 B).

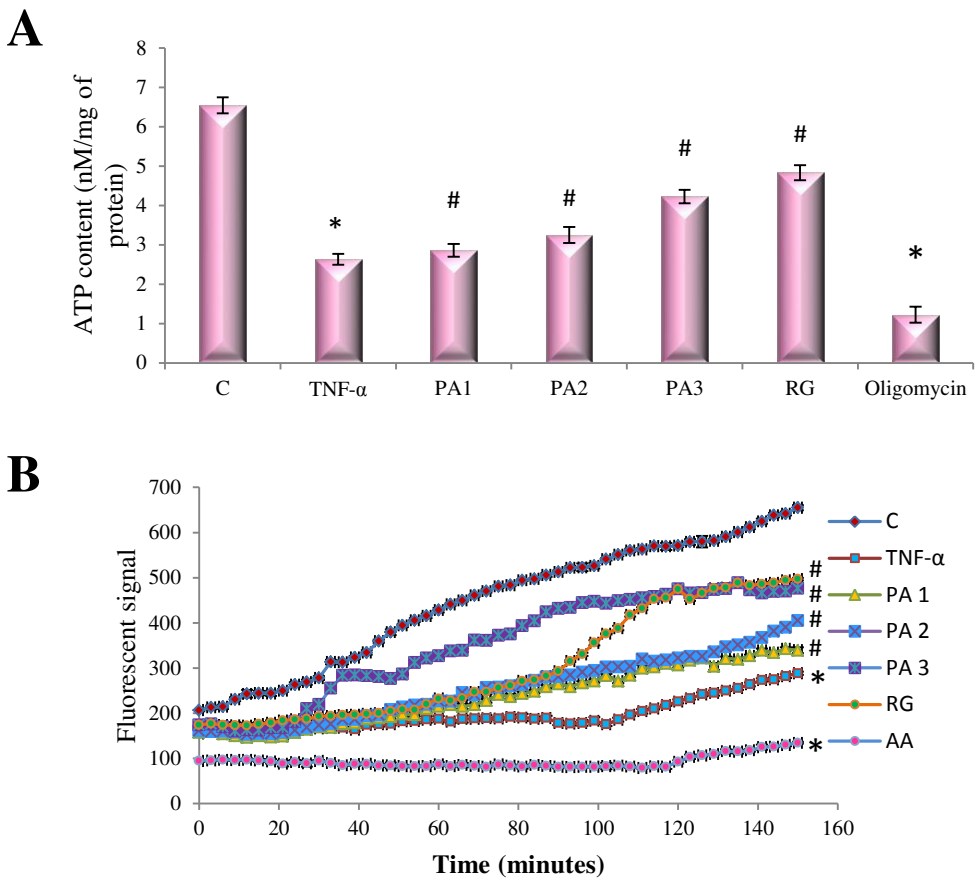


Figure 5.6. ATP production and O_2 consumption in various groups: A) ATP content in various groups. C-control, TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100nM, Oligomycin- Oligomycin 8 µM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group. **B)** Changes in oxygen consumption in various groups. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM, AA-Antimycin A. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

5.3.8. Effect of PA on TNF-α induced alterations in gene expression of PGC-1α, NRF1, Tfam, mtDNA copy number and mitochondrial biogenesis in TNF-α treated 3T3-L1 adipocytes

The expression of genes associated with mitochondrial biogenesis like PGC-1α, NRF1 and Tfam were done by RT-PCR. The results showed that TNF-α treatment caused a significant reduction in the expression of the genes ($p \leq 0.05$) and treatment with PA or RG significantly restored the gene expressions ($p \leq 0.05$; Figure 5.7 A). In TNF-α treated cells, the expression of PGC-1α, NRF1 and Tfam were decreased by

6.20, 4.06 and 3.27 fold compared to the control group. On treatment with PA or RG the expression levels of these genes were increased significantly compared to the TNF-α treated group (for PGC-1α, the expression was increased by 1.58, 2.70, 3.65 and 4.57 fold; for NRF1, the expression was increased by 2.07, 2.71, 3.00 and 3.10 fold and with Tfam, the expression was increased by 1.42, 2.11, 2.44 and 2.68 fold with 5, 10 and 30 μM of PA and 100 nM of RG respectively). qRT-PCR data also showed that TNF-α treatment caused a significant reduction in the expression of mtDNA copy number (decreased by 4.68 fold compared to the control group; $p \leq 0.05$) and on treatment with PA or RG the expression level of mtDNA was restored significantly ($p \leq 0.05$) by 1.22, 1.43, 2.16 and 3.25 fold compared with TNF-α treated group for 5, 10 and 30 μM of PA and 100 nM of RG respectively (Figure 5.7 B).

Effect of PA on TNF-α induced alteration in mitochondrial biogenesis was studied by using Abcam MitoBiogenesis In-Cell ELISA kit. The kit utilize the measurement of subunit I of complex IV (COX-1) which is mitochondrial DNA-encoded, and subunit of complex II (SDH-A) which is nuclear DNA-encoded and used as the control. The results of mitochondrial biogenesis studies revealed that TNF-α caused a significant reduction in mtDNA-encoded COX-1 expression (1.67 fold decrease compared with the control group; $p \leq 0.05$), and PA and RG treatment significantly increased the expression ($p \leq 0.05$) by 1.29, 1.43, 1.51 and 1.53 fold compared with TNF-α treated group for 5, 10 and 30 μM of PA and 100 nM of RG respectively (Figure 5.7 C).

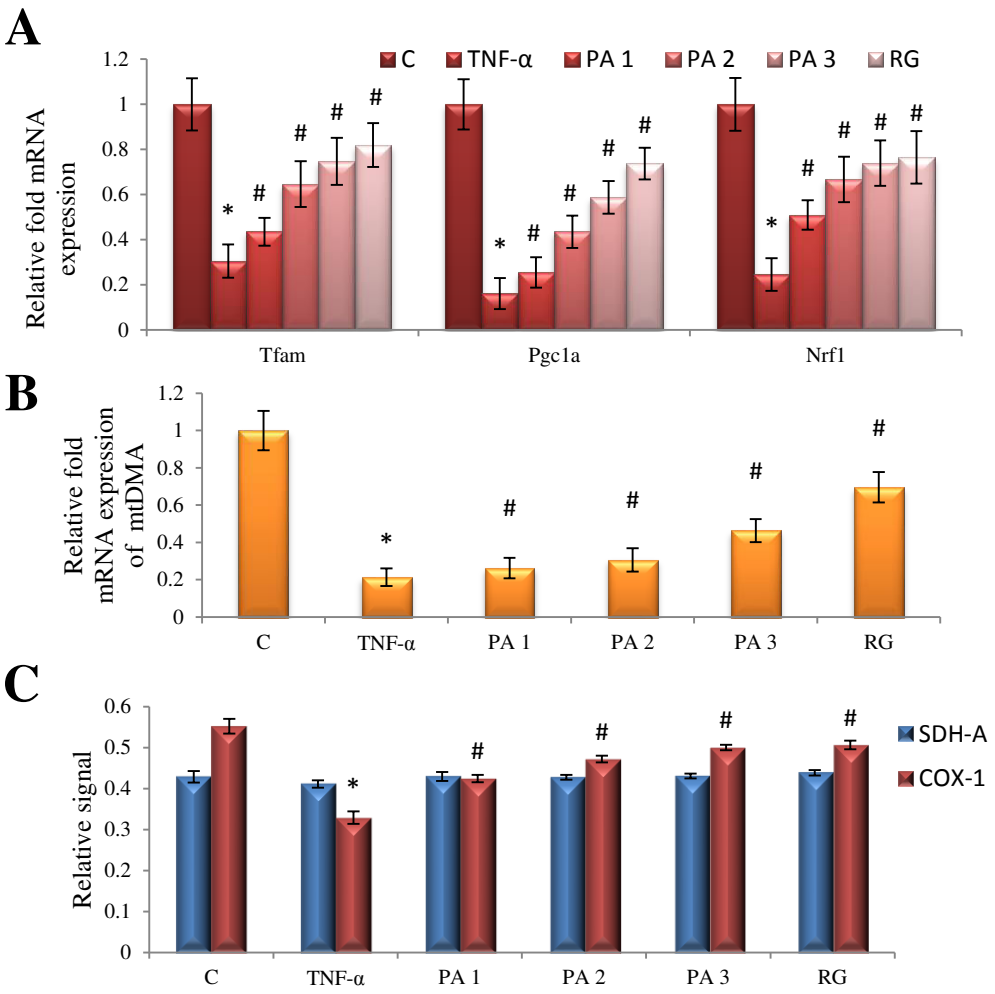


Figure 5.7. Expression of mitochondrial biogenesis markers in various experimental groups: A) Relative mRNA level of genes Tfam, PGC-1α, NRF1 and mtDNA. **B)** mitochondrial DNA copy number determined by qRT-PCR. **C)** Mitochondrial biogenesis represented by the activity of COX-1 and SDH-A. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Data are expressed as the mean ± SD, n = 3. * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

5.3.9. Effect of PA on the expression of mitochondrial fission-fusion proteins in insulin resistant model of 3T3-L1 adipocytes

Western blot analysis showed that the expression of mitochondrial fission protein FIS1 was increased and the expression of mitochondrial fusion protein OPA1 was decreased in TNF-α treated group. The expression of OPA1 (a mitochondrial fusion marker protein) was decreased significantly ($p \leq 0.05$) in TNF-α group compared to the control by 1.62 fold (Figure 5.8 A, B) and on treatment with PA and RG, the expression was increased significantly by 1.16, 1.19, 1.32 and 1.40 fold ($p \leq 0.05$) compared to the TNF-α treated group for 5, 10 and 30 µM of PA and 100 nM of

RG respectively. The expression of FIS1 was increased significantly ($p \leq 0.05$) by 1.50 fold in TNF-α treated group and on treatment with PA and RG, the expression was reduced significantly ($p \leq 0.05$) by 1.15, 1.23, 1.32 and 1.33 fold compared to the TNF-α treated group for 5, 10 and 30 µM of PA and 100 nM of RG respectively. But the expression levels of the fusion protein MFN2 and fission protein DRP1 were not significantly changed. (Figure 5.8 A, B).

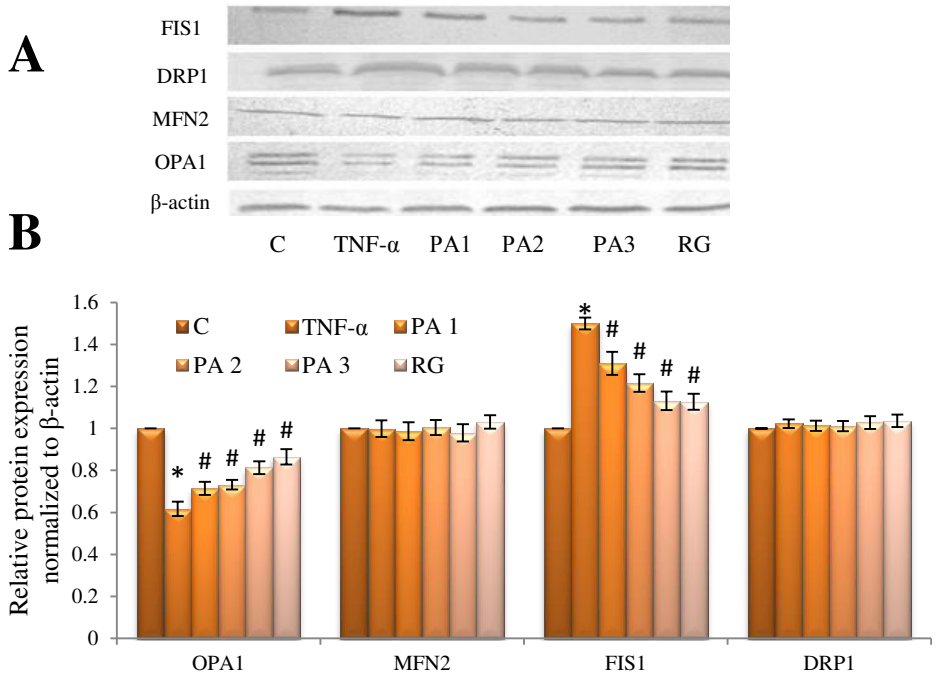


Figure 5.8. Effect of punicic acid on TNF-α induced alterations in mitochondrial dynamics in 3T3-L1 adipocytes: A) Representative immunoblot of FIS1, DRP1, MFN2 and OPA1. **B)** Densitometric quantification of western blot of protein level normalised to β-actin. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Data are expressed as the mean ± SD (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α treated group.

5.3.10. Effect of PA on expression of ER stress markers in TNF-α induced insulin resistant model in 3T3-L1 adipocytes

Effect of TNF-α treatment on the expression of ER stress markers were studied by qRT-PCR and immunoblotting. TNF-α significantly ($p \leq 0.05$) increased expression of ER chaperon Grp78/BiP mRNA (increased by 3.80 fold compared to the control group), and treatment with PA and RG significantly reduced ($p \leq 0.05$) the mRNA expression level of Grp78/BiP (decreased by 1.61, 1.90, 2.01 and 1.78 fold compared to the TNF-α treated group with 5, 10 and 30 µM of PA and 100 nM of RG respectively; Figure 5.9 A). Immunoblot analysis of Grp78/BiP also showed a

significant increase in the expression of the protein in TNF-α treated group (increased by 2.43 fold compared to the control group; $p \leq 0.05$) and on treatment with PA or RG the expression level of Grp78/BiP protein was reduced significantly ($p \leq 0.05$) by 1.92, 2.02, 2.25 and 2.11 fold compared to the TNF-α treated group with 5, 10 and 30 µM of PA and 100 nM of RG respectively; Figure 5.9 B, C).

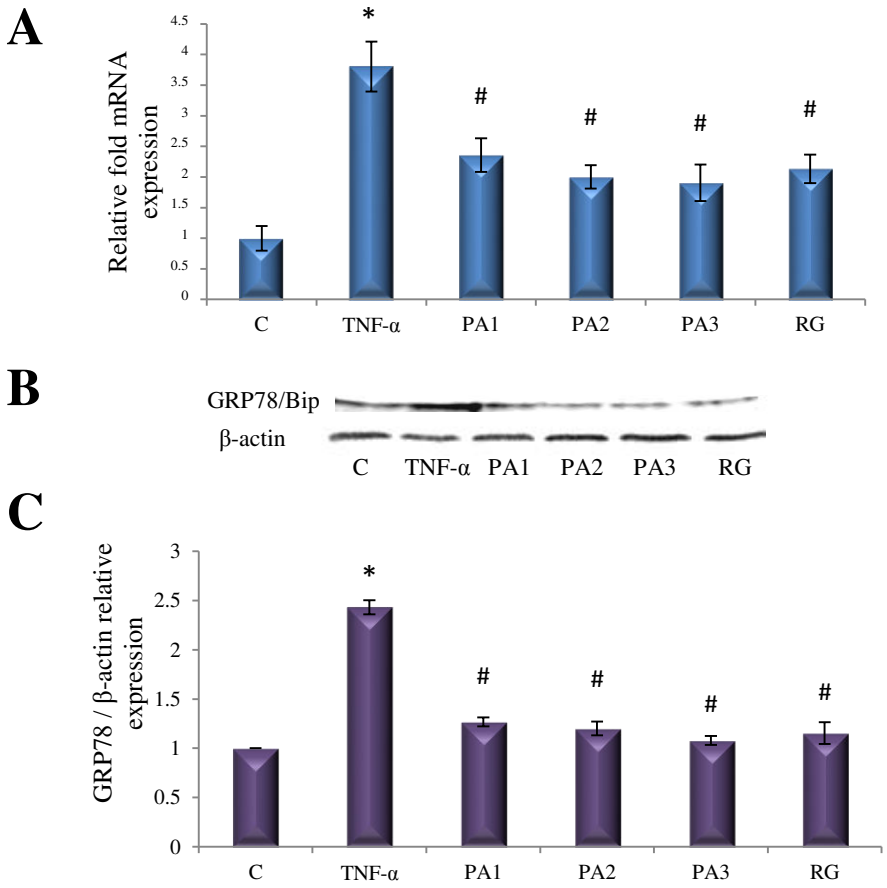


Figure 5.9. The expression of Grp78/BiP in control and treated groups: A) Relative mRNA expression of Grp-78 normalized to β-actin **B)** The immunoblot analysis of GRP78/BiP **C)** Densitometic analysis of GRP78 normalized to β-actin. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α +punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α+rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group.

Also, our western blot analysis showed that the expression of another ER stress marker ERO1-Lα was found to be increased significantly (increased by 2.03 fold; $p \leq 0.05$) in TNF-α treated group compared to the control group and on treatment with PA or RG, the expression of ERO1-Lα was found to be reduced in a dose dependent manner (decreased by 1.12, 1.22, 1.28 and 1.31 fold compared to the TNF-α treated

group with 5, 10 and 30 µM of PA and 100 nM of RG respectively; $p \leq 0.05$; Figure 5.10 A, B).

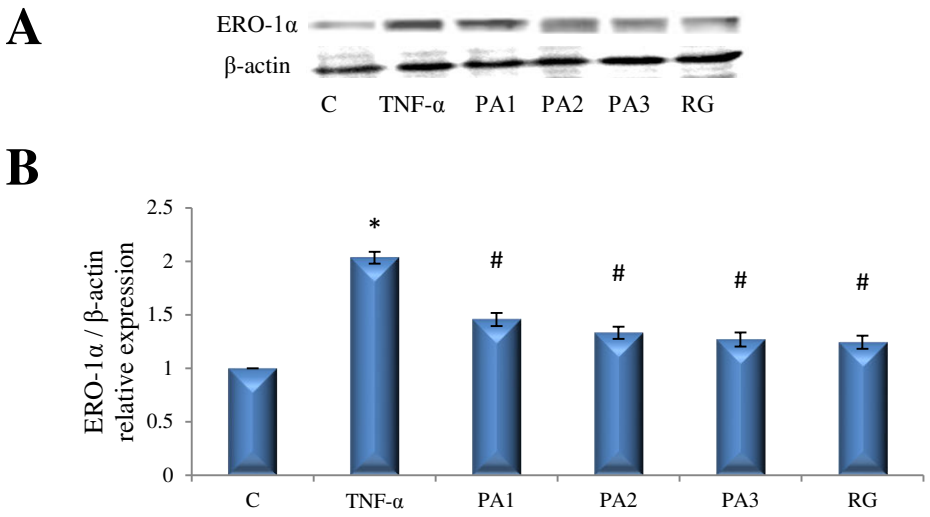


Figure 5.10 The expression of ERO-1α in control and treated groups: A) The immunoblot analysis of ERO-1α **B)** Densitometic analysis of ERO-1α normalized to β-actin. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α +punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group.

TNF-α treatment also caused a marked elevation in the expression of UPR sensors. The expression of UPR sensors IRE-1α, PERK and ATF6 were increased significantly ($p \leq 0.05$) by 2.49, 2.64 and 2.53 fold, compared to the control group, and PA or RG treatment significantly decreased ($p \leq 0.05$) the expression of these proteins. ATF6 level was decreased by 1.86, 2.03, 2.17 and 2.10 fold, IRE-1α level was decreased by 1.85, 2.05, 2.18 and 2.38 fold and PERK level was decreased by 1.83, 1.98, 2.09 and 2.20 fold compared to the TNF-α treated group for 5, 10 and 30 µM of PA and 100 nM of RG respectively (Figure 5.11 A, B).

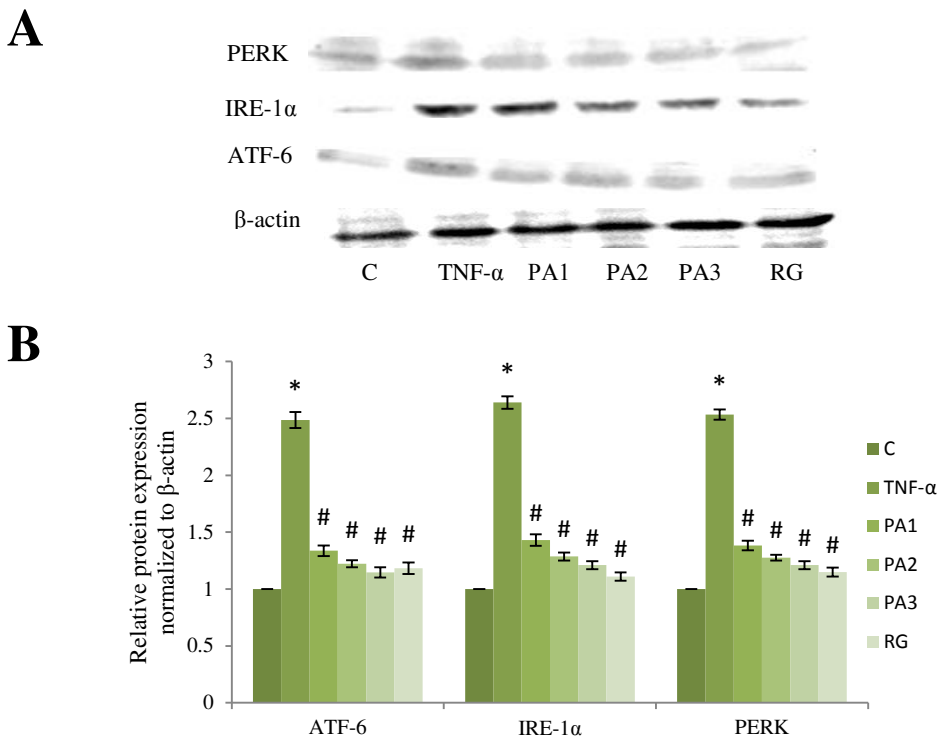


Figure 5.11. Immunoblot analysis of unfolded protein response markers in control and TNF-α treated cells: A) The immunoblot of PERK, IRE-1α and ATF-6 **B)** Densitometic analysis of protein level of PERK, IRE-1α and ATF-6 normalized to β-actin. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α +punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α+rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group.

CHOP is an important protein involved in ER stress mediated apoptosis pathway. Our qRT-PCR analysis showed that TNF-α treatment caused a significant increase (increased by 4.23 fold; $p \leq 0.05$) in the expression of CHOP mRNA compared to the control group, and on treatment with PA and RG, the expression of CHOP was decreased significantly ($p \leq 0.05$) by 1.23, 1.50, 1.69 and 1.68 fold compared to the TNF-α treated group for 5, 10 and 30 µM of PA and 100 nM of RG respectively (Figure 5.12 A). Also, immunoblot analysis showed that TNF-α treatment caused a significant increase in the expression of the protein ($p \leq 0.05$) by 3.76 fold compared to the control group and on treatment with PA and RG, the expression of CHOP was reduced significantly ($p \leq 0.05$) by 2.32, 2.60, 2.83 and 2.84 fold compared to the TNF-α treated group with 5, 10 and 30 µM of PA and 100 nM of RG respectively (Figure 5.12 B, C).

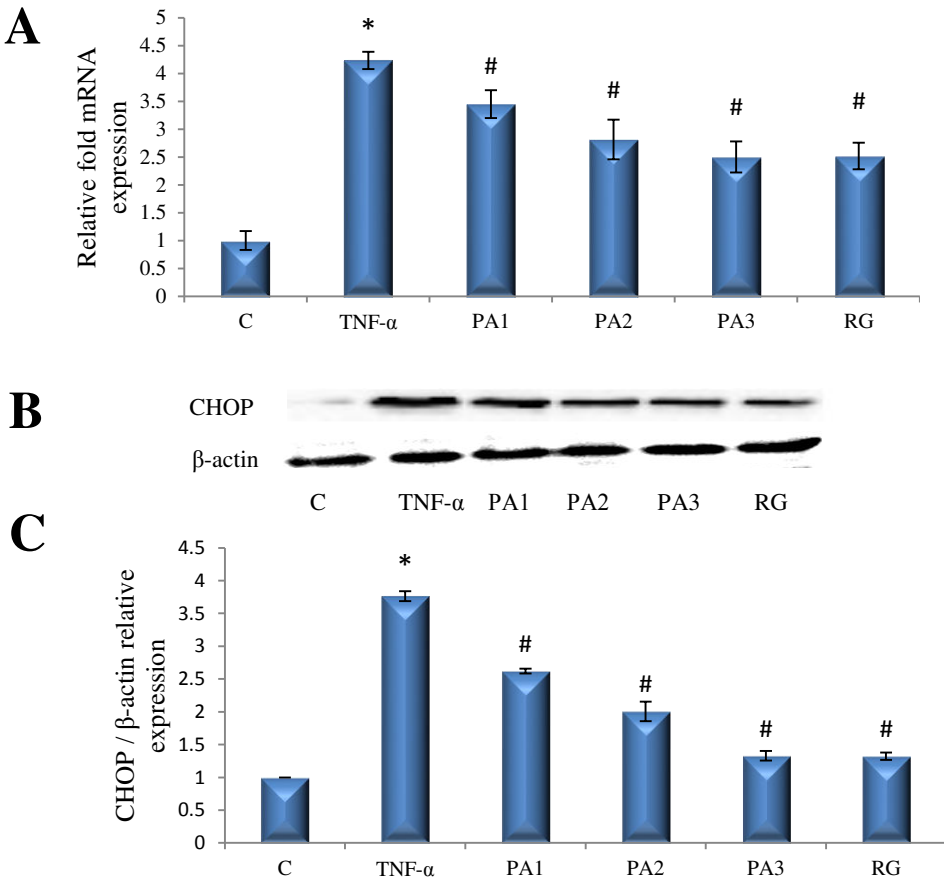


Figure 5.12. Protein and mRNA level expression of CHOP in control and treated cells: A) Relative mRNA expression of CHOP normalized to β-actin **B)** The immunoblot analysis of CHOP **C)** Densitometric analysis protein level of CHOP normalized to β-actin. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α +punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α+rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group.

5.3.11. Effect of PA on intracellular Ca^{2+} homeostasis in TNF-α induced insulin resistant model of 3T3-L1 adipocytes

TNF-α caused an increase in intracellular Ca^{2+} overload in 3T3-L1 adipocytes, which was evident from increased blue fluorescence of Fura-2AM compared to control group. In TNF-α induced insulin resistant model of 3T3-L1 adipocytes, the fluorescence was significantly increased ($p \leq 0.05$) by 2.34 fold compared to the control group. Treatment with different doses of PA and RG decreased the fluorescence compared to the TNF-α treated group. The fluorescence of PA and RG treated groups were decreased significantly ($p \leq 0.05$) by1.22, 1.65 and 1.49 fold for 10 and 30 µM of PA and 100 nM of RG respectively (Figure 5.13 A, B). The results suggest that PA

positively modulate Ca^{2+} homeostasis in TNF-α induced insulin resistant model of 3T3-L1 adipocytes.

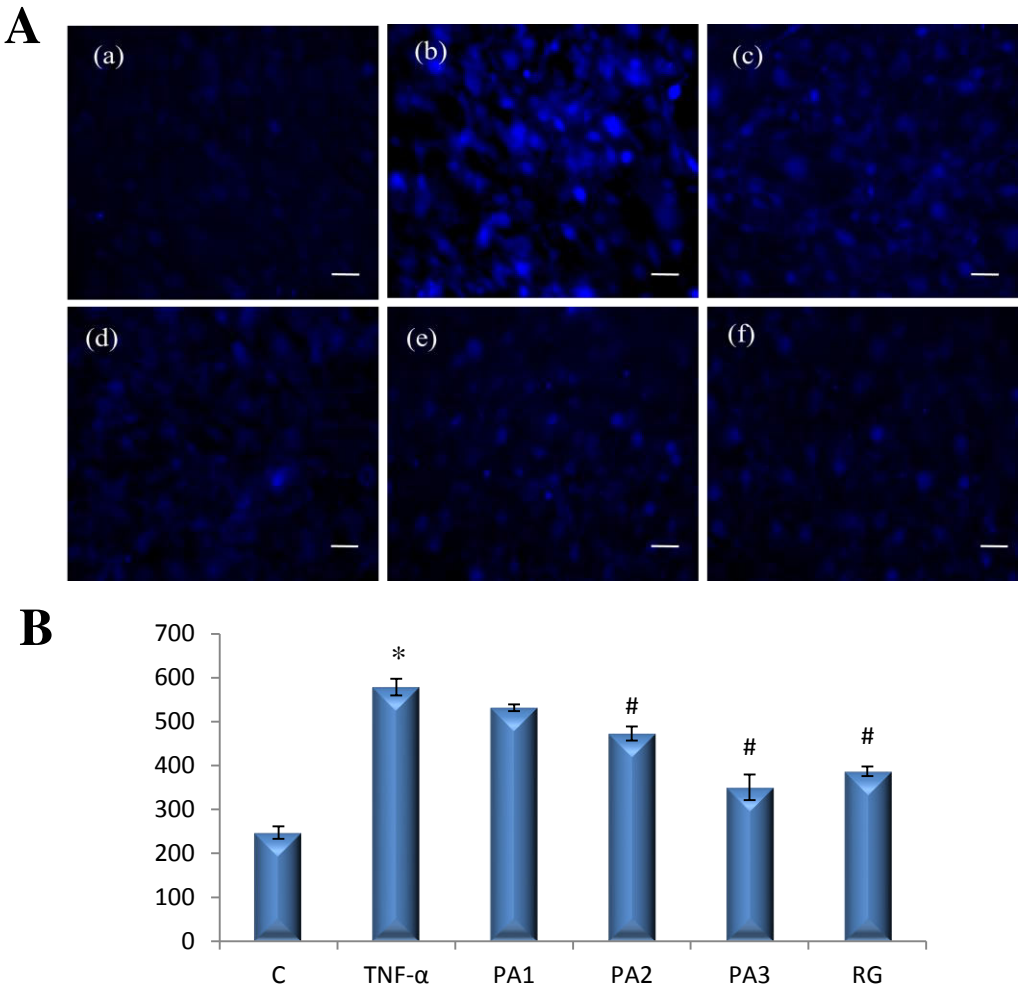


Figure 5.13. Intracellular Ca^{2+} overload in different experimental groups: (A) Representative fluorescent microscopic images of 3T3-L1 cells stained with Fura-2AM. (a) control, (b) TNF-α treated group, (c) TNF-α + punicic acid 5 µM, (d) TNF-α + punicic acid 10 µM, (e) TNF-α + punicic acid 30 µM, (f) TNF-α+ rosiglitazone 100 nM. Scale bar corresponds to 100 µm. **(B) Relative fluorescence intensity of Fura-2AM**. C-control, TNF-α- TNF-α treated group, PA1- TNF-α + punicic acid 5 µM, PA2- TNF-α + punicic acid 10 µM, PA3- TNF-α + punicic acid 30 µM, RG- TNF-α + rosiglitazone 100 nM. Values are means, with standard deviations represented by vertical bars (n=6). * $p \leq 0.05$ relative to control group; # $p \leq 0.05$ relative to TNF-α alone treated group.

5.4. Discussion

In this chapter, we studied the effect of PA on TNF-α induced oxidative stress, mitochondrial dysfunction and ER stress in 3T3-L1 adipocytes. Increased oxidative stress is considered as a major factor leading to the development of IR, pancreatic β-cell dysfunction, impaired glucose tolerance, and T2DM. Reactive oxygen species are associated with obesity related chronic inflammation, adiponectin reduction, and other

metabolic dysfunctions (Furukawa et al., 2004). TNF-α induced IR was found to be associated with increased ROS generation in 3T3-L1 adipocytes (Chen et al., 2010). Our study also demonstrated that treatment of 3T3-L1 adipocytes with 10 ng/mL of TNF-α for 24 hours caused a significant increase in overall ROS. PA and RG treatment significantly down regulated ROS level in TNF-α treated cells. Surplus ROS can also oxidize lipids, proteins and DNAs which are considered as important markers of oxidative damage. We observed an increase in lipid peroxidation and protein carbonyls in TNF-α treated cells and PA and RG treatment significantly reduced the level of oxidation of lipids and proteins, indicating a protection from oxidative stress.

The levels of various antioxidant enzymes like SOD, catalse, GR and GPx were found to be reduced in oxidative stress and associated metabolic complications, which may increase oxidative stress in the cell. GSH is one of the major non-enzymatic antioxidant and depletion of GSH leads to the impairment of cellular defence against ROS and may lead to oxidative injury. In the present study, we observed a decrease in the activity of this innate antioxidant system. PPARγ agonists are reported to have antioxidant potential and are reported to improve the expression of antioxidant enzymes (Polvani et al., 2012). On treatment with PA and RG the expression of antioxidant enzymes were increased, thus ameliorating oxidative stress in TNF-α induced insulin resistant model of 3T3-L1 adipocytes.

Mitochondrial dysfunction can contribute to the development of IR in skeletal muscles, liver, pancreas etc (Chen et al., 2010; Hoehn et al., 2009; Shibasaki et al., 2003). In insulin insensitivity, there is a decrease in mitochondrial O_2 consumption, ATP production, mitochondrial biogenesis and alterations in mitochondrial dynamics (Jheng et al., 2012; Kim et al., 2008; Kusminski and Scherer, 2012). Induction of insulin resistance by TNF-α in 3T3-L1 adipocytes caused an increase in overall cell ROS production and dissipation in mitochondrial transmembrane potential (Chen et al., 2010). Also, TNF-α treatment caused a reduction in oxidative phosphorylation and ATP production (Samavati et al., 2008). Mitochondrial oxidative stress can damage the activity of the Fe-S protein aconitase and the activity of aconitase is found to be reduced in high fat fed rat hearts (Relling et al., 2006; Sivitz and Yorek., 2010). In consistent with these reports, our results showed that TNF-α treatment caused significant alterations in mitochondrial transmembrane potential, reduction in ATP

production, O_2 consumption, increased mitochondrial superoxide generation, overall increase in cellular ROS generation and a decrease in aconitase activity. PPARγ plays an important role in energy metabolism because of its effects on mitochondrial function and ATP production (Landreth et al., 2008). Treatment with PA or RG significantly restored these parameters indicating improved mitochondrial function in PA or RG treated groups.

TNF-α treatment caused a reduction in the expression of genes associated with mitochondrial biogenesis and thus contributes to mitochondrial dysfunction (de Pauw et al., 2009). Mitochondrial biogenesis is regulated by various factors like PGC-1α, NRFs and Tfam (Liu et al., 2009). PPARγ agonists increase mitochondrial biogenesis in cultured adipocytes and also in diabetic models (Jeon et al., 2012; Joseph et al., 2012; Rong et al., 2007; Wilson-Fritch et al., 2003). In patients with T2D, pioglitazone treatment increased mitochondrial copy number and expression of factors involved in mitochondrial biogenesis like PGC-1α and Tfam and improves insulin sensitivity (Bogacka et al., 2005). Our results showed that in TNF-α induced insulin resistant model of 3T3-L1 adipocytes, there is a reduction in the expression of genes associated with mitochondrial biogenesis including Tfam, PGC-1α, NRF1 and mtDNA copy number. Also mtDNA-encoded COX-1 protein level was depleted in TNF-α treated group indicating a decline in mitochondrial biogenesis. In adipocytes and neuroblastoma PPARγ can activate the expression of PGC-1α and can promote mitochondrial biogenesis (Hondares et al., 2006; Miglio et al., 2009). Our results suggest that PA like RG increased the expression of PGC-1α in a PPARγ dependent way, which in turn activated the expression of Nrf1 and Tfam; mtDNA copy number and increased mitochondrial biogenesis, contributing partly to the enhancement of insulin sensitivity.

Mitochondria are dynamic organelle that undergoes continuous fission and fusion in a balanced way to maintain mitochondrial morphology and function (Chan, 2006). The rates of mitochondrial fission and fusion are finely regulated by the mitochondrial fusion proteins MFN1, MFN2, and OPA1 and by the mitochondrial fission proteins DRP1 and FIS1 (Belenguer and Pellegrini, 2013). OPA1 mediates the fusion of inner membrane and MFN1/2 mediates the fusion of outer mitochondrial membrane (Song et al., 2009).

A high mitochondrial fusion-fission ratio causes the development of elongated, interconnected mitochondria which are considered to be the functional mitochondria (Jheng et al., 2012). TNF-α treatment is reported to increase OPA1 cleavage and decreased the total cellular level of OPA1 in 3T3-L1 adipocytes (Hahn et al., 2014). Mouse embryonic fibroblasts lacking the expression of MFN1 and MFN2 have altered mitochondrial membrane potential and respiration rate (Chen et al, 2005). An increase in MFN2 levels in TNF-α treated 3T3-L1 adipocytes was also reported (Chen et al., 2010). Increased amount of fission proteins are reported in cardiovascular system in hyperglycaemic condition (Yu et al., 2008), in acute kidney injury (Brooks et al., 2009) and also in cardiac ischemia/reperfusion injury (Ong et al., 2010). Hahn et al. reported that TNF-α treatment did not affect the expression level of mitochondrial fission proteins DRP1, or mitofilin in adipocytes (Hahn et al., 2014). Our results clearly showed that TNF-α treatment increased the expression of mitochondrial fission protein FIS1 and decreased the expression of fusion protein OPA1, indicating a high fission-fusion ratio. But the level of fission protein DRP1 and fusion protein MFN2 were not changed upon TNF-α treatment.

A high mitochondrial fission-fusion ratio results in highly fragmented mitochondria, which are capable of producing more ROS and facilitate mitophagy (Archer, 2013). TNF-α treatment increased oxidative stress in 3T3-L1 adipocytes (Hahn et al., 2014) and mitochondrial oxidative stress can lead to an imbalance in mitochondrial fission-fusion protein levels leading to mitochondrial fragmentation (Wu et al., 2011). PPARγ and PPARα agonists are reported to protect neuronal mitochondria by modulating mitochondrial fission and fusion events by directly inducing the expression of several mitochondrial-related proteins like uncoupled protein (UCP), DRP1, MFN, Tfam etc. Additionally, the PPARs-mediated calcium balance also contributed to mitochondrial dynamics alterations (Zolezzi et al., 2013). Since altered mitochondrial fission or fusion is found to be associated with insulin resistance in tissues like adipocytes and skeletal muscles (Chen et al., 2010; Hahn et al., 2014; Jheng et al., 2012), improving mitochondrial dynamics can also contribute to the enhancement of insulin sensitivity. Our results showed that PPARγ agonists PA or RG treatment reduced the expression of the fission protein FIS1 and increased the

expression of fusion protein OPA1, in TNF-α treated 3T3-L1 adipocytes, normalizing the fission fusion ratio.

Mitochondria and ER are physically and functionally interconnected organelle and the alterations in their physiology and function during hyperglycemia and type 2 DM can be inter related. TNF-α induced oxidative stress and ER stress are important hallmarks in the pathophysiology of insulin resistance (Cawthorn and Sethi, 2008). Oxidative stress can augment the expression of ER stress genes and UPR. ER provides an unique oxidizing environment for protein folding which favours the formation of disulphide bonds (Malhotra and Kaufman, 2007). ROS production can interfere with protein folding by inactivation of PDI/ERO1 thiol-disulphide exchange reactions and/or by causing aberrant disulphide bond formation.

We found an increase in the expression of ERO-1α in insulin resistant model, which is an indication of ROS induced ER stress in TNF-α treated 3T3-L1 adipocytes. In addition, TNF-α treatment also augmented the expression of molecular chaperon GRP78 and UPR transducers, PERK, IRE1α and ATF-6. Also, the expression of CHOP, which is involved in ER stress mediated apoptosis, is also increased in TNF-α treated insulin resistant model. ROS can cause an efflux of Ca^{2+} ions from the ER into the cytoplasm. The increased concentrations of calcium in the cytoplasm may cause an entry of Ca^{2} into the nucleus as well as into the mitochondria which may increase the production of ROS, and the increased levels of ROS in the mitochondria may cause a further increase in Ca^{2+} leakage from the ER (Moserova and Kralova 2012). In TNF-α induced insulin resistant model, the level of intracellular Ca^{2+} level was also found to be increased.

PPARγ agonists are known to have inhibitory effect on ER stress. In diabetic mice, pioglitazone, a PPARγ agonist is known to enhance insulin release from islets cells by alleviating ER stress (Evans-Molina et al., 2009). Pioglitazone is also found to reduce ER stress in the liver of diabetic mice (Yoshiuchi et al., 2009). PPARγ agonist troglitazone and pioglitazone were found to reduce ROS induced ER stress and apoptosis in pancreatic β cells (Chung et al., 2011). Our results showed that PA protected the cells against TNF-α induced ER stress by ameliorating overall ROS and down-regulating the expression of various ER stress markers and also by decreasing intracellular Ca^{2+} overload.

In brief results from chapter 5 demonstrated the role of partial PPARγ agonist PA in ameliorating TNF-α induced oxidative stress, mitochondrial dysfunctions and ER stress in TNF-α induced insulin resistant model of 3T3-L1 adipocytes. PA restored mitochondrial dysfunction in TNF-α induced insulin resistant model by improving mitochondrial biogenesis, energetics, transmembrane potential and dynamics. Considering the role of PA in improving mitochondrial dysfunction and ER stress associated with IR, this study provides an insight into the possible application for PA as a PPARγ agonist in improving insulin sensitivity and thus to the management of T2DM and MS.

Chapter 6

Summary and Conclusion

The prevalence of metabolic syndrome is increasing worldwide. Metabolic syndrome is characterized by the co-occurrence of a number of clinical features including obesity, insulin resistance, dyslipidemia etc. These abnormalities lead to the development of type 2 diabetes mellitus and heart diseases. The most prominent characteristic feature of metabolic syndrome is insulin resistance, which is characterized by glucose intolerance or impaired glucose tolerance. Adipose tissue, liver and skeletal muscles are the primary organs involved in glucose and lipid metabolism, and these organs play a pivotal role in systemic insulin resistance. In insulin resistance, there is impairment in insulin mediated glucose disposal in these insulin sensitive tissues, leading to hyperglycemia. Hyperglycemia causes more secretion of insulin from the pancreatic β-cells causing hyperinsulineamia. Hyperinsulineamia and hyperglycemia causes a malfunctioning of the β-cells, leading to type 2 diabetes mellitus.

Adipose tissue has an important role in the pathogenesis of insulin resistance. In obesity and insulin resistance, there is a chronic low grade inflammation in adipocytes, leading to an increase in the secretion of pro-inflammatory cytokines including TNF-α, IL-6, IFN-γ and MCP-1 causing vascular inflammation leading to hypertension and atherosclerosis. A dysfunctional adipose tissue causes abnormalities in lipid storage, leading to increased lipolysis and increased free fatty acids in the circulation, leading to increased lipid accumulation in liver and muscle. This causes a decreased utilization of glucose in these tissues, leading to systemic insulin resistance.

The main perspectives for the management of obesity and insulin resistance are lifestyle modifications and pharmacological interventions. The pharmacological interventions include agents that can improve insulin sensitivity by decreasing the glucotoxicity. The currently available anti-hyperglycemic drugs have different mode of actions and include sufonylureas, biguanides, alpha glucosidase inhibitors, dipeptidylpeptidase-IV (DPP-IV) inhibitors, thiazolidinediones (agonists of PPARγ) etc. Thiazolidinediones or glitazones are a class of drugs, which are ligand activated transcription factors, belonging to nuclear receptor superfamily. Thiazolidinediones

are PPARγ agonists, which mediate their action by binding with the ligand binding sites of PPARγ. The families of PPARs include three members, PPARα, PPARβ/δ and PPARγ. They play an essential role in energy metabolism; however, they differ in their distribution and they have distinct yet overlapping roles. PPARγ is mainly expressed in adipose tissue and muscles and is mainly involved in glucose and lipid metabolism. PPARα is expressed predominantly in the liver, and to a lesser extent, in muscle, heart, bone, and is involved in energy metabolism. PPARδ is ubiquitously expressed in whole body and regulate energy expenditure.

Glitazones are PPARγ agonists used to treat insulin resistance, and this class of drugs can increase insulin sensitivity by increasing glucose uptake in adipocytes, skeletal muscles and liver and also by decreasing hepatic glucose output. Rosiglitazone and pioglitazone are important glitazones used in the treatment of insulin resistance and type 2 diabetes mellitus. Apart from the beneficial effects, these full PPARγ agonists are reported with certain side effects, including obesity, edema, heart failure etc. So there is a need for the development of safer PPARγ modulators, like partial, selective or pan PPARγ agonists and/or partial PPARγ agonists of natural origin. Punicic acid is a poly unsaturated fatty acid found in the pomegranate seed oil, and it is reported to have PPARγ and PPARα ligand binding activity. The aim of the present study is to characterize PA as a partial PPARγ agonist and to study the effect of PA on insulin resistance in 3T3-L1 adipocytes.

In order to characterize PA as a partial PPARγ agonist, effect of PA on differentiation and triglyceride accumulation in 3T3-L1 adipocytes were evaluated. Adipocyte differentiation was confirmed by oil red O assay. Effect of PA on specific markers of adipocyte differentiation like C/EBPα and CD36 were also studied. Adipocyte differentiation assay proved PA as a partial PPARγ agonist, since the expression of adipocyte differentiation markers and extent of lipid accumulation was found to be less compared to the full PPARγ agonist, rosiglitazone. Molecular docking study and TR-FRET assay also proved that PA behaves more like a partial PPARγ agonist compared with RG. PA also improved the expression of insulin sensitive markers like IRS2, PI3K, pAKT, PPARγ and GLUT4 and also increased the translocation of GLUT4 to the plasma membrane; all were comparable to the PPARγ full agonist rosiglitazone. Also, PA increased insulin stimulated glucose uptake in 3T3-

L1 adipocytes in a dose-dependent manner. The results from the first chapter indicate that PA behaves as a partial PPARγ agonist and has better insulin sensitizing property in 3T3-L1 adipocytes.

In order to see the effect of PA on insulin resistance in 3T3-L1 adipocytes, PA was screened against TNF-α induced insulin resistance in 3T3-L1 adipocytes. TNF-α induced insulin resistance was found to be associated with an increase in the expression of serine phosphorylated IRS1, SOCS3 and PTP1B. TNF-α induced insulin resistance was also found to be associated with a reduction in the expression of GLUT4 and PPARγ. TNF-α induced insulin resistance was found to be associated with inflammation in adipocytes, increased translocation of NFκB to the nucleus, increased JNK phosphorylation and increased secretion of inflammatory cytokines. PA treatment decreased the expression of insulin resistant markers and ameliorated inflammation and thereby enhanced insulin sensitivity in 3T3-L1 adipocytes.

TNF-α induced insulin resistance was found to be associated with increased cellular ROS and a reduction in various antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidaase and glutathione reductase. Increased ROS causes a dysfunction in mitochondria, which is characterized by altered mitochondrial membrane potential, increased mitochondrial superoxide production, decreased ATP content and decreased oxygen consumption. Insulin resistance was also found to be associated with decreased mitochondrial biogenesis characterized by decreased expression of biogenesis markers like PGC1α, NRF1, Tfam and also mtDNA copy number. TNF-α induced resistance was also found to be associated with an alteration in the expression of proteins associated with mitochondrial dynamics. The expression of mitochondrial fission protein FIS1 was increased and the expression of mitochondrial fusion protein OPA1 was decreased leading to an increase in mitochondrial fission rate. Altered mitochondrial fission-fusion rate can lead to the development of dysfunctional mitochondria. PA treatment improved mitochondrial parameters associated with insulin resistance and thus can contribute to the enhancement of insulin sensitivity. TNF-α induced insulin resistance was also found to be associated with ER stress, which is characterized by increased expression of ER stress markers like GRP78/Bip, ERO-1α and CHOP. Also insulin resistance increased the expression of UPR sensors like PERK, ATF6 and IRE-1α. ER stress is also characterized by an increased intracellular

Ca^{2+} overload. PA treatment ameliorated the expression of ER stress markers leading to enhancement of insulin sensitivity.

Overall results proved that PA improved insulin sensitivity by decreasing the expression of various insulin resistant markers and inflammatory markers. PA also ameliorated inflammation in 3T3-L1 adipocytes by decreasing the translocation of NFκB to the nucleus. PA improved insulin resistance by ameliorating mitochondrial dysfunctions and ER stress. PA was characterized as a partial PPARγ agonist with better insulin sensitizing property. Since punicic acid behaves as a partial PPARγ agonist and enhances insulin resistance in 3T3-L1 adipocytes, it can be considered as an effective lead for the management of metabolic syndrome and insulin resistance.

References

Aebi H (1984). Catalase in vitro. Methods Enzymol, 105: 121-126.

Ahlers NHE, Dennison AC, O'Neill LA (1954). Spectroscopic examination of punicic acid. Nature, 173: 1045-1046.

Ahmad F, Li PM, Meyerovitch J, Goldstein BJ (1995). Osmotic loading of neutralizing antibodies demonstrates a role for protein-tyrosine phosphatase 1B in negative regulation of the insulin action pathway. J Biol Chem, 270: 20503-20508.

Ahmadian M, Suh JM, Hah N, Liddle C, Atkins AR, Downes M, Evans RM (2013). PPARγ signalling and metabolism: the good, the bad and the future. Nat Med, 99: 557-566.

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 23: 6541-6551.

Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, Gaffney P, Reese CB, MacDougall CN, Harbison D, Ashworth A, Bownes M (1997). 3-Phosphoinositide-dependent protein kinase-1(PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Curr Biol, 10: 776-789.

Allen T, Zhang F, Moodie SA, Clemens LE, Smith A, Gregoire F, Bell A, Muscat GE, Gustafson TA (2006). Halofenate is a selective peroxisome proliferator-activated receptor γ modulator with antidiabetic activity. Diabetes, 55: 2523-2533.

Amri EZ, Bonino F, Ailhaud G, Abumrad NA, Grimaldi PA (1995). Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes. Homology to peroxisome proliferator-activated receptors. J Biol Chem, 270: 2367-2371.

Andreadis EA, Tsourous GI, Tzavara CK, Georgiopoulos DX, Katsanou PM, Marakomichelakis GE, Diamantopoulos EJ (2007). Metabolic syndrome and incident cardiovascular morbidity and mortality in a Mediterranean hypertensive population. Am J Hypertens, 20: 558-564.

Archer SL (2013). Mitochondrial dynamics--mitochondrial fission and fusion in human diseases. N Engl J Med, 369: 2236-2251.

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y (1999). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun, 257: 79-83.

Aroor AR, DeMarco VG (2014). Oxidative stress and obesity: the chicken or the egg? Diabetes, 63: 2216-2218.

Austin S, Medvedev A, Yan ZH, Adachi H, Hirose T, Jetten AM (1998). Induction of the nuclear orphan receptor ROR gamma during adipocyte differentiation of D1 and 3T3-L1 cells. Cell Growth Diff, 9: 267-276.

Bailey CJ (1992). Biguanides and NIDDM. Diabetes Care, 15: 755-772.

Bailey CJ, Turner RC (1996). Metformin. N Engl J Med, 334: 574-579.

Balunas MJ, Kinghorn AD (2005). Drug discovery from medicinal plants. Life Sci, 78: 431-441.

Bamba V, Rader DJ (2007). Obesity and atherogenic dyslipidemia. Gastroenterology, 132: 2181-2190.

Bassaganya-Riera J, Guri AJ, Hontecillas R (2011). Treatment of obesity-related complications with novel classes of naturally occurring PPAR agonists. J Obes, doi:10.1155/2011/897894.

Bassaganya-Riera J, Skoneczka J, Kingston DG, Krishnan A, Misyak SA, Guri AJ, Pereira A, Carter AB, Minorsky P, Tumarkin R, Hontecillas R (2010). Mechanisms of action and medicinal applications of abscisic acid. Curr Med Chem, 17: 467-478.

Bastaki S (2005). Diabetes mellitus and its treatment. Int J Diabetes Metab, 13: 111-134.

Belenguer P, Pellegrini L (2013). The dynamin GTPase OPA1: more than mitochondria? Biochim Biophys Acta, 1833: 176-183.

Bennett BL, Satoh Y, Lewis AJ (2003). JNK: a new therapeutic target for diabetes. Curr Opin Pharmacol, 3: 420-425.

Benomar Y, Gertler A, De Lacy P, Crépin D, Ould Hamouda H, Riffault L, Taouis M (2013). Central resistin overexposure induces insulin resistance through Toll-like receptor 4. Diabetes, 62: 102-114.

Boden G (1997). Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes, 46: 3-10.

Boden G (2008). Obesity and free fatty acids. Endocrinol Metab Clin North Am, 37: 635-646.

Boden G, Duan X, Homko C, Molina EJ, Song W, Perez O, Cheung P, Merali S (2008). Increase in endoplasmic reticulum stress-related proteins and genes in adipose tissue of obese insulin-resistant individuals. Diabetes, 57: 2438-2444.

Bogacka I, Xie H, Bray GA, Smith SR (2005). Pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue in vivo. Diabetes, 54: 1392-1399.

Bonora E, Targher G (2012). Increased risk of cardiovascular disease and chronic kidney disease in NAFLD. Nat Rev Gastroenterol Hepatol, 9: 372-381.

Boura-Halfon S, Zick Y (2009). Phosphorylation of IRS proteins, insulin action, and insulin resistance. Phosphorylation of IRS proteins, insulin action, and insulin resistance. Am J Physiol Endocrinol Metab, doi: 10.1152/ajpendo.90437.2008.

Braissant O, Foufelle F, Scotto C, Dauça M, Wahli W (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, beta, and gamma in the adult rat. Endocrinology, 137: 354-366.

Brand MD, Nicholls DG (2011). Assessing mitochondrial dysfunction in cells. Biochem J, 435: 297-312.

Brooks C, Wei Q, Cho SG, Dong Z (2009). Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. J Clin Invest, 119: 1275-85.

Brown JD, Plutzky J (2007). Peroxisome proliferator-activated receptors as transcriptional nodal points and therapeutic targets. Circulation, 115: 518-533.

Burgermeister E, Schnoebelen A, Flament A, Benz J, Stihle M, Gsell B, Rufer A, Ruf A, Kuhn B, Märki HP, Mizrahi J, Sebokova E, Niesor E, Meyer M (2006). A novel partial agonist of peroxisome proliferator-activated receptor-γ (PPARγ) recruits PPARγ-coactivator-1α, prevents triglyceride accumulation, and potentiates insulin signaling in vitro. Mol Endocrinol, 4: 809-830.

Buttar HS, Li T, Ravi N (2005). Prevention of cardiovascular diseases: Role of exercise, dietary interventions, obesity and smoking cessation. Exp Clin Cardiol, 10: 229-249.

Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature, 415: 92-96.

Cameron AJ, Shaw JE, Zimmet PZ (2004). The metabolic syndrome: prevalence in worldwide populations. Endocrinol Metab Clin North Am, 33: 351-375.

Camp HS, Ren D, Leff T (2002). Adipogenesis and fat-cell function in obesity and diabetes. Trends Mol Med, 8: 442-447.

Cawthorn WP, Sethi JK (2008). TNF-alpha and adipocyte biology. FEBS Lett, 582: 117-131.

Cefalu WT (2001). Insulin resistance: cellular and clinical concepts. Exp Biol Med, 226: 13-26.

Chaldakov GN, Stankulov IS, Hristova M, Ghenev PI (2003). Adipobiology of disease: adipokines and adipokine-targeted pharmacology. Curr Pharm Des, 9: 1023-1031.

Chan DC (2006). Mitochondrial fusion and fission in mammals. Annu Rev Cell Dev Biol, 22: 79-99.

Chen H, Chomyn A, Chan DC (2005). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J Biol Chem, 280: 26185-26192.

Chen L, Chen R, Wang H, Liang F (2015). Mechanisms Linking Inflammation to Insulin Resistance. Int J Endocrinol, doi: 10.1155/2015/508409.

Chen XH, Zhao YP, Xue M, Ji CB, Gao CL, Zhu JG, Qin DN, Kou CZ, Qin XH, Tong ML, Guo XR (2010). TNF-alpha induces mitochondrial dysfunction in 3T3-L1 adipocytes. Mol Cell Endocrinol, 328: 63-69.

Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, Staels B (1998). Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. J Biol Chem, 273: 25573-25580.

Cho MC, Lee K, Paik SG, Yoon DY (2008). Peroxisome Proliferators-Activated Receptor (PPAR) Modulators and Metabolic Disorders. PPAR Res, doi: 10.1155/2008/679137.

Cho N, Momose Y (2008). Peroxisome proliferator activated receptor gamma agonists as insulin sensitizers: from the discovery to recent progress. Curr Top Med Chem, 25: 1483-1507.

Choi SS, Cha BY, Lee YS, Yonezawa T, Teruya T, Nagai K, Woo JT (2009). Magnolol enhances adipocyte differentiation and glucose uptake in 3T3-L1 cells. Life Sci, 84: 908-914.

Chuang CC, Bumrungpert A, Kennedy A, Overman A, West T, Dawson B, McIntosh MK (2011). Grape powder extract attenuates tumor necrosis factor αmediated inflammation and insulinresistance inprimary cultures of human adipocytes. J Nutr Biochem, 22: 89-94.

Chuang CC, Martinez K, Xie G, Kennedy A, Bumrungpert A, Overman A, Jia W, McIntoshMK (2010). Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor-alpha mediated inflammation and insulin resistance in primary human adipocytes**.** Am J Clin Nutr**,** 92: 1511-1521.

Chung SS, Kim M, Lee JS, Ahn BY, Jung HS, Lee HM, Park KS (2011). Mechanism for antioxidative effects of thiazolidinediones in pancreatic β-cells. Am J Physiol Endocrinol Metab, 301: 912-921.

Coburn CT, Knapp FF Jr, Febbraio M, Beets AL, Silverstein RL, Abumrad NA (2000). J Biol Chem, 275: 32523-32529.

Cragg GM, Newman DJ (2013). Natural products: a continuing source of novel drug leads. Biochim Biophys Acta, 1830: 3670-3695.

Crimmins NA, Martin LJ (2007). Polymorphisms in adiponectin receptor genes ADIPOR1 and ADIPOR2 and insulin resistance. Obes Rev, 8: 419-423.

Dalle Grave R, Calugi S, Centis E, Marzocchi R, El Ghoch M, Marchesini G (2010). Lifestyle modification in the management of the metabolic syndrome: achievements and challenges. Diabetes Metab Syndr Obes, 2: 373-385.

Davi G, Santilli F, Patrono C (2010). Nutraceuticals in diabetes and metabolic syndrome. Cardiovasc Ther, 28: 216-226.

David M, Richard JS (1983). In: Methods of enzymatic analysis. Bergmeyer J, Marianne Grab. eds. Verlag Chemie Weinheim Deer Field, Beach Floride, p. 358.

de Luca C, Olefsky JM (2008). Inflammation and insulin resistance. FEBS Lett, 582: 97-105.

De Pauw A, Tejerina S, Raes M, Keijer J, Arnould T (2009). Mitochondrial (dys)function in adipocyte (de)differentiation and systemic metabolic alterations. Am J Pathol, 175: 927-939.

Desroches S, Lamarche B (2007). The evolving definitions and increasing prevalence of the metabolic syndrome. Appl Physiol Nutr Metab, 32: 23-32.

Diaz-Delfín J, Morales M, Caelles C (2007). Hypoglycemic action of thiazolidinediones/peroxisome proliferator-activated receptor gamma by inhibition of thec-Jun NH2-terminal kinase pathway. Diabetes, 56: 1865-1871.

Diep QN, Touyz RM, Schiffrin EL (2000). Docosahexaenoic acid, a peroxisome proliferator-activated receptor-alpha ligand, induces apoptosis in vascular smooth muscle cells by stimulation of p38 mitogen-activated protein kinase. Hypertension, 36: 851-855.

Eckel RH, Grundy SM, Zimmet PZ (2005). The metabolic syndrome. Lancet, 365: 1415-1428.

Egerod FL, Nielsen HS, Iversen L, Thorup I, Storgaard T, Oleksiewicz MB (2005). Biomarkers for early effects of carcinogenic dual-acting PPAR agonists in rat urinary bladder urothelium in vivo. Biomarkars, 10: 295-309.

E-Haggar SM, Mostafa TM (2015). Adipokines and biochemical changes in Egyptian obese subjects: possible variation with sex and degree of obesity. Endocrine, 48: 878-85.

Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP (1999). Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science, 283: 1544-1548.

Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E (2001). SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. J Biol Chem, 276: 47944-47949.

Emoto M, Langille SE Czech MP (2001). A role for kinesin in insulin-stimulated GLUT4 glucose transporter translocation in 3T3-L1 adipocytes. J Biol Chem, 276: 10677-10682.

Enas EA, Mohan V, Deepa M, Farooq S, Pazhoor S, Chennikkara H (2007). The metabolic syndrome and dyslipidemia among Asian Indians: a population with high rates of diabetes and premature coronary artery disease. J Cardiometab Syndr, 2: 267-275.

Engvall E, Perlmann P (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry, 8: 871-874.

Escobar-Henriques M, Anton F (2013). Mechanistic perspective of mitochondrial fusion: tubulation vs. fragmentation. Biochim Biophys Acta, 1833:162-175.

Evans RM, Barish GD, Wang YX (2004). PPARs and the complex journey to obesity. Nat Med, 10: 355-361.

Evans-Molina C, Robbins RD, Kono T, Tersey SA, Vestermark GL, Nunemaker CS, Garmey JC, Deering TG, Keller SR, Maier B, Mirmira RG (2009). Peroxisome proliferator-activated receptor gamma activation restores islet function in diabetic mice through reduction of endoplasmic reticulum stress and maintenance of euchromatin structure. Mol Cell Biol, 29: 2053-2067.

Fagerberg B, Edwards S, Halmos T, Lopatynski J, Schuster H, Stender S, Stoa-Birketvedt G, Tonstad S, Halldórsdóttir S, Gause-Nilsson I (2005). Tesaglitazar, a novel dual peroxisome proliferator-activated receptor alpha/gamma agonist, dose-dependently improves the metabolic abnormalities associated with insulin resistance in a non-diabetic population. Diabetologia, 48: 1716-1725.

Fanelli C, Calderone S, Epifano L, De Vincenzo A, Modarelli F, Pampanelli S, Perriello G, De Feo P, Brunetti P, Gerich JE (1993). Demonstration of a critical role for free fatty acids in mediating counterregulatory stimulation of gluconeogenesis and suppression of glucose utilization in humans. J Clin Invest, 92: 1617-1622.

Farmer SR (2006). Transcriptional control of adipocyte formation. Cell Metab, 4: 263-273.

Fernandez-Veledo S, Nieto-Vazquez I, Rondinone CM, Lorenzo M (2006). Liver X receptor agonists ameliorate TNF alpha-induced insulin resistance in murine brown adipocytes by downregulating protein tyrosine phosphatase-1B gene expression. Diabetologia, 49: 3038-3048.

Ferrannini E, Barrett EJ, Bevilacqua S, DeFronzo RA (1983). Effect of fatty acids on glucose production and utilization in man. J Clin Invest, 72: 1737-1747.

Flamment M, Hajduch E, Ferré P, Foufelle F (2012). New insights into ER stress-induced insulin resistance. Trends Endocrinol Metab, 23: 381-390.

Flier JS, Cook K., Usher P, Spiegelman BM (1987). Severely impaired adipsin expression in genetic and acquired obesity. Science, 237: 405-408.

Fonseca-Alaniz MH, Takada J, Alonso-Vale MIC, Lima FB (2007). Adipose tissue as an endocrine organ: from theory to practice. J Pediatr, 83: 192-203.

Ford ES, Giles WH, Dietz WH (2002). Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA, 287: 356-359.

Forman BM, Chen J, Evans RM (1996). The peroxisome proliferator-activated receptors: ligands and activators. Ann N Y Acad Sci, 804: 266-275.

Frayn KN (2001). Adipose tissue and the insulin resistance syndrome. Proc Nutr Soc, 60: 375-380.

Fu Y, Luo N, Klein RL, Garvey WT (2005). Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. J lipid Res, 46: 1369-1379.

Fujishiro M, Gotoh Y, Katagiri H, Sakoda H, Ogihara T, Anai M, Onishi Y, Ono H, Abe M, Shojima N, Fukushima Y, Kikuchi M, Oka Y, Asano T (2003). Three mitogen-activated protein kinases inhibit insulin signaling by different mechanisms in 3T3-L1 adipocytes. Mol Endocrinol, 17: 487-497.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest, 114: 1752-1761.

Gil A, Olza J, Gil-Campos M, Gomez-Llorente C, Aguilera CM (2011). Is adipose tissue metabolically different at different sites? Int J Pediatr Obes, 1: 13-20.

Giralt M, Villarroya F (2013). White, brown, beige/brite: different adipose cells for different functions? Endocrinology, 154: 2992-3000.

Glide AJ, Fruchart J, Staels B (2006). Peroxisome Proliferator-Activated Receptors at the crossroads of obesity, diabetes, and cardiovascular disease. J Am Coll Cardiol, 48: 24-32.

Gomes EC, Silva AN, de Oliveira MR (2012). Oxidants, antioxidants, and the beneficial roles of exercise-induced production of reactive species. Oxid Med Cell Longev, doi: 10.1155/2012/756132.

Goossens GH (2008). The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav, 94: 206-218.

Graham TL, Mookherjee C, Suckling KE, Palmer CN, Patel L (2005). The PPAR delta agonist GW0742X reduces atherosclerosis in LDLR(-/-) mice. Atheroscerosis, 181: 29-37.

Grant RW, Dixit VD (2013). Mechanisms of disease: inflammasome activation and the development of type 2 diabetes. Front Immunol, doi: 10.3389/fimmu.2013.00050.

Greenberg AS, Obin MS (2006). Obesity and the role of adipose tissue in inflammation and metabolism. Am J Clin Nutr, 83: 461-465.

Gregoire FM, Smas CM, Sul HS (1998). Understanding adipocyte differentiation. Physiol Rev, 78: 784-809.

Gregor MF, Hotamisligil GS (2007). Adipocyte stress: the endoplasmic reticulum and metabolic disease. J Lipid Res, 48: 1905-1914.

Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C (2004). Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation, 109: 433-438.

Grygiel-Górniak B (2014). Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications-a review. Nutr J, doi: 10.1186/1475-2891-13-17.

Gual P, Le Marchand-Brustel Y, Tanti JF (2005). Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. Biochimie, 87: 99-109.

Guasch L, Sala E, Valls C, Blay M, Mulero M, Arola L, Pujadas G, Garcia-Vallvé S (2011). Structural insights for the design of new PPARgamma partial agonists with high binding affinity and low transactivation activity. J Comput Aided Mol Des, 25: 717-728.

Guasch L, Sala E, Mulero M, Valls C, Salvadó MJ, Pujadas G, Garcia-Vallvé S (2013). Identification of PPAR gamma partial agonists of natural (II): in silico prediction in natural extracts with known antidiabetic activity. Plos One, doi: 10.1371/journal.pone.0055889.

Guilherme A, Virbasius JV, Puri V, Czech MP (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol, 9: 367-377.

Gum RJ, Gaede LL, Koterski SL, Heindel M, Clampit JE, Zinker BA, Trevillyan JM, Ulrich RG, Jirousek MR, Rondinone CM (2003). Reduction of protein tyrosine phosphatase 1B increases insulin-dependent signaling in ob/ob mice. Diabetes, 52: 21-28.

Guo S (2014). Insulin signaling, resistance, and the metabolic syndrome: insights from mouse models into disease mechanisms. J Endocrinol, doi: 10.1530/JOE-13-0327.

Gupta R, Deedwania PC, Gupta A, Rastogi S, Panwar RB, Kothari K (2004). Prevalence of metabolic syndrome in an Indian urban population. Int J Cardiol, 97: 257-261.

Hafizi M, Bakar A (2015). Mitochondrial dysfunction enhances lipolysis and intracellular lipid accumulation in 3T3-L1 adipocytes. Int J Pharm Med Biol Sci, 4: 65-69.

Hagberg H, Mallard C, Rousset CI, Thornton C (2014). Mitochondria: hub of injury responses in the developing brain. Lancet Neurol, 13: 217-232.

Hahn WS, Kuzmicic J, Burrill JS, Donoghue MA, Foncea R, Jensen MD, Lavandero S, Arriaga EA, Bernlohr DA (2014). Proinflammatory cytokines differentially regulate adipocyte mitochondrial metabolism, oxidative stress and dynamics. Am J Physiol Endocrinol Metab, doi: 10.1152/ajpendo.00422.2013.

Halberg N, Wernstedt-Asterholm I, Scherer PE (2008). The adipocyte as an endocrine cell. Endocrinol Metab Clin North Am, 37: 753-768.

Han D, Ybanez MD, Ahmadi S, Yeh K, Kaplowitz N (2009). Redox regulation of tumor necrosis factor signaling. Antioxid Redox Signal, 11: 2245-2263.

Han MS, Jung DY, Morel C, Lakhani SA, Kim JK, Flavell RA, Davis RJ (2013). JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation. Science, 339: 218-222.

Hao J, Shen W, Yu G, Jia H, Li X, Feng Z, Wang Y, Weber P, Wertz K, Sharman E, Liu J (2010). Hydroxytyrosol promotes mitochondrial biogenesis and mitochondrial function in 3T3-L1 adipocytes. J Nutr Biochem, 21: 634-644.

Harding HP, Zhang Y, Ron D (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature, 397: 271-274.

Heilbronn LK, Gan SK, Turner N, Campbell LV, Chisholm DJ (2007). Markers of mitochondrial biogenesis and metabolism are lower in overweight and obese insulin-resistant subjects. J Clin Endocrinol Metab, 92: 1467-1473.

Henke BR, Blanchard SG, Brackeen MF, Brown KK, Cobb JE, Collins JL, Harrington WW Jr, Hashim MA, Hull-Ryde EA, Kaldor I, Kliewer SA, Lake DH, Leesnitzer LM, Lehmann JM, Lenhard JM, Orband-Miller LA, Miller JF, Mook RA Jr, Noble SA, Oliver W Jr, Parks DJ, Plunket KD, Szewczyk JR, Willson TM (1998). N-(2-Benzoylphenyl)-L-tyrosine PPARgamma agonists. Discovery of a novel series of potent antihyperglycemic and antihyperlipidemic agents. J Med Chem, 41: 5020-5036.

Higgins LS, Depaoli AM (2010). Selective peroxisome proliferator-activated receptor gamma (PPARgamma) modulation as a strategy for safer therapeutic PPARgamma activation. Am J Clin Nut, 91: 267-272.

Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS (2002). A central role for JNK in obesity and insulin resistance. Nature, 420: 333-336.

Hoehn KL, Salmon AB, Hohnen-Behrens C, Turner N, Hoy AJ, Maghzal GJ, Stocker R, Van Remmen H, Kraegen EW, Cooney GJ, Richardson AR, James DE (2009). Insulin resistance is a cellular antioxidant defense mechanism. Proc Natl Acad Sci, 106: 17787-17792.

Hondares E, Mora O, Yubero P, Rodriguez de la Concepción M, Iglesias R, Giralt M, Villarroya F (2006). Thiazolidinediones and rexinoids induce peroxisome proliferator-activated receptor-coactivator (PGC)-1alpha gene transcription: an autoregulatory loop controls PGC-1alpha expression in adipocytes via peroxisome proliferator-activated receptor-gamma coactivation. Endocrinology, 147: 2829-2838.

Hontecillas R, O'Shea M, Einerhand A, Diguardo M, Bassaganya-Riera J (2009). Activation of PPAR α and γ by punicic acid ameliorates glucose tolerence and suppresses obesity related inflammation. J Am Coll Nutr, 28: 184-195.

Hotamisligil GS (2006). Inflammation and metabolic disorders. Nature, 444: 860-867.

Hotamisligil GS (2008). Inflammation and endoplasmic reticulum stress in obesity and diabetes. Int J Obes, doi: 10.1038/ijo.2008.238.

Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM (1996). IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science, 271: 665-668.

Hotamisligil GS, Shargill NS, Spiegelman BM (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science, 259: 87-91.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM (1995). Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest, 95: 2409-2415.

Houstis N, Rosen ED, Lander ES (2006). Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature, 440: 944-948.

Howard JK, Flier JS (2006). Attenuation of leptin and insulin signaling by SOCS proteins. Trends Endocrinol Metab, 17: 365-371.

Hsu KC, Chen YF, Lin SR, Yang JM (2011). iGEMDOCK: a graphical environment of enhancing iGEMDOCK using pharmacological interactions and post-screening analysis. BMC Bioinformatics, doi:10.1186/1471-2105-12-S1-S33.

Hsu WH, Lee BH, Liao TH, Hsu YW, Pan TM (2012). Monascus-fermented metabolite monascin suppresses inflammation via PPAR-γ regulation and JNK inactivation in THP-1 monocytes. Food Chem Toxicol, 50:1178-1186.

Huang PL (2009). A comprehensive definition for metabolic syndrome. Dis Model Mech, 2: 231-237.

Hwang CS, Loftus TM, Mandrup S, Lane MD (1997). Adipocyte differentiation and leptin expression. Annu Rev Cell Dev Biol, 13: 231-259.

Hwang CS, Mandrup S, MacDougald OA, Geiman DE, Lane MD (1996). Transcriptional activation of the mouse obese (ob) gene by CCAAT/enhancer binding protein alpha. Proc Natl Acad Sci U S A, 93: 873-877.

Inoue I, Shino K, Noji S, Awata T, Katayama S (1998). Expression of peroxisome proliferator-activated receptor alpha (PPAR alpha) in primary cultures of human vascular endothelial cells. Biochem Biophys Res Commun, 246: 370-374.

Ishizuka K, Usui I, Kanatani Y, Bukhari A, He J, Fujisaka S, Yamazaki Y, Suzuki H, Hiratani K, Ishiki M, Iwata M, Urakaze M, Haruta T, Kobayashi M (2007). Chronic tumor necrosis factor-alpha treatment causes insulin resistance via insulin receptor substrate-1 serine phosphorylation and suppressor of cytokine signaling-3 induction in 3T3-L1 adipocytes. Endocrinology, 148: 2994-3003.

Issemann I, Prince RA, Tugwood JD, Green S (1993). The retinoid X receptor enhances the function of the peroxisome proliferator activated receptor. Biochimie, 75: 251-256.

Itoh T, Fairall L, Amin K, Inaba Y, Szanto A, Balint BL, Nagy L, Yamamoto K, Schwabe JW (2008). Structural basis for the activation of PPARgamma by oxidized fatty acids. Nat Struct Mol Biol, 15: 924-931.

Jain RG, Phelps KD, Pekala PH (1999). Tumor necrosis factor-alpha initiated signal transduction in 3T3-L1 adipocytes. J Cell Physiol, 179: 58-66.

James SR, Downes CP, Gigg R, Grove SJ, Holmes AB, Alessi DR (1996). Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. Biochem J, 315: 709-713.

Jeon MJ, Leem J, Ko MS, Jang JE, Park HS, Kim HS (2012). Mitochondrial dysfunction and activation of iNOS are responsible for the palmitate induced decrease in adiponectin synthesis in 3T3L1 adipocytes. Exp Mol Med, 44: 562-570.

Jheng HF, Tsai PJ, Guo SM, Kuo LH, Chang CS, Su IJ, Chang CR, Tsai YS (2012). Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistane in skeletal muscle. Mol Cell Biol, 32: 309-319.

Jiang G, Dallas-Yang Q, Biswas S, Li Z, Zhang BB (2004). Rosiglitazone, an agonist of peroxisome-proliferator-activated receptor gamma (PPARgamma), decreases inhibitory serine phosphorylation of IRS1 in vitro and in vivo. Biochem J, 377: 339-346.

Jiang G, Dallas-Yang Q, Li Z, Szalkowski D, Liu F, Shen X, Wu M, Zhou G, Doebber T, Berger J, Moller DE, Zhang BB (2002). Potentiation of insulin signaling in tissues of Zucker obese rats after acute and long-term treatment with PPARgamma agonists. Diabetes, 51: 2412-2419.

Jin SM, Kim KS, Lee SY, Gong CH, Park SK, Shin JS, Park CG, Kim SJ (2011). The sequential combination of a JNK inhibitor and simvastatin protects porcine islets from peritransplant apoptosis and inflammation. Cell Transplantat, 20: 1139-1151.

Joh YG, Kim SJ, Christie WW (1995). The structure of the triacyglycerols, containing punicic acid, in the seed oil of Trichosanthes kirilowii. J Am Oil Chem Soc, 72: 1037-1042.

Joseph AM, Joanisse DR, Baillot RG, Hood DA (2012). Mitochondrial dysregulation in the pathogenesis of diabetes: potential for mitochondrial biogenesis-mediated interventions. Exp Diabetes Res, doi: 10.1155/2012/642038.

Jung UJ, Choi MS (2014). Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. Int J Mol Sci, 15: 6184-223.

Kakkar P, Das B, Viswanathan PN (1984). A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys, 21: 130-132.

Kalekar SA, Munshi RP, Bhalerao SS, Thatte UM (2013). Insulin sensitizing effect of 3 Indian medicinal plants: an in vitro study. Indian J Pharmacol, 45: 30-33.

Kanatani Y, Usui I, Ishizuka K, Bukhari A, Fujisaka S, Urakaze M, Haruta T, Kishimoto T, Naka T, Kobayashi M. (2007). Effects of pioglitazone on suppressor of cytokine signaling 3 expression: potential mechanisms for its effects on insulin sensitivity and adiponectin expression. Diabetes, 56: 795-803.

Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest, 116: 1494-1505.

Kaufman M, Wiesman Z (2007). Pomegranate oil analysis with emphasis on TOF/MS triacylglycerol fingerprinting. J Agric Food Chem, 55: 10405-10413.

Kaur J (2014). A comprehensive review on metabolic syndrome. Cardiol Res Pract, doi: 10.1155/2014/943162.

Kendall DM, Rubin CJ, Mohideen P, Ledeine JM, Belder R, Gross J, Norwood P, O'Mahony M, Sall K, Sloan G, Roberts A, Fiedorek FT, DeFronzo RA (2006). Improvement in glycemic control, triglycerides, and HDL cholesterol levels with

muraglitazar, a dual (alpha/gamma) peroxisome proliferator-activated receptor activator, in patients with type 2 diabetes inadequately controlled with metformin monotherapy: A double blind, randomized, pioglitazone-comparative study. Diabetes Care, 29: 1016-1023.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R, Simsolo RB (1995). The expression of tumor necrosis factor in human adipose tissue. Regulation by obesity, weight loss, and relationship to lipoprotein lipase. J Clin Invest, 95: 2111-2119.

Kershaw EE, Flier JS (2004). Adipose tissue as an endocrine organ. J Clin Endocrinol Metab, 89: 2548-56**.**

Kim JA, Montagnani M, Koh KK, Quon MJ (2006). Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation, 113: 1888-1904**.**

Kim JA, Wei Y, Sowers JR (2008). Role of mitochondrial dysfunction in insulin resistance. Circ Res, 102: 401-414.

Kim W, Egan JM (2008). The role of incretins in glucose homeostasis and diabetes treatment. Pharmacol Rev, 60: 470-512.

Kohlroser J, Mathai J, Reichheld J, Banner BF, Bonkovsky HL (2000). Hepatotoxicity due to troglitazone: report of two cases and review of adverse events reported to the United States Food and Drug Administration. Am J Gastroenterol, 95: 272-276.

Krey G, Braissant O, L'Horset F, Kalkhoven E, Perroud M, Parker MG, Wahli W (1997). Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol Endocrinol, 11: 779-791.

Kubota N, Terauchi Y, Kubota T, Kumagai H, Itoh S, Satoh H, Yano W, Ogata H, Tokuyama K, Takamoto I, Mineyama T, Ishikawa M, Moroi M, Sugi K, Yamauchi T, Ueki K, Tobe K, Noda T, Nagai R, Kadowaki T (2006). Pioglitazone ameliorates insulin resistance and diabetes by both adiponectin-dependent and independent pathways. J Biol Chem, 281: 8748-8755.

Kuhn C, Arapogianni NE, Halabalaki M, Hempel J, Hunger N, Wober J, Skaltsounis AL, Vollmer G (2011). Constituents from Cistus salvifolius (Cistaceae) activate

peroxisome proliferator activated receptor γ but not δ and stimulate glucose uptake by adipocytes. Planta Med, 77: 346-353.

Kusminski CM, Scherer PE (2012). Mitochondrial dysfunction in white adipose tissue. Trends Endocrinol Metab, 23: 435-443.

Kwon H, Pessin JE (2013). Adipokines mediate inflammation and insulin resistance. Front Endocrinol, doi: 10.3389/fendo.2013.00071.

Laaksonen DE, Niskanen L, Nyyssönen K, Lakka TA, Laukkanen JA, Salonen JT (2008). Dyslipidaemia as a predictor of hypertension in middle-aged men. Eur Heart J, 29: 2561-2568.

Lagathu C, Yvan-Charvet L, Bastard JP, Maachi M, Quignard-Boulangé A, Capeau J, Caron M (2006). Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes. Diabetologia, 49: 2162-2173.

Lai E, Bikopoulos G, Wheeler MB, Rozakis-Adcock M, Volchuk A (2008). Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic β-cells. Am J Physiol Endocrinol Metab, 294: 540-550.

Lai E, Teodoro T, Volchuk A (2007). Endoplasmic reticulum stress: signaling the unfolded protein response. Physiology, 22: 193-201.

Lamont BJ, Drucker DJ (2008). Differential antidiabetic efficacy of incretin agonists versus DPP-4 inhibition in high fat fed mice. Diabetes, 57: 190-198.

Landreth G, Jiang Q, Mandrekar S, Heneka M (2008). PPARgamma agonists as therapeutics for the treatment of Alzheimer's disease. Neurotherapeutics, 5: 481-489.

Lanuza-Masdeu J, Arévalo MI, Vila C, Barberà A, Gomis R, Caelles C (2013). In vivo JNK activation in pancreatic β-cells leads to glucose intolerance caused by insulin resistance in pancreas. Diabetes, 62: 2308-2317.

Lau DC, Dhillon B, Yan H, Szmitko PE, Verma S (2005). Adipokines: molecular links between obesity and atheroslcerosis. Am J Physiol Heart Circ Physiol, 288: 2031-2041.

Laudet V, Hanni C, Coll J, Catzeflis F, Stehelin D (1992). Evolution of the nuclear receptor gene superfamily. EMBO J, 11: 1003-1013.

Lebovitz HE, Banerji MA (2004). Treatment of insulin resistance in diabetes mellitus. Eur J Pharmacol, 490: 135-146.

Lee BH, Hsu WH, Liao TH, Pan TM (2011). The Monascus metabolite monascin against TNF-α-induced insulin resistance via suppressing PPAR-γ phosphorylation in C2C12 myotubes. Food Chem Toxicol, 49: 2609-2617.

Lee JM, Kim SS, Cho YS (2012). The Role of PPARγ in Helicobacter pylori Infection and Gastric Carcinogenesis. PPAR Res, doi: 10.1155/2012/687570.

Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ (2004). Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell, 15: 5001-5011.

Lee YM, Choi JS, Kim MH, Jung MH, Lee YS, Song J (2006). Effects of dietary genistein on hepatic lipid metabolism and mitochondrial function in mice fed high-fat diets. Nutrition, 22: 956-964.

Leonardini A, Laviola L, Perrini S, Natalicchio A, Giorgino F (2009). Cross-Talk between PPARgamma and Insulin Signaling and Modulation of Insulin Sensitivity. PPAR Res, doi: 10.1155/2009/818945.

Lewis SN, Brannan L, Guri AJ, Lu P, Hontecillas R, Bassaganya-Riera J, Bevan DR (2011). Dietary α eleostearic acid ameliorates experimental inflammatory bowel disease in mice by activating peroxisome proliferator-activated receptor γ. Plos One, doi:10.1371/journal.pone.0024031.

Liao Z, Wu Z, Wu M (2012). Cirsium japonicum flavones enhance adipocyte differentiation and glucose uptake in 3T3-L1 cells. Bio Pharm Bull, 35:855-860.

Lima LM, Trachez MM, Chaves de Araujo JS, da Silva JS , do Amaral DS, Sudo RT , Barreiro EJ , Sudo GZ (2014). Novel Partial Agonist of PPAR-Gamma for Treatment of Diabetic Neuropathy in Rat. Diabetes Metab, doi.org/10.4172/2155-6156.1000392

Liu J, Shen W, Zhao B, Wang Y, Wertz K, Weber P, Zhang P (2009). Targeting mitochondrial biogenesis for preventing and treating insulin resistance in diabetes and obesity: Hope from natural mitochondrial nutrients. Adv Drug Deliv Rev, 61:1343-1352.

Lorenzo M, Fernández-Veledo S, Vila-Bedmar R, Garcia-Guerra L, De Alvaro C, Nieto-Vazquez I (2008). Insulin resistance induced by tumor necrosis factor-alpha in myocytes and brown adipocytes. J Anim Sci, 86: 94-104.

Lu IL, Huang CF, Peng YH, Lin YT, Hsieh H-P (2006). Structure-based drug design of a novel family of PPARgamma partial agonists: virtual screening, X-ray crystallography, and in vitro/in vivo biological activities. J Med Chem, 49: 2703-2712.

Lumeng CN, Bodzin JL, Saltiel AR. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Investig, 117: 175-184.

Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, Prigaro BJ, Wood JL, Bhanot S, MacDonald MJ, Jurczak MJ, Camporez JP, Lee HY, Cline GW, Samuel VT, Kibbey RG, Shulman GI (2014). Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature, 510: 542-546.

Magkos F, Sidossis LS (2007). Recent advances in the measurement of adiponectin isoform distribution. Curr Opin Clin Nutr Metab Care, 10: 571-575.

Mahindroo N, Wang CC, Liao CC, Huang CF, Lu IL, Lien TW, Peng YH, Huang WJ, Lin YT, Hsu MC, Lin CH, Tsai CH, Hsu JT, Chen X, Lyu PC, Chao YS, Wu SY, Hsieh HP (2006). Indol-1-yl acetic acids as peroxisome proliferator activated receptor agonists: design, synthesis, structural biology, and molecular docking studies. J Med Chem, 49: 1212-1216.

Makki K, Froguel P, Wolowczuk I (2013). Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. ISRN Inflamm, doi: 10.1155/2013/139239.

Malhotra JD, Kaufman RJ (2007). The endoplasmic reticulum and the unfolded protein response. Semin Cell Dev Biol, 18: 716-731.

Mandard S, Müller M, Kersten S (2004). Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci, 61: 393-416.

Martin SS, Qasim A, Reilly MP (2008). Leptin resistance: a possible interface of inflammation and metabolism in obesity-related cardiovascular disease. J Am Coll Cardiol, 52: 1201-1210.

Matough FA, Budin SB, Hamid ZA, Alwahaibi N, Mohamed J (2012). The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos Univ Med J, 12: 5-18.

Matthaei S, Stumvoll M, Kellerer M, Häring HU (2000). Pathophysiology and pharmacological treatment of insulin resistance. Endocr Rev, 21: 585-618.

Mentreddy SR (2007). Medicinal plant species with potential antidiabetic properties. Sci Food Agric, 87: 743-750.

Miglio G, Rosa AC, Rattazzi L, Collino M, Lombardi G, Fantozzi R (2009). PPARgamma stimulation promotes mitochondrial biogenesis and prevents glucose deprivation-induced neuronal cell loss. Neurochem Int, 55; 496-504.

Misra A, Khurana L (2009). The metabolic syndrome in South Asians: epidemiology, determinants, and prevention. Metab Syndr Relat Disord, 7: 497-514.

Mohan V, Gokulakrishnan K, Deepa R, Shanthirani CS, Datta M (2005). Association of physical inactivity with components of metabolic syndrome and coronary artery disease-the Chennai Urban Population Study (CUPS no. 15). Diabet Med, 22: 1206-1211.

Mohan V, Shanthirani S, Deepa R, Premalatha G, Sastry NG, Saroja R (2001). Intra-urban differences in the prevalence of the metabolic syndrome in southern India-the Chennai Urban Population Study (CUPS No. 4). Diabet Med, 18: 280-287.

Moller DE, Berger JP (2003). Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. Int J Obes Relat Metab Disord, 27: 17-21.

Morino K, Petersen KF, Shulman GI (2006). Molecular mechanism of insulin resistance in humans and their potential links with mitochondrial dysfunction. Diabetes, 55: 9-15.

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ (2009). AutoDock4 and Autodock Tools 4: Automated docking with selective receptor flexibility. J Comput Chem, 16: 2785-2791.

Moserova I, Kralova J (2012). Role of ER stress response in photodynamic therapy: ROS generated in different subcellular compartments trigger diverse cell death pathways. PLoS One, doi: 10.1371/journal.pone.0032972.

Munigoti SP, Harinarayan CV (2014). Role of Glitazars in atherogenic dyslipidemia and diabetes: Two birds with one stone? Indian J Endocrinol Metab, 18: 283-287.

Nakatani Y, Kaneto H, Kawamori D, Hatazaki M, Miyatsuka T, Matsuoka TA, Kajimoto Y, Matsuhisa M, Yamasaki Y, Hori M (2004). Modulation of the JNK pathway in liver affects insulin resistance status. J Biol Chem, 279: 45803-45809.

Naowaboot J, Chung CH, Pannangpetch P, Choi R, Kim BH, Lee MY, Kukongviriyapan U (2012). Mulberry leaf extract increases adiponectin in murine 3T3-L1 adipocytes. Nutr Res, 32: 39-44.

Niehaus WG Jr, Samuelsson B (1968). Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem, 6: 126-130.

Nielsen LL, Young AA, Parkes DG (2004). Pharmacology of exenatide (synthetic exendin-4): a potential therapeutic for improved glycemic control of type 2 diabetes. Regul Pept, 117: 77-88.

Nio Y, Yamauchi T, Iwabu M, Okada-Iwabu M, Funata M, Yamaguchi M, Ueki K, Kadowaki T (2012). Monocyte chemoattractant protein-1 (MCP-1) deficiency enhances alternatively activated M2 macrophages and ameliorates insulin resistance and fatty liver in lipoatrophic diabetic A-ZIP transgenic mice. Diabetologia, 55: 3350-3358.

Ntambi JM, Kim YC (2000). Adipocyte differentiation and gene expression. J Nutr, 130: 3122-3126.

Okami N, Narasimhan P, Yoshioka H, Sakata H, Kim GS, Jung JE, Maier CM, Chan PH (2013). Prevention of JNK phosphorylation as a mechanism for rosiglitazone in neuroprotection after transient cerebral ischemia: activation of dual specificity phosphatase. J Cereb Blood Flow Metab, 33: 106-114.

Olefsky JM (2000). Treatment of insulin resistance with peroxisome proliferator–activated receptor γ agonists. J Clin Invest, 106: 467-472.

Olson AL (2012). Regulation of GLUT4 and insulin-dependent glucose flux. ISRN Mol Biol, doi:10.5402/2012/856987.

Ong SB, Subrayan S, Lim SY, Yellon DM, Davidson SM, Hausenloy DJ (2010). Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. Circulation, 121: 2012-2022.

Oslowski CM, Urano F (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol, 490: 71-92.

Otto TC, Lane MD (2005). Adipose development: from stem cell to adipocyte. Crit Rev Biochem Mol Biol, 40: 229-242.

Ouchi N, Parker JL, Lugus JJ, Walsh K (2011). Adipokines in inflammation and metabolic disease. Nat Rev Immunol, 11: 85-97.

Ozcan L, Tabas I (2012). Role of endoplasmic reticulum stress in metabolic disease and other disorders. Annu Rev Med, 63: 317-328.

Pantoja C, Huff JT, Yamamoto KR (2008). Glucocorticoid signaling defines a novel commitment state during adipogenesis in vitro. Mol Biol Cell, 19: 4032-4041.

Parikh RM, Mohan V (2012). Changing definitions of metabolic syndrome. Indian J Endocrinol Metab, 16: 7-12.

Park YW, Zhu S, Palaniappan L, Heshka S, Carnethon MR, Heymsfield SB (2003). The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988-1994. Arch Intern Med, 163: 427-436.

Patel DK, Prasad SK, Kumar R, Hemalatha S (2012). An overview on antidiabetic medicinal plants having insulin mimetic Property. Asian Pac J Trop Biomed, 4: 320-330.

Pawlak M, Lefebvre P, Staels B (2012). General molecular biology and architecture of nuclear receptors. Curr Top Med Chem, 12: 486-504.

Penumetcha M, Santanam N (2012). Nutraceuticals as ligands of PPARγ. PPAR Res, doi:10.1155/2012/858352.

Peraldi P, Xu M, Spiegelman BM (1997). Thiazolidinediones block tumor necrosis factor-alpha-induced inhibition of insulin signalling. J Clin Invest, 100: 1863-1869.

Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK (2005). Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. Diabetes, 54: 2939–2945

Pochetti G, Godio C, Mitro N, Caruso D, Galmozzi A, Scurati S, Loiodice F, Fracchiolla G, Tortorella P, Laghezza A, Lavecchia A, Novellino E, Mazza F, Crestani M (2007). Insights into the mechanism of partial agonism: crystal structures of the peroxisome proliferator-activated receptor gamma ligand-binding

domain in the complex with two enantiomeric ligands. J Biol Chem, 282: 17314-17324.

Polvani S, Tarocchi M, Galli A (2012). PPARγ and Oxidative Stress: Con(β) Catenating NRF2 and FOXO. PPAR Res, doi: 10.1155/2012/641087.

Rabe K, Lehrke M, Parhofer KG, Broedl UC (2008). Adipokines and insulin resistance. Mol Med. 14: 741-751.

Rahman MM, McFadden G (2011). Modulation of NF-κB signalling by microbial pathogens. Nat Rev Microbiol, 9: 291-306.

Ramaa CS, Shirode AR, Mundada AS, Kadam VJ (2006). Nutraceuticals- an emerging era in the treatment and prevention of cardiovascular diseases. Curr Pharm Biotechnol, 7: 15-23.

Ramachandran A, Snehalatha C, Latha E, Satyavani K, Vijay V (1998). Clustering of cardiovascular risk factors in urban Asian Indians. Diabetes Care, 21: 967-971.

Raman P, Kaplan BL, Thompson JT, Vanden Heuvel JP, Kaminski NE (2011). 15-deoxy$\Delta^{12,14}$- prostaglandin J2-glycerol ester, a putative metabolite of 2 arachidonyl glycerol, activates peroxisome proliferator activated receptor γ. Mol Pharmacol, 80: 201-209.

Rau O, Wurglics M, Dingermann T, Abdel-Tawab M, Schubert-Zsilavecz M (2006). Screening of herbal extracts for activation of the human peroxisome proliferator activated receptor. Pharmazie, 61: 952-956.

Reaven GM (1995). Pathophysiology of insulin resistance in human disease. Physiol Rev, 75: 473-486.

Reilly MP, Lehrke M, Wolfe ML, Rohatgi A, Lazar MA, Rader DJ (2005). Resistin is an inflammatory marker of atherosclerosis in humans. Circulation, 111: 932-939.

Reilly MP, Rader DJ (2003). The metabolic syndrome: more than the sum of its parts? Circulation, 108: 1546-1551.

Relling DP, Esberg LB, Fang CX, Johnson WT, Murphy EJ, Carlson EC, Saari JT, Ren J (2006). High-fat diet induced juvenile obesity leads to cardiomyocyte dysfunction and up regulation of Foxo3a transcription factor independent of lipotoxicity and apoptosis. J Hypertens, 24: 549-561.

Remels AH, Langen RC, Gosker HR, Russell AP, Spaapen F, Voncken JW, Schrauwen P, Schols AM (2009). PPARgamma inhibits NF-kappaB-

dependent transcriptional activation in skeletal muscle, Am J Physiol Endocrinol Metab, 297: 174-183.

Richter B, Bandeira-Echtler E, Bergerhoff K, Lerch C (2008). Emerging role of dipeptidyl peptidase-4 inhibitors in the management of type 2 diabetes. Vasc Health Risk Manag, 4: 753-68.

Riemer J, Bulleid N, Herrmann JM (2009). Disulfide formation in the ER and mitochondria: two solutions to a common process. Science, 324: 1284-1287.

Rizvi SI, Mishra N (2013). Traditional Indian medicines used for the management of diabetes mellitus. J Diab Res, doi.org/10.1155/2013/712092.

Roberts CK, Sindhu KK (2009). Oxidative stress and metabolic syndrome. Life Sci, 84: 705-712.

Romeo GR, Lee J, Shoelson SE (2012). Metabolic syndrome, insulin resistance, and roles of inflammation--mechanisms and therapeutic targets. Arterioscler Thromb Vasc Biol, 32: 1771-1776.

Ron D, Walter P (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol, 8: 519-529.

Rong JX, Qiu Y, Hansen MK, Zhu L, Zhang V, Xie M (2007). Adipose mitochondrial biogenesis is suppressed in db/db and high-fat diet-fed mice and improved by rosiglitazone. Diabetes, 56: 1751-1760.

Ronti T, Lupattelli G, Mannarino E (2006). The endocrine function of adipose tissue: an update. Clin Endocrinol, 64: 355-365.

Rosen ED, MacDougald OA (2006). Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol, 7: 885-896.

Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM (2000). Transcriptional regulation of adipogenesis. Genes Dev, 14: 1293-307.

Rotruck, JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG Hoekstra WG (1973) Selenium: Biochemical role as a component of glutathione peroxidase. Science, 179: 588-590.

Rotter V, Nagaev I, Smith U (2003). Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. J Biol Chem, 278: 45777-45784.

Ruan H, Hacohen N, Golub TR, Van Parijs L, Lodish HF (2002). Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. Diabetes, 51: 1319-1336.

Rubenstrunk A, Hanf R, Hum DW, Fruchart JC, Staels B (2007). Safety issues and prospects for future generations of PPAR modulators. Biochim Biophy Acta, 1771: 1065-1081.

Ruskovska T, Bernlohr DA (2013). Oxidative stress and protein carbonylation in adipose tissue - implications for insulin resistance and diabetes mellitus. J Proteomics, 30: 323-334.

Sakamoto Y, Naka A, Ohara N, Kondo K, Iida K (2014). Daidzein regulates proinflammatory adipokines thereby improving obesity-related inflammation through PPARγ. Mol Nutr Food Res, 58: 718-726.

Salam NK, Huang TH, Kota BP, Kim MS, Li Y, Hibbs DE Hibbbs (2008). Novel PPAR gamma agonists identified from a natural products library; a virtual screening, induced fit docking and biological assay study. Chem Oil Drug Des, 71: 57-70.

Sale EM, Sale GJ (2008). Protein kinase B: signalling roles and therapeutic targeting. Cell Mol Life Sci, 65: 113-127.

Saltiel AR (2000). Series introduction: the molecular and physiological basis of insulin resistance: emerging implications for metabolic and cardiovascular diseases. J Clin Invest, 106: 163-164.

Saltiel AR, Olefsky JM (1996). Thiazolidinediones in the treatment of insulin resistance and type II diabetes. Diabetes, 45: 1661-1669.

Samavati L, Lee I, Mathes I, Lottspeich F, Hüttemann M (2008). Tumor necrosis factor alpha inhibits oxidative phosphorylation through tyrosine phosphoryoation at subunit I of cytochrome c oxidase. J Biol Chem, 283: 21134-31144.

Samuel VT, Shulman GI (2012). Mechanisms for insulin resistance: common threads and missing links. Cell, 148: 852-857.

Sarabia V, Lam L, Burdett E, Leiter LA, Klip A (1992). Glucose transport in human skeletal muscle cells in culture. Stimulation by insulin and metformin. J Clin Invest, 90: 1386-1395.

Sartipy P, Loskutoff DJ (2003). Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc Nat Acad Sci U S A, 100: 7265-7270.

Sassano G, Sanderson P, Franx J, Groot P, Straalen JV, Bassaganya-Riera J (2009). Analysis of pomegranate seed oil for the presence of jacaric acid. J Sci Food Agric, 89: 1046-1052.

Schipper HS, Prakken B, Kalkhoven, E, Boes, M (2012). Adipose tissue-resident immune cells: Key players in immunometabolism. Trends Endocrinol Metab, 23: 407-415.

Schroder M, Kaufman RJ (2005). The mammalian unfolded protein response. Annu Rev Biochem, 74: 739-789.

Scott PH, Brunn GJ, Kohn AD, Roth RA, Law-rence JC (1998). Evidence of insulin-stimulated phos-phorylation and activation of the mammalian tar-get of rapamycin mediated by a protein kinase B signaling pathway. Proc Natl Acad Sci U S A, 95: 7772-7777.

Seo AY, Joseph AM, Dutta D, Hwang JC, Aris JP, Leeuwenburgh C (2010). New insights into the role of mitochondria in aging: mitochondrial dynamics and more. J Cell Sci, 123: 2533-2542.

Serrano-Marco L, Barroso E, El Kochairi I, Palomer X, Michalik L, Wahli W, Vázquez-Carrera M (2012). The peroxisome proliferator-activated receptor (PPAR) β/δ agonist GW501516 inhibits IL-6-induced signal transducer and activator of transcription 3 (STAT3) activation and insulin resistance in human liver cells. Diabetologia, 55: 743-751.

Seufert J, Kieffer TJ, Leech CA, Holz GG, Moritz W, Ricordi C, Habener JF (1999). Leptin suppression of insulin secretion and gene expression in human pancreatic islets: implications for the development of adipogenic diabetes mellitus. J Clin Endocrinol Metab, 84: 670-676.

Shi H, Tzameli I, Bjorbaek C, Flier JS (2004). Suppressor of cytokine signaling 3 is a physiological regulator of adipocyte insulin signaling. J Biol Chem, 279: 34733-34740.

Shibasaki M, Takahashi K, Itou T, Bujo H, Saito Y (2003). A PPAR agonist improves TNF- alpha-induced insulin resistance of adipose tissue in mice. Biochem Biophys Res Commun, 309: 419-424.

Shimojo Y, Kosaka K, Shirasawa T (2007). Effect of Ganoderma lucidum extract on adipocyte differentiation and adiponectin gene expression in the murine preadipocytes cell line, 3T3-L1. Phytother Res, 25: 202-207.

Shiraki T, Kamiya N, Shiki S, Kodama TS, Kakizuka A, Jingami H (2005). Alpha,beta-unsaturated ketone is a core moiety of natural ligands for covalent binding to peroxisome proliferator-activated receptor gamma. J Biol Chem, 280: 14145-14153.

Shoelson SE, Lee J, Goldfine AB (2006). Inflammation and insulin resistance. J Clin Invest, 116: 1793-1801.

Shulman AI, Mangelsdorf DJ (2005). Retinoid X receptor heterodimers in the metabolic syndrome. N Engl J Med, 353: 604-615.

Silverstein RL, Febbraio M (2009). CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis and behaviour. Sci Signal, doi: 10.1126/scisignal.272re3.

Sivitz WI, Yorek MA (2010). Mitochondrial dysfunction in diabetes: from molecular mechanisms to functional significance and therapeutic opportunities. Antioxid Redox Signal, 12: 537-577.

Smith U, Gogg S, Johansson A, Olausson T, Rotter V, Svalstedt B (2001). Thiazolidinediones (PPARgamma agonists) but not PPAR alpha agonists increase IRS-2 gene expression in 3T3-L1 and human adipocytes. FASEB J, 15: 215-220.

Soares AF, Nosjean O, Cozzone D, D'Orazio D, Becchi M, Guichardant M, Ferry G, Boutin JA, Lagarde M, Geloen A (2005). Covalent binding of 15-deoxy-delta12,14-prostaglandin J2 to PPARgamma. Biochem Biophys Res Commun, 337: 521-525.

Song Z, Ghochani M, McCaffery JM, Frey TG, Chan DC (2009). Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. Mol Biol Cell, 20: 3525-3532.

Staels B, Fruchart JC (2005). Therapeutic roles of peroxisome proliferator-activated receptor agonists. Diabetes, 54: 2460-2470.

Stephens JM, Lee J, Pilch PF (1997). Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem, 272: 971-976.

Stephens JM, Morrison RF, Wu Z, Farmer SR (1999). PPARγ ligand-dependent induction of STAT1, STAT5A, and STAT5B during adipogenesis. Biochem Biophys Res Commun, 262: 216-222.

Stephens JM, Pekala PH (1991). Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. J Biol Chem, 266: 21839-21845.

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT (1997). Dual role of phosphatidylinositol-3, 4,5-trisphosphate in the activation of protein kinase B. Science, 5325: 567-570.

Summers SA, Garza LA, Zhou H, Birnbaum MJ (1998). Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by cer amide. Mol Cell Boil, 9: 5457-5464.

Szulinska M, Musialik K, Suliburska J, Lis I, Bogdanski P (2014). The effect of L-arginine supplementation on serum resistin concentration in insulin resistance in animal models. Eur Rev Med Pharmacol Sci, 18: 575-580.

Tangvarasittichai S (2015). Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. World J Diabetes, 6: 456-480.

Tanti JF, Jager J (2009). Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. Curr Opin Pharmacol, 9: 753-762.

Tateya S, Kim F, Tamori Y (2013). Recent advances in obesity-induced inflammation and insulin resistance. Front Endocrinol (Lausanne), doi: 10.3389/fendo.2013.00093.

Tenenbaum A, Motro M, Fisman EZ (2005). Dual and pan-peroxisome proliferator-activated receptors (PPAR) co-agonism: the bezafibrate lessons. Cardiovasc Diabetol, doi:10.1186/1475-2840-4-14.

Tilg H, Moschen AR (2006). Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol, 6: 772-783.

Todaro GJ , Green H (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol, 17: 299-313.

Tontonoz P, Hu E, Devine J, Beale EG, Spiegelman BM (1995). PPARγ2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. Mol Cell Biol, 15: 351-357.

Trayhurn P, Wood IS (2004). Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr, 92: 347-55.

Trujillo ME, Scherer PE (2006). Adipose tissue-derived factors: impact on health and disease. Endocr Rev, 27: 762-778.

Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S (2011). The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. J Adv Pharm Technol Res, 2: 236-240.

Ursini F, Maiorino M, Brigelius-Flohé R (1995). The diversity of glutathione peroxidase. Meth Enzymol, 252: 38-63.

Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS (1997). Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature, 389: 610-614.

van der Kallen CJ, van Greevenbroek MM, Stehouwer CD, Schalkwijk CG (2009). Endoplasmic reticulum stress-induced apoptosis in the development of diabetes: is there a role for adipose tissue and liver? Apoptosis, 14: 1424-1434.

Viladomiu M, Hontecillas R, Lu P, Bassaganya-Riera J (2013). Preventive and prophylactic mechanisms of action of pomegranate bioactive constituents. J Evid Based Complement Alternat Med, doi.org/10.1155/2013/789764 64.

Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F (2012). Cellular and molecular mechanisms of metformin: an overview. Clin Sci (Lond), 122: 253-270.

Wada T, Hoshino M, Kimura Y, Ojima M, Nakano T, Koya D, Tsuneki H, Sasaoka T (2011). Both type I and II IFN induce insulin resistance by inducing different isoforms of SOCS expression in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab, 300: 1112-1123.

Wang J, Green PS, Simpkins JW (2001). Estradiol protects against ATP depletion, mitochondrial membrane potential decline and the generation of reactive oxygen species induced by 3-nitroproprionic acid in SK-N-SH human neuroblastoma cells. J Neurochem, 77: 804-811.

Wang L, Waltenberger B, Pferschy-Wenzig EM, Blunder M, Liu X, Malainer C, Blazevic T, Schwaiger S, Rollinger JM, Heiss EH, Schuster D, Kopp B, Bauer R, Stuppner H, Dirsch VM, Atanasov AG (2014). Natural product agonists of peroxisome proliferator-activated receptor gamma (PPARγ): a review. Biochem Pharmacol, 92: 73-89.

Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM, Ron D (1996). Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol Cell Biol, 16: 4273-80.

Wassink, AM, Olijhoek JK, Visseren,FL (2007). The metabolic syndrome: metabolic changes with vascular consequences. Eur J Clin Invest, 37: 8-17.

Watkins PB, Whitcomb RW (1998). Hepatic dysfunction associated with troglitazone. New England J Med, 338: 916-917.

Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr (2003). Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest, 112: 1796-1808.

Weydert CJ, Cullen JJ (2010). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc, 5: 51-66.

Wilborn C, Beckham J, Campbell B, Harvey T, Galbreath M, La Bounty P, Nassar E, Wismann J, Kreider R (2005). Obesity: prevalence, theories, medical consequences, management, and research directions. J Int Soc Sports Nutr, 9: 4-31.

Wilcox G (2005). Insulin and insulin resistance. Clin Biochem Rev, 26: 19-39.

Willson TM, Brown PJ, Sternbach DD, Henke BR (2000). The PPARs: from orphan receptors to drug discovery. J Med Chem, 43: 527-550.

Wilson AP (2000). Cytotoxicity and viability assays. In: J.R.W. Masters (Eds) Animal cell culture: A practical approach. 2nd ed. Oxford: Oxford University Press, 175-219.

Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloro S, Czech M, Corvera S (2003). Mitochondrial biogenesis and remodelling during adipogenesis and in response to the insulin sensitizer rosiglitazone. Mol Cell Biol, 23: 1085-1094.

Wise LS, Green H (1979). Participation of one isozyme of cytosolic glycero phosphate dehydrogenase in the adipose conversion of 3T3 Cells. J Biol Chem, 254: 273-275.

Wouters BG, Koritzinsky M (2008). Hypoxia signalling through mTOR and the unfolded protein response in cancer. Nat Rev Cancer, 8: 851-864.

Wu J, Boström P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G, Huang K, Tu H, van Marken Lichtenbelt WD, Hoeks J, Enerbäck S, Schrauwen P, Spiegelman BM (2012). Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell, 150: 366-376.

Wu S, Zhou F, Zhang Z, Xing D (2011). Mitochondrial oxidative stress causes mitochondrial fragmentation via differential modulation of mitochondrial fission-fusion proteins. FEBS J, 278: 941-954.

Xu C, Bailly-Maitre B, Reed JC (2005). Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest, 115: 2656-2664.

Xue X, Piao JH, Nakajima A, Sakon-Komazawa S, Kojima Y, Mori K, Yagita H, Okumura K, Harding H, Nakano H (2005). Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha. J Biol Chem, 280: 33917-33925.

Yamauchi T, Kamon J, Waki H, Murakami K, Motojima K, Komeda K, Ide T, Kubpta N,Terauchi Y, Tobe K, Miki H, Tsuchida A, Akanuma Y, Nagai R, Kimura S, Kadowaki T (2001). The mechamisms by which both heterozygous peroxisome proliferator-activated receptor γ (PPARγ) deficiency and PPARγ agonist improve insulin resistance. J Boil Chem, 276: 41245-41254.

Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, Kawamoto S, Kubota N, Kubota T, Ito Y, Kamon J, Tsuchida A, Kumagai K, Kozono H, Hada Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Awazawa M, Takamoto I, Froguel P, Hara K, Tobe K, Nagai R, Ueki K, Kadowaki T (2007). Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. Nat Med, 13: 332-339.

Yan J, Zhao Y, Suo S, Liu Y, Zhao B (2012). Green tea catechins ameliorate adipose insulin resistance by improving oxidative stress. Free Radic Biol Med, 52: 1648-1657.

Yang Y, Shang W, Zhou L, Jiang B, Jin H, Chen M (2007). Emodin with PPARγ ligand-binding activity promotes adipocyte differentiation and increases glucose uptake in 3T3-Ll cells. Biochem Biophys Rese Commun, 353: 225-230.

Yang Z, Hulver M, McMillan RP, Cai L, Kershaw EE, Yu L, Xue B, Shi H (2012). Regulation of insulin and leptin signaling by muscle suppressor of cytokine signaling 3 (SOCS3). PLoS One, doi: 10.1371/journal.pone.0047493.

Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell, 107: 881-891.

Yoshiuchi K, Kaneto H, Matsuoka TA, Kasami R, Kohno K, Iwawaki T, Nakatani Y, Yamasaki Y, Shimomura I, Matsuhisa M (2009). Pioglitazone reduces ER stress in the liver: direct monitoring of in vivo ER stress using ER stress-activated indicator transgenic mice. Endocr J, 56: 1103-1111.

Yu T, Sheu SS, Robotham JL, Yoon Y (2008). Mitochondrial fission mediates high glucose-induced cell death through elevated production of reactive oxygen species. Cardiovasc Res, 79: 341-351.

Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, and Shoelson SE (2001). Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science, 293: 1673-1677.

Zabolotny JM, Kim YB, Welsh LA, Kershaw EE, Neel BG, Kahn BB (2008). Protein-tyrosine phosphatase 1B expression is induced by inflammation in vivo. J Biol Chem, 283: 14230-14241.

Zhang F, Lavan BE, Gregoire FM (2007). Selective Modulators of PPAR-gamma Activity: Molecular Aspects Related to Obesity and Side-Effects. PPAR Res, doi:10.1155/2007/32696.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994). Positional cloning of the mouse obese gene and its human homologue. Nature, 372: 425-432.

Zhang Z, Zhao M, Li Q, Zhao H, Wang J, Li Y (2009). Acetyl-l-carnitine inhibits TNF-alpha-induced insulin resistance via AMPK pathway in rat skeletal muscle cells. FEBS Lett, 538: 470-474.

Zhiyong L, Zhihua W, Mingjiang W (2012). Cirsium japonicum flavones enhance adipocyte differentiation and glucose uptake in 3T3-L1 cells. Biol Pharm Bull, 35: 855-860.

Zolezzi JM, Silva-Alvarez C, Ordenes D, Godoy JA, Carvajal FJ, Santos MJ, Inestrosa NC (2013). Peroxisome Proliferator-Activated Receptor (PPAR) gamma and PPARalpha agonists modulate mitochondrial fusion-fission dynamics: Relevance to reactive oxygen species (ROS)-related neurodegenerative disorders? PLoS One, doi: 10.1371/journal.pone.0064019.

List of Publications

1. **Anusree SS**, Nisha VM, Priyanka A, Raghu KG (2015). Insulin resistance by TNF-α is associated with mitochondrial dysfunction in 3T3-L1 adipocytes and is ameliorated by punicic acid, a PPARγ agonist. **Molecular and Cellular Endocrinology**, 413: 120-128.
2. **Anusree SS**, Priyanka A, Nisha VM, Arya A Das, Raghu KG (2014). An in vitro study reveals nutraceutical potential of punicic acid relevant to diabetes via enhanced GLUT4 expression and adiponectin secretion. **Food and Function**, 5: 2590-2601.
3. Nisha VM, Priyanka A, **Anusree SS**, Raghu KG (2014). (-)-Hydroxycitric acid attenuates endoplasmic reticulum stress-mediated alterations in 3T3-L1 adipocytes by protecting mitochondria and downregulating inflammatory markers. **Free Radical Research**, 48: 1386-1396.
4. Priyanka A, Nisha VM, **Anusree SS**, Raghu KG (2014). Bilobalide attenuates hypoxia induced oxidative stress, inflammation, and mitochondrial dysfunctions in 3T3 L1 adipocytes via its antioxidant potential. **Free Radical Research**, 48: 1206-1217.
5. Priyanka A, **Anusree SS**, Nisha VM, Raghu KG (2014). Curcumin improves hypoxia induced dysfunctions in 3T3-L1 adipocytes by downregulating oxidative stress, inflammation, and mitochondrial alterations. **Biofactors**, 40: 513-523.
6. Nisha VM, **Anusree SS**, Priyanka A, Raghu KG (2014). Apigenin and quercetin ameliorate mitochondrial alterations by tunicamycin-induced ER stress in 3T3-L1 adipocytes. **Applied Biochemistry Biotechnology**, 174: 1365-1375.
7. Shyni GL, Kavitha S, Indu S, Arya AD, **Anusree SS**, Vineetha VP, Vandana S, Sundaresan A, Raghu KG (2014). Chebulagic acid from Terminalia chebula enhances insulin mediated glucose uptake in 3T3-L1 adipocytes via PPARγ signaling pathway. **Biofactors,** 40: 646-657.
8. Prathapan A, Mukesh Kumar Singh, **Anusree SS**, DR Soban Kumar, A Sundaresan, KG Raghu (2011). Antiperoxidative, free radical scavenging and metal chelating activities of *Boherhaavia diffusa* L. **Food Biochemistry**, 35: 1548-1554.

Presentations in Scientific conferences

- Oral presentation on "Punicic acid enhances adipocyte differentiation, glucose uptake and inhibits oxidative stress in adipocytes- an *in vitro* cell line based study". International Conference on Phytochemicals in Health and Disease: Challenges and Future Opportunities (ICPHD), Annamalai University, January 23-25, 2013.
- Poster presentation on "Punicic acid enhances GLUT4 expression and translocation in 3T3-L1 adipocytes via PPAR gamma & PI3 kinase dependent mechanism". International conference on recent advances in research and treatment of human diseases & 4th annual meeting of Indian Academy of Biomedical Sciences, CSIR-IICT, Tarnaka, Hyderabad, India. January 9-11, 2015.