PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-GAMMA P465L MUTATION IN DIABETES AND ATHEROSCLEROSIS

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ABSTRACT

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(Under the direction of Dr. Nobuyo Maeda, PhD)

The dominant-negative P467L mutation in peroxisome proliferator activated receptor-γ (PPARγ) was identified in insulin resistant patients with hyperglycemia and lipodystrophy. However, mice carrying the corresponding *Pparg-P465L* mutation have normal insulin sensitivity, with mild hyperinsulinemia. I hypothesized that murine *Pparg-P465L* mutation leads to covert insulin resistance, masked by hyperinsulinemia, to retain normal plasma glucose. I introduced in $Pparg^{P465L/+}$ mice, *Ins2-Akita* mutation that causes islet apoptosis to lower plasma insulin. Unlike $Ins2^{Akita/+}$ littermates, male $Pparg^{P465L/+}Ins2^{Akita/+}$mice have drastically reduced life-span. $Pparg^{P465L/+}Ins2^{Akita/+}$ females have aggravated hyperglycemia, smaller islets, reduced plasma insulin and impaired insulin sensitivity. Enhanced gluoconeogenesis, due to reduced plasma insulin, in $Pparg^{P465L/+}Ins2^{Akita/+}$ females compared to $Ins2^{Akita/+}$ littermates, contributes to their fasting hyperglycemia. While liver and skeletal muscles remain sensitive, adipose tissue is resistant to insulin. $Pparg^{P465L/+}Ins2^{Akita/+}$ mice have smaller fat depots composed of larger adipocytes suggesting impaired lipid storage with subsequent hepatomegaly and hypertriglyceridemia.

The increasing prevalence of obesity and insulin resistance and its negative impact on atherosclerotic cardiovascular disease is of major concern. In the second part of my study, I hypothesized that the L/+ mutation will worsen atherosclerosis in *Apoe-/-* mice. Despite a consistent increase in blood pressure, $PPAR\gamma^{P465L/+}$ mutation did not affect plaque size in *Apoe-/-* mice fed normal chow or high-fat-diet. Gene expression studies revealed significantly increased ABCA-1 and decreased CD-36 in $PPAR\gamma^{P465L/+;}$*Apoe-/-* macrophages compared to *Apoe-/-* littermates, suggesting reduced lipid accumulation. Cultured $PPAR\gamma^{P465L/+;}$*Aoe-/-* macrophages, had a significant reduction in DiI-labeled-VLDL uptake, compared to *Apoe-/-* macrophages. It is likely that balance between increased blood pressure with its pro-atherogenic potential and macrophage athero-protective phenotype results in an unchanged plaque. Bone marrow transfer experiments to investigate the role of $PPAR\gamma^{P465L/+}$ mutation specifically in the macrophage showed a trend towards decrease in plaque size in mice reconstituted with $PPAR\gamma^{P465L/+;}$*Apoe-/-* macrophages.

Thus, I have established the causative link between PPARγP465L mutation and insulin resistance phenotype, consolidating the crucial role of PPARγ in diabetes. PPARγP465L mutation, however, does not change the atherosclerotic plaque size in *Apoe-/-* mice, and warrants further investigation.

ACKNOWLEDGMENTS

The author wishes to Last few years in Chapel Hill have been wonderful. From a sheltered life in India at my parent's house, I moved to a new country, to a new work environment. Nobuyo gave me an opportunity of a life time by accepting me as a member of her lab. This one step changed the entire course of my life. While everyone in my medical school class was looking out for residency positions, I embarked on a research adventure. The Smithies-Maeda lab has been the best starting point any one can ask for. I came in with absolute lack of research experience and am very grateful to Nobuyo for taking a chance on me. Yau-Sheng Tsai was my graduate student mentor and he taught me the basic lab techniques. Yau has been a good influence on me – meticulous and careful – against my rushing self. He taught me to think, to be patient and to eat sushi!

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Mike and Jen have been ever supportive and I have enjoyed going to their awesome house and hanging out in the yard numerous times. Feng and Svetlana are my best girl-friends, the category I am tempted to put Lance and Ray in as well. I value the guidance and friendship of Kumar, Jose, Matt (honorary member of out look-alike board), Takashi (ex member), and Sylvie. I have learnt a lot from Heather, Hirofumi, Masao, Yi, Ron, John H, Dr. Kim and Shin-Ja. Thanks also to Marcus, Ruri, Randy, Chih-Hong, Yukako, Hind (ex-member), Cassandra; I can not imagine this lab without them. Special thanks to Sabrina and Taylor for the opportunity to mentor them.

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in-laws for granted. I am lucky to have them as a part of my life and also my brother Neeraj, his wife Deepa, my sister-in-law Yogita and her husband Salil….yes, it is quite confusing having two Salils in the family! I have dedicated this thesis to my two little nephews – Siddharth and Arnav, though not with any intent to put pressure on them. I just want to wish them good luck in whatever they choose to do in life.

Coming to Chapel Hill also got me my best friend – my husband, Salil. Without undermining the science and the degree, finding Salil has been the best thing that ever happened to me in Chapel Hill. He has been a constant support and guide in everything I do……at times unsolicited. I do not think I can ever thank him enough for all the things he has done and will do in years to come.

As I am about to leave the lab, the thought of leaving all these people makes me sad. The time spent here with all my friends is very valuable and I will hold them all close to my heart forever.

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LIST OF ABBREVIATIONS

9-HODE	9-hydroxy octadecadienoic acid
13-HODE	13-hydroxy octadecadienoic acid
15d-PGJ2	15d-Prostaglandin J2
15-HETE	15-hydroxy eicosatetraenoic acid
ABCA-1	ATP-binding cassette transporter
AdipoR	Adiponectin receptor
AF	Activation function
aP2	Adipocyte fatty acid binding protein
AP1	Activator protein 1
BMT	Bone marrow transfer
BP	Blood pressure
CCL2	Chemokine CC-chemokine ligand
CCR2	CC chemokine receptor 2
C/EBP	CCAAT-enhancer binding protein
DBD	DNA-binding Domain
DiI	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
eNOS	Endothelial nitric oxide synthase
GF	Perigonadal fat
GK	Glucokinase

GLUT-2	Glucose transporter - 2
GLUT-4	Glucose transporter – 4
GSIS	Glucose stimulated insulin secretion
hPPAR	Human Peroxisome Proleferator Activated Rceeptor
H&E	Hematoxylin and eosin
HDL	High-density lipoprotein
HFD	High fat diet
IBMX	3-isobutyl-1-methylxanthine
IF	Inguinal fat
iNOS	Inducible nitric oxide synthase
IPITT	Intra-peritoneal insulin tolerance test
IRE	Inflammatory response element
IRS-2	Insulin receptor substrate – 2
LBD	Ligand-binding Domain
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LDLRKO	Low-density lipoprotein receptor knock out
mPPAR	Mouse Peroxisome Proleferator Activated Rceeptor
mRNA	Messenger ribonucleic acid
MetS	Metabolic syndrome

MCP-1	Monocyte chemoattractant protein
NCoR	Nuclear receptor corepressor
NEFA	Non-esterified fatty acid/ Free fatty acid
NF-κB	Nuclear factor – κB
NO	Nitric oxide
NS	Non-significant
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PEPCK	Phosphoenol pyruvate carboxykinase
PPRE	Peroxisome Proliferator Response Element
PPARα	Peroxisome Proleferator Activated Rceeptor – alpha
PPARβ/δ	Peroxisome Proleferator Activated Rceeptor – beta/delta
PPARγ	Peroxisome Proleferator Activated Rceeptor – gamma
ROS	Reactive oxygen species
RXR	Retinoid X Receptor
SREBP	Steroid receptor element binding protein
STZ	Streptozotocin
TG	Triglyceride
TZD	Thiazolidinedione
UTR	Untranslated region

VLDL Very low-density lipoprotein

VSMC Vascular smooth muscle cell

xPPAR Xenopus Peroxisome Proleferator Activated Rceeptor

Chapter 1

BACKGROUND

The nuclear hormone receptor super-family consists of several structurally related members that have a diverse array of biological functions [1]. Some of the prominent members of this family include the estrogen, progesterone, glucocorticoid and thyroid hormone receptors. These molecules share a structural organization, which consists of an N-terminal, DNA-binding domain and a C-terminal, ligand-binding domain. The nuclear hormone receptors bind to their specific ligands and transcriptionally control numerous genes in several different gene networks. The liganded nuclear hormone receptors recognize a unique transcription enhancer sequence, known as the hormone response element, and regulate the transcription of downstream genes. In addition to their dedicated ligands, nuclear hormone receptors work with several co-activator and co-repressor proteins and transcription factors to induce or suppress their target genes.

Peroxisome Proliferator Activated Receptors (PPARs) belong to the superfamily of nuclear hormone receptors and three major isoforms, encoded by separate genes, have been discovered to date - PPARα, PPARδ/β and PPARγ [2]. In contrast to the typical members, such as estrogen receptor with its known exclusive ligands, PPARs are considered as orphan receptors since the unique, high-affinity, endogenous ligands for the PPAR isoforms remain elusive.

1.1 Discovery of PPARs

Continued exposure to hypolipidemic drugs, such as clofibrate, certain herbicides and plasticizers, was known to cause peroxisome proliferation and induce microsomal compartment in mouse and rat liver parenchymal cells eventually leading to hepatic tumors [3, 4]. These peroxisome proliferators did not damage DNA and are structurally quite different from each other. This led to a proposition by Reddy and Rao in 1986 that the responses to peroxisome proliferators and stimulation of genes encoding fatty acid oxidation enzymes is brought about by soluble receptor/s [5]. The experimental evidence for this potential receptor mediated mechanism was provided by previous work of Goldfischer and Reddy in 1984, who demonstrated specific soluble binding sites for peroxisome proliferators in liver and kidney cell extracts [6]. Eventually, a mouse receptor mPPAR (mouse peroxisome proliferator activated receptor) was isolated and was shown to be activated by peroxisome proliferators [7]. In 1992, Dreyer *et al* cloned three novel members from the nuclear hormone receptor superfamily that were structurally and functionally related to mPPAR. These new sequences isolated from *Xenopus laevis* were named xPPARα, β, and γ. These novel members were shown to be transcription factors and mediated the peroxisome proliferator-induced response of acyl coenzyme A (acyl-coA) oxidase gene, an important enzyme in the peroxisomal fatty acid oxidation. The human peroxisome proliferator activated receptor (hPPAR) was soon cloned from a human liver and fat cell cDNA library [8, 9]. Similar to Xenopus and mouse PPAR, the hPPAR was also shown to transactivate the acyl-coA oxidase PPAR response element with the prototypical peroxisome proliferators, including clofibrate,

nafenopin and WY-14643 [8]. Subsequently, the presence and differential expression of the three PPAR isoforms was confirmed in mice and humans [8, 10, 11].

1.2 Peroxisome Proliferator Activated Receptor-γ

Among the three PPAR isoforms, PPARγ is essential for adipocyte differentiation, glucose and lipid metabolism, inflammation, and regulation of cell growth [12]. PPARγ functions as a transcription factor and is most abundantly expressed in adipocytes and is also found in liver, large intestine, β cells of pancreatic islets, macrophages, skeletal muscle and kidney [13, 14]. Thiazolidinedione drugs, such as Rosiglitazone, which were already in use as insulin sensitizers for treating diabetic patients, were found to be specific, high affinity ligands for PPARγ.

1.3 PPARγ Gene and Protein Structure

PPARγ exists as four protein isoforms - PPARγ1 to PPARγ4 - derived from a single gene through differential promoter usage and different leader exons [15]. The PPARG gene is located on chromosome 3 in humans and is composed of nine exons which span more than 100 kb [14, 16]. All four transcripts contain six common exons (exons 1-6). The human PPARγ1 mRNA is coded by 8 exons. Exon A1 and A2 comprise the 5'-untranslated region of human PPARγ1 and are followed by exons 1 to 6. PPARγ2 consists of an additional 28 amino acids, which are encoded by exon B. As shown in figure 1.1, mouse *Pparg* is located on chromosome 6 and extends more than 105 kb [17]. Mouse PPARγ-2 has an additional 30 amino acids in the N-terminus of the molecule

compared to mouse PPARγ1. Mouse PPARγ1 is composed of eight exons (A1, A2 and 1-6), whereas mouse PPARγ2 consists of seven exons (B1, 1-6) [17].

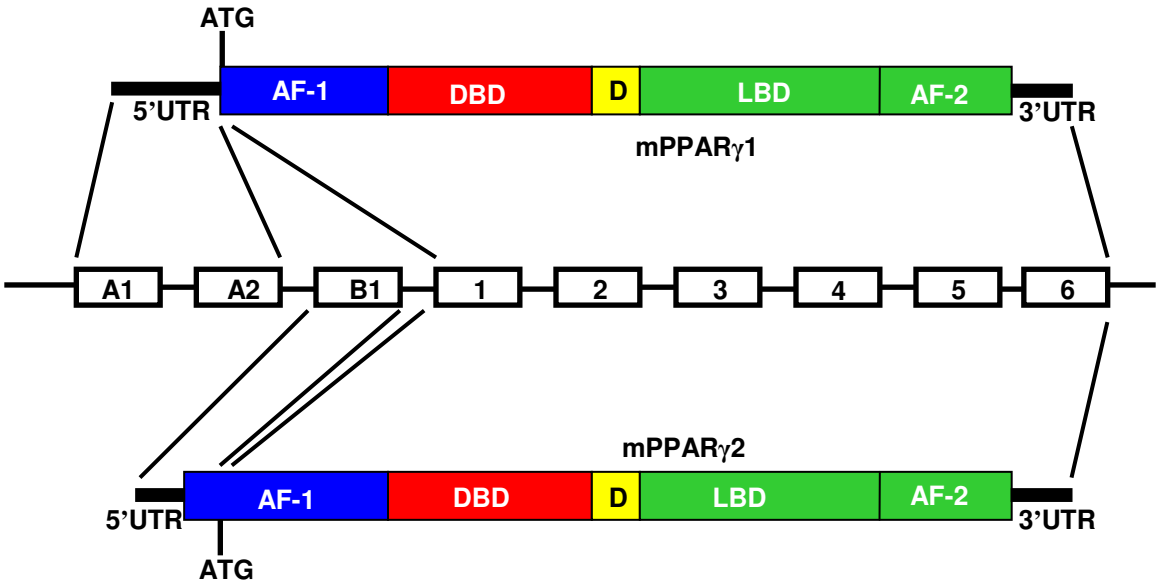

Figure 1.1: Schematic of the mouse PPARγ gene and mRNA structures of mouse PPARγ1 and mouse PPARγ2. The 5'-UTR of m PPARγ1 is encoded by exon A1 and A2. Exon B1 codes for the 5'-UTR of m PPARγ2 and 30 additional amino acids in the N-terminal of the protein. (adapted from Zhu,Y *et al* PNAS, 1995, ref#17)

Considering the relative amounts of transcripts, PPARγ2 is the most important form of the protein and is almost exclusively expressed in the adipose tissue with some expression in the pancreatic islets [18]. PPARγ1 and PPARγ3 are more ubiquitously expressed. Little information is available about the PPARγ4 isoform, however it seems to be important in adipocytes.

The human and mouse PPARγ proteins demonstrate a 99% similarity and 95% identity in their amino acids [14]. PPARγ is one of the typical members of the nuclear receptor superfamily. Thus, the protein structure consists of an N-terminal domain, which has a ligand-independent transcriptional activation function (AF-1), a DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) with a ligand-dependent transcriptional activation function (AF-2) [19, 20]. The D site is a docking domain for cofactors, which can be different co-activator and co-repressor proteins.

1.4 PPARγ Ligands

Many compounds, like polyunsaturated fatty acids and prostaglandins, bind to PPARγ [21]. Since these compounds exist in cells at much lower concentrations than necessary for effective PPARγ binding, their ability to activate PPARγ with significant downstream effects and consequently their role as a true endogenous ligand are debatable. Out of all the known nuclear receptors, PPARs have a ligand binding domain with a very large volume, which measures about 1300 $Å^3$[22]. This large ligand binding domain allows PPARγ to bind to a wide variety of ligands, which vary in size and shapes. Thus, polyunsaturated fatty acids, oxidized fatty acids, eicosanoids, and prostaglandin

J2 are some of the compounds known to be the natural ligands of PPARγ (Table 1.1) [23, 24]. PPARγ is activated by synthetic compounds, including Thiazolidinediones (TZD), with higher affinity and kd values at nanomolar levels [25]. TZDs are one of the principle drugs in the treatment of diabetes mellitus [21] and are known to increase insulin sensitivity and improve hyperglycemia by up-regulating glucose transporters GLUT-2 and GLUT-4 [26, 27].

PPARγ agonists		Binding affinity
Synthetic	Thiazolidinedione Rosiglitazone Pioglitazone Troglitazone Ciglitazone	High (Nanomolar)
Natural/ Endogenous	15d-PGJ2 9-HODE 13-HODE 15-HETE oxLDL	Medium (low Micromolar)
Natural/Endogenous	Linoleic acid Arachidonic acid	Low (high Micromolar to Millimolar)

Table 1.1: PPARγ agonsists and their relative binding affinity. 15d-PGJ2, 15d-prostaglandin J2; 9-HODE, 9-hydroxy octadecadienoic acid; 13-HODE, 13-hydroxy octadecadienoic acid; 15-HETE, 15-hydroxy eicosatetraenoic acid; oxLDL, oxidized low density lipoprotein (adapted from Bishop-Bailey 2003, ref#28).

1.5 Mechanism of Action of PPARγ

Transactivation: PPARγ regulates the function of its target genes via activation of transcription. It heterodimerizes with Retinoid X Receptor-α (RXRα) and binds to the Peroxisome Proliferator Response Element (PPRE) in the promoter region of the target genes [21]. Most PPREs are degenerate variants of a direct repeat of the hormone response element sequence (AGGTCA) separated by one base pair [29]. In the absence of the ligand, the PPARγ /RXR heterodimer is bound to co-repressor proteins, such as histone deacetylases and chromatin-modifying factors. This complex binds to the PPRE in the regulatory region of the target gene leading to active silencing of transcription. Binding of a ligand to the LBD causes a conformational change in PPARγ, release of repressor molecules, and recruitment of co-activator/s, resulting in an induction in the transcription of the downstream genes (Figure 1.2). The co-activators include proteins which modify chromatin structure and bridge PPARγ to the transcription machinery. The majority of these downstream genes such as Glut-4, Glut-2, adipocyte fatty acid binding protein (aP2), phosphoenolpyruvate carboxykinase (PEPCK) and insulin receptor substrate (IRS-2) are involved in glucose and lipid metabolism [21]. Thus, mutations in PPARγ may alter the glucose homeostatic and adipogenic signals and contribute to metabolic disorders.

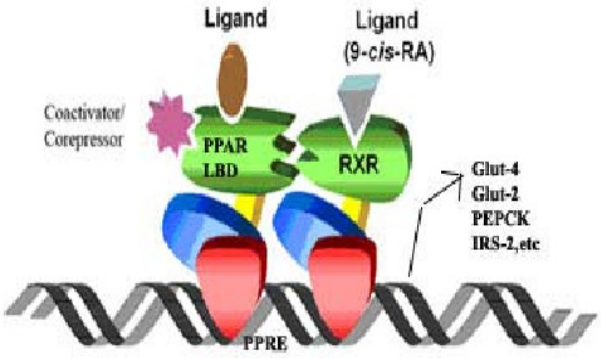

Figure 1.2: Schematic of PPARγ transactivation. Upon ligand binding in the presence of co-activator proteins, the PPARγ/ RXR heterodimer binds to the PPRE of its target genes to induce transcription (adapted from Tsai *et al,* 2005, ref#30).

Transrepression: In addition to the direct regulation of transcription, another mechanism of action of PPARγ termed transrepression has been proposed and comprehensively studied in macrophages [31]. In the transrepression pathway, PPARγ has been shown to interact with other transcription factors, such as NF-κB and AP1. An inflammatory stimulus induces the transcription of pro-inflammatory genes via transcription factors, such as NF-κB andAP1, due to association of these factors with the inflammatory response element (IRE) [31]. PPARγ with its ligand forms a part of an inhibitory complex (IC) that reduces the expression of the pro-inflammatory genes and exerts an anti-inflammatory action (Figure 1.3). In this pathway, PPARγ does not need to form a heterodimer with RXR and does not require a PPRE. The inhibitory complex consists of post-transcriptionally modified PPARγ, SUMO (small ubiquitin-like modifier)-protein ligases, ubiquitin-conjugating enzymes, and proteins that link these with co-repressors to suppress NF-κB and AP1 [32]. The first step of the trans-repression pathway begins with ligand-dependent SUMOylation of the PPARγ ligand binding domain, which targets PPARγ to nuclear receptor corepressor (NCoR)-histone deacetylase -3 (HDAC3) complexes on the promoter of inflammatory genes. This prevents recruitment of the ubiquitylation/19S proteosome machinery that is responsible for a signal-dependent removal of corepressor complexes required for gene activation. Thus, NCoR complexes are not cleared from the promoter and the transcription of the target pro-inflammatory genes is suppressed [31].

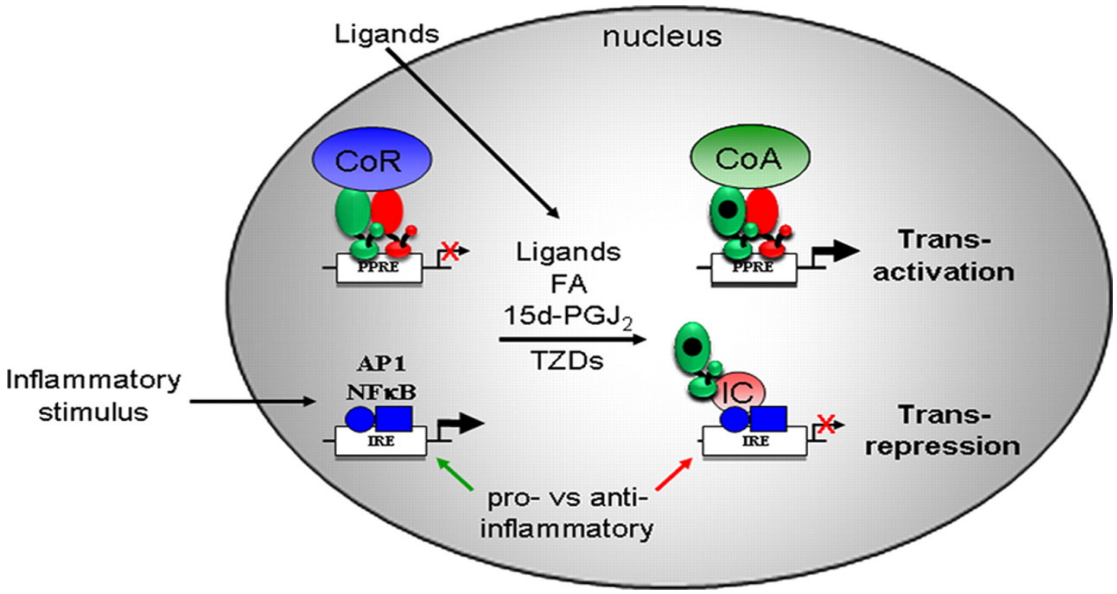

Figure 1.3: Mechanism of action of PPARγ: transrepression pathway by which PPARγ regulates the expression of inflammatory genes is primarily characterized in the macrophage (Ketsawatsomkron, 2010, ref#32).

The functional impact caused by PPARγ is complicated by the fact that more than one compound has been shown to act as a ligand and multiple compounds are known to act as co-activators and co-repressors. Unique conformational changes in response to a particular ligand may allow for the recruitment of a specific group of co-activators, resulting in the induction of a subset of genes in response to that ligand. The relative availability of the ligands and co-activators in a particular cell type and under specific metabolic states may also contribute to the resulting transactivation/ transrepression of the target genes.

1.6 Biological Functions of PPARγ

PPARγ in adipogenesis: PPARγ is essential for the formation of adipose tissue in vivo [12, 33] . In addition, PPARγ has been shown to have a role in glucose metabolism and insulin sensitivity, inflammation and growth regulation. Below, I discuss a few of the biological processes in which PPARγ plays a critical role, thus highlighting the importance of this molecule in metabolism.

PPARγ PPARγ is crucial for the differentiation of adipose tissue and for the deposition of triglycerides in adipocytes. PPARγ is expressed early in adipogenesis in the fibroblast-like preadipocytes that are destined to become mature adipocytes. PPARγ expression is brought about by transcription factors C/EBPβ and C/EBPδ with availability of insulin and steroid hormones [34, 35]. In a well defined positive feed back loop, C/EBPα increases the expression of PPARγ in the pre-adipocytes (Figure 1.4).

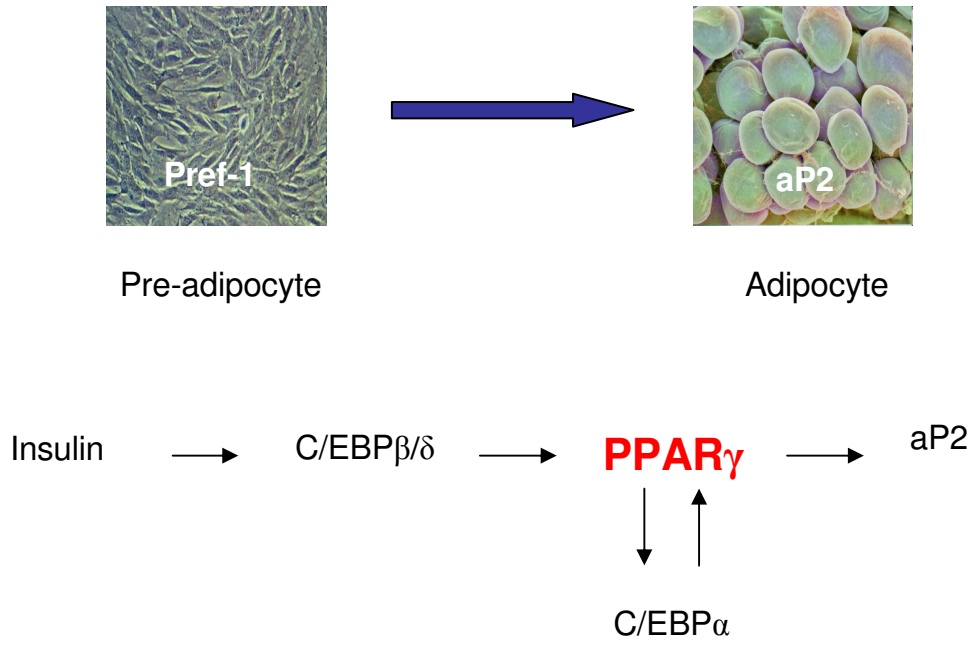

Figure 1.4: Schematic of adipocyte differentiation from a fibroblast like pre-adipocyte cell. Pref-1 is a pre-adipocyte specific gene, aP2, adiponectin are adipocyte specific genes.

The over expression of PPARγ by itself can induce the conversion of cultured fibroblasts (NIH-3T3 cells) into adipocytes [36]. Additionally, PPARγ and C/EBPα can together induce trans-differentiation of myoblasts into adipocytes [37]. Consistent with these over-expression in-vitro studies, Rosen *et al* reported that when embryonic stem cells lacking both copies of PPARγ were subjected to an adipose differentiation protocol, no adipocytes developed [38]. PPARγ knockout pups, rescued by tetraploid aggregation to circumvent placental pathology, have complete absence of adipose tissue (Table 1.3#1) [12]. Mice with 25% normal PPARγ expression have a significantly reduced adipose tissue mass [39] (Table 1.3#4) and a severe lipodystrophy phenotype has been demonstrated in mice specifically lacking PPARγ2, the isoform predominantly expressed in white adipose tissue [40] (Table 1.3#3).

A number of point mutations in PPARγ have been reported to occur within human populations. These mutations, which affect adipose tissue distribution, further confirm the crucial role of PPARγ in adipose physiology. For example, heterozygous P467L and V290M mutations in the ligand binding domain of PPARγ were identified among insulin resistant patients. The mutations act in a dominant-negative fashion, since heterozygous presence of this mutation reduces transactivation by PPARγ to significantly less than 50%. These patients have normal total adipose tissue weights, however, the fat distribution is altered resulting in increased intra-peritoneal fat deposits and a decrease in subcutaneous fat [51].

Gene	Function	Reference
Adipose Tissue		
aP2/ Adipose fatty acid binding protein	Intracellular fatty acid binding	Wu, 1995, Genes Dev
Adiponectin	Adipose-derived, insulin sensitizing cytokine	Iwaki, 2003, Diabetes
Acyl CoA synthetase	Fatty acid transport, lipogenesis and catabolism	Martin, 1997, JBC
FATP-1/ Fatty acid transporter, member 1	Regulation of intracellular fatty acid concentration	Martin, 1997, JBC
PEPCK/ Phosphoenolpyruvate carboxykinase	Glyceroneogenesis	Tontonoz, 1995, Mol Cell Biol
Macrophage		
ABCA-1/ATP-binding cassette protein-1	Choleserol efflux	Akiyama, 2002, Mol Cell Biol
CD-36/ Scavenger receptor	Lipid uptake	Akiyama, 2002, Mol Cell Biol
Apolipoprotein E	Receptor-mediated lipoprotein uptake	Akiyama, 2002, Mol Cell Biol
CCR2/ Chemokine Receptor 2	Monocyte recruitement to vessel wall	Han, 200, JCI
Liver		
Glucokinase	Glucose transporter	Kim, 2004, Diabetes
Pancreas		
Glut-2	Glucose sensor in β-cells	Kim, 2000, Diabetes
Glucokinase	Glucose sensor in β-cells	Kim, 2000, Diabetes
Pdx-1	B-cell differentiation factor	Gupta, 2008, JBC

Skeletal Muscle		
Glut-4	Insulin-responsive glucose transporter	Ezaki, 1997, Biochem Biophys Res Commun Fernyhough, 2007, Domest Anim Endocrinol

Table 1.2: PPARγ target genes.

Mouse Model	Reference	Phenotype
1. PPARγ -/-	Barak Y, 1999, Mol Cell [12]	- Embryonic lethal due to placental and cardiac defects - Pups rescued by tetraploid aggregation with wild type placentas had absence of all types of adipose tissue
2. PPARγ +/- with HFD	Kubota N, 1999, Mol Cell [55]	- Absence of adipocyte hypertrophy in response to HFD - Protection against HFD induced insulin resistance via leptin hypersecretion
3. PPARγ2 exonB -/-	Koutnikova, 2003, PNAS [40]	- Predominantly white adipose tissue knockdown of PPARγ - Growth retardation, severe lipodystrophy, hyperlipidemia - mild glucose intolerance due to compensatory effective lipid oxidation in skeletal muscle
4. PPARγ c/- (25% of WT expression)	Tsai YS, 2009, Mol Endocrinol [39]	- Reduced body weight and reduced total fat mass due to selective reduction in perigonadal fat depot - Insulin resistance - Dyslipidemia, increased ectopic lipid deposition in skeletal muscle
5. Adipose-specific PPARγ -/-	He W, 2003, PNAS [52]	- Adipocyte hypocellularity and hypertrophy, increased plasma NEFA and TG, decreased plasma leptin and adiponectin - Increased hepatic gluconeogensis and insulin resistance, corrected by TZD - No insulin resistance in skeletal muscle
6. Liver-specific PPARγ -/-	Gavrilova O, 2003, JBC [53]	- Increased adipocity, insulin resistance, hyperlipidemia and fat intolerance - Worsened hyperlipidemia, TG clearance and skeletal muscle insulin resistance on AZIP lipoatrophic background
7. Skeletal muscle-specific	Hevener AL, 2003, Nat Med [54]	- Glucose intolerance and insulin resistance - Not improved by TZDs

PPARγ -/-		

8. B-cell-specific PPARγ -/-	Rosen ED, 2003, MCB [61]	- Significant islet hyperplasia on normal chow diet - Markedly blunted expansion of islets in response to HFD - No difference in plasma glucose and glucose homeostasis compared to WT
9. Macrophage-specific PPARγ -/-	Odegaard JI, 2007, Nature [82]	- Impaired alternative macrophage activation - Diet induced obesity and insulin resistance
10. Endothelium and bone marrow-specific PPARγ -/-	Kanda, 2009, JCI [92]	- Decreased adiposity and increased insulin sensitivity on HFD - Significant dyslipidemia, not corrected by TZDs

Table 1.3: Adipose tissue and insulin sensitivity related phenotypes in various PPARγ mutant mice.

PPARγ in Insulin Secretion and Insulin Sensitivity: The increased occurrence of insulin resistance in obese individuals, the use of TZDs as anti-diabetic drugs, and the role of PPARγ in pre-adipocyte differentiation and triglyceride deposition in adipocytes suggest a link between PPARγ and maintenance of normal plasma glucose. As stated earlier, many of the genes involved in peripheral insulin sensitivity are downstream targets of PPARγ (Table 1.2). For example, Glucose Transporter 4 (GLUT-4), which is transcriptionally activated by PPARγ, is a major insulin-responsive glucose transporter in skeletal muscle and adipose tissue, organs which are responsible for the post-prandial clearance of glucose from the plasma [50].

Mice that have reduced PPARγ expression globally or have tissue-specific PPARγ deficiency show altered insulin sensitivity. A global PPARγ deletion is embryonic lethal [12]; however, a specific deletion in PPARγ2, which is mostly expressed in adipose tissue, results in severe lipodystrophy, hyperlipidemia and a mild glucose intolerance [40] (Table 1.3#3). Glucose intolerance is mild in these mice due to a compensatory increase in lipid oxidation by skeletal muscle. The phenotypes of various PPARγ mutant mouse models have been discussed in Table 1.3 – "Adipose tissue and insulin sensitivity related phenotypes in various PPARγ mutant mice".

The tissue-specific knockouts of PPARγ have also highlighted the contribution of individual organs to the maintenance of normal insulin sensitivity globally at the animal level and specifically in individual tissues. The adipose tissue-specific PPARγ knockout mice display a reduced buffering capacity for lipids in the PPARγ deficient adipocytes, resulting in hyperlipidemia on

normal chow and hepatic steatosis with high fat feeding. In addition, these mice had a insulin resistance phenotype restricted to the liver and increased hepatic gluconeogenesis [52] (Table 1.3#5). Glucose uptake in the skeletal muscle of adipose-specific PPARγ deficient mice was not affected. Mice lacking PPARγ specifically in the liver display hyperlipidemia, increased adiposity and global insulin resistance. These mice, however, responded to hypoglycemic and hypolipidemic properties of rosiglitazone, consistent with the idea that the adipose tissue is the primary site of action of the PPARγ agonist TZDs. When liver PPARγ was ablated from lipoatrophic A-ZIP/F-1 mice (lacking adipose tissue), there was worsening of hyperlipidemia and insulin resistance, but reduced hepatic steatosis compared to liver specific PPARγ knock-out, non A-ZIPF-1 mice. Further, in A-ZIP/F-1 mice lacking PPARγ in liver, hypoglycemic and hypolipidemic action of PPARγ agonsist rosiglitazone was abolished, indicating that in the absence of adipose tissue, hepatic PPARγ is essential for rosiglitazone action [53] (Table 1.3#6). Hevener *et al* showed that the deficiency of PPARγ in skeletal muscle causes progressive insulin resistance in this tissue [54] (Table 1.3#7).

Based on these mouse models of PPARγ, several important deductions can be made. Adipose tissue emerges as the primary site of action of PPARγ agonists and optimal levels of PPARγ are essential for lipid deposition in the adipocytes. This buffering function compartmentalizes the excess lipids in adipose tissue and protects the liver from lipid toxicity, which can interfere with maintenance of insulin sensitivity. Second to adipose tissue, PPARγ is essential for the appropriate amount of lipid accumulation in the hepatocyte and also for glucose transport into the cell.

Compared to adipose tissue, skeletal muscles express lower levels of PPARγ. However, skeletal muscle is the single most important organ for post-prandial glucose uptake in response to insulin. Glut-4, the major glucose transporter in skeletal muscle is transcriptionally activated by PPARγ. Thus, the absence of PPARγ in skeletal muscle affects glucose handling and insulin sensitivity.

Surprisingly, mice with a heterozygous deficiency in PPARγ were protected from a high fat diet induced insulin resistance [55] (Table 1.3#2). However, Tsai *et al* recently examined the effects of quantitative changes in PPARγ levels on insulin sensitivity in mice. We generated mice with a global reduction in PPARγ levels to 25% of wild type by destabilizing its 3'-UTR and demonstrated that this level of reduction results in smaller adipose tissue mass, hyperlipidemia, increased lipid deposition in skeletal muscle and insulin resistance [39] (Table 1.3#4).

Mutations in PPARγ are rare events, but seem to have a considerable impact on insulin sensitivity. Among others, loss of function mutations P467L and V290M in human patients are associated with reduced insulin sensitivity [56]. On the other hand, a fairly common P12A polymorphism with reduced PPARγ activity is associated with improved insulin sensitivity [57].

In addition to peripheral tissues, PPARγ appears to play a role in regulating glucose uptake in the beta cells of the pancreas. Treatment of rat primary islets for three days with Troglitazone (a TZD drug) resulted in an increase in GLUT-2 expression [26]. Glucose transporter GLUT-2, which is considered to be the glucose sensor in the pancreatic islets, has a functional PPRE [26]. Thus, PPARγ can potentially increase insulin secretion from the β-cells of the pancreatic islets in

response to increased concentration of glucose. Supporting this possibility, Xu *et al* demonstrated that PPARγ activation increased Glut2 expression, increased glucose stimulated insulin secretion (GSIS), increased fatty acid oxidation and decreased accumulation of triglycerides in INS-1 cells [58, 59]. In contrast, Ravnskjaer *et al* demonstrated that when PPARγ is over-expressed in INS-1E cells, it increases fatty acid uptake and deposition of triglycerides in these cells. This, in turn, results in a reduction in glucose stimulated insulin secretion from these cells. They propose that in conditions of hyperglycemia and/or obesity, β-cells over-express PPARγ, resulting in increased triglyceride accumulation and impaired insulin secretion [60]. Thus, the specific effect of PPARγ activation on GSIS is still debatable.

Rosen *et al* generated mice lacking PPARγ specifically in the β-cells of the pancreatic islets by crossing mice with floxed PPARγ and mice carrying a Cre transgene driven by the rat insulin promoter (Table1.3#8) [61]. The mice lacking PPARγ selectively in the β-cells have a significant islet hyperplasia on a normal chow diet. Additionally, the normal expansion of islets in response to high fat diet is markedly blunted in these mice. However, there was no difference in plasma insulin levels compared to wild type mice and the glucose homeostasis remained unchanged from wild type regardless of the diet. Recent in-vivo data suggest that PPARγ activation can improve islet function in diabetic mice [62]. Evans-Mollina showed that when diabetic mice (due to high fat diet or due to deficiency in leptin receptor) were treated with Pioglitazone by oral gavage for 4-6 weeks, there was significant reduction in their hyperglycemia. This was attributed to increased circulating insulin levels in mice and enhanced glucose stimulated insulin secretion demonstrated

in-vitro. The improved islet function was associated with significant up-regulation of islet genes, including Ins1/2 and Glut-2. The authors showed a 2-3 fold increase in euchromatin marker histone H3 dimethyl-Lys4 on the Ins1/2 and Glut-2 promoters and increased nuclear occupancy of islet methyltransferase Set7/9. Using in-vitro experiments, these favorable effects on islet gene chromatin structure were shown to be due to reduced endoplasmic reticulum stress in the presence of Pioglitazone. Although the direct mechanism by which PPARγ regulates insulin secretion is not entirely clear, there is convincing data implicating an involvement of PPARγ in insulin secretion from pancreatic islets.

PPARγ in Blood Pressure Regulation: In addition to its role as an important regulator of adiposity, PPARγ has been shown to play a role in the maintenance of blood pressure. Patients heterozygous for a P467L mutation in PPARγ have hypertension at an early age. Although the individual mutations are a rare occurrence, hypertension is associated with many of the point mutations (P467L, V290M, F388L, R425C) in the ligand binding domain of PPARγ, thus consolidating its role in the regulation of blood pressure [51, 63, 64]. Calnek *et al* showed that treatment of cultured human endothelial cells with PPARγ ligands 15d-PGJ_2 or ciglitazone increased release of vasodilator nitric oxide (NO), without increasing endothelial nitric oxide synthase (eNOS) expression [65]. Further, PPARγ specific siRNA and PPARγ antagonist GW9662 significantly reduced 15d-PGJ_2, ciglitazone and rosiglitazone induced NO release from cultured endothelial cells, establishing that the effect of these compounds is mediated by PPARγ [66]. In addition to these in-vitro studies, two independent groups demonstrated the involvement

of PPARγ in blood pressure regulation in mice. Using mice selectively deficient in PPARγ in the endothelial cells, Nicol *et al* showed that the basal levels of blood pressure on normal chow diet are not different in these mice when compared to wild type controls. However, there is a significant increase in blood pressure in high fat fed mice lacking PPARγ in their endothelial cells and this increase is not corrected by rosiglitazone treatment [67] (Table 1.4#4). In another study, Kleinhenz *et al* showed that endothelium specific PPARγ knockout mice have elevated baseline blood pressure due to reduction in the production of vasodilator nitric oxide and impaired aortic ring expansion in response to acetylcholine.

Mouse Model	Reference	Phenotype
1. Embryo-specific PPARγ-/- (Trophoblast spared)	Duan, 2007, JCI [86]	- Hypotension not corrected by salt loading - Vasculature more sensitive to endothelium-dependant relaxation caused by muscarinic stimulation - Impaired VSMC contraction in response to alpha-adrenergic agents
2. PPARγP465L/+	Tsai, 2004, JCI [84] Beyer, 2008, Hypertension [93]	- Increased BP - Intact response of aorta to Ach, increased contraction to endothelin 1, serotonin and $PGF_{2\alpha}$ - Impaired Ach-induced dilatation in cerebral blood vessels and increased superoxide - Reduced maximally dilated diameter of cerebral arteriole, increased wall thickness suggestive of hypertrophy and remodelling
3. Endothelium-specific PPARγ-/-	Kleinhenz, 2009 AJPHCP [68]	- High baseline BP but similar increase in SBP (compared to WT) in response to angII infusion - Impaired aortic ring relaxation to ACh
4. Endothelium-specific PPARγ-/-	Nicol J, 2005, Am J Hypertens [67]	- No increase in BP with normal chow and high salt diet - Significant elevation of SBP with HFD, not corrected by rosiglitazone
5. Endothelium and bone marrow-specific PPARγ-/-	Kanda, 2009, JCI [92]	- Increased BP on HFD - Impaired carotid artery relaxation in HF fed mice

6. Endothelium-specific PPARγP465L/+ & V290M/+	Beyer, 2008, Circ Res [94]	- Impaired Ach induced dilatation of basilar artery after 12 week HFD, improved by treatment with superoxide scavanger - Slight elevation in baseline BP, augmented pressor response to angII
7. VSMC-specific PPARγ-/-	Chang, 2009, Circulation [71]	- Reduced BP - Reduced NE-stimulated aortic contraction, increased β-adrenergic agonists stimulated vasorelaxation
8. VSMC-specific PPARγP465L/+ & V290M/+	Halabi, 2008, Cell Metab [70]	- Systolic hypertension - Impaired aortic ring reactivity to endothelium-derived or exogenous NO - Increased vasoconstrictor response to serotonin, endothelin 1 but modest decrease with PE Vascular hypertrophy and remodeling of micro-vasculature

Table 1.4: Vascular and Blood Pressure Phenotypes in PPARγ Mutant Mice Ach, acetylcholine; ang II, angiotensin II, BP, blood pressure; HFD, high fat diet; NO, nitric oxide; PE, phenylephrine; SBP, systolic blood pressure; VSMC, vascular smooth muscle cell.

This phenotype was also associated with an increase in the parameters of oxidative stress in blood, such as reactive oxygen species (ROS), plasma concentration of cysteine (Cys), cystine (CySS) and the Cys/CySS redox potential (Table 1.4#3) [68]. Thus, PPARγ in the endothelial cells is essential for blood pressure regulation and its absence can result in a hypertensive phenotype. In addition to the endothelium, vascular smooth muscle cells are shown to express PPARγ [69]. Halabi *et al* demonstrated that mice expressing the dominant negative P465L and V290M mutations in PPARγ, specifically in the vascular smooth muscle cells, have elevated blood pressure levels. These mice show a severely impaired aortic reactivity to endothelial-derived or exogenous nitric oxide (Table 1.4#8) [70]. Based on this data, PPARγ in vascular smooth muscle cell is necessary to mediate vasodilator signals originating in the endothelium, such as NO release. However, lack of PPARγ in vascular smooth muscle cell results in reduced blood pressure (Table 1.4#7) [71]. These mice had a reduced aortic contraction in response to nor-epinephrine and an increase in β-adrenergic agonist mediated vasodilatation. Several mouse models, as discussed in Table#1.4, titled "Vascular and Blood Pressure Phenotypes in PPARγ Mutant Mice", have been generated to understand the mechanism by which PPARγ affects blood pressure. These models highlight the actions of PPARγ in endothelium and vascular smooth muscle cells, the two cell types which can modulate flow and pressure in the blood vessels. Taken together, these in vitro and in vivo experiments suggest that an increase in PPARγ activity has a beneficial effect on vascular function. In addition to studying the effect of PPARγ on blood pressure regulation, these mouse models can also be used successfully to study the effects of alterations in PPARγ activity on

blood pressure and the indirect effect of the change in blood pressure on chronic disease processes, such as atherosclerosis.

PPARγ in Macrophage Function and Atherosclerosis: An oxidized alkyl phospholipid, hexadecyl azelaoyl phosphatidylcholine, a component of oxidized low density lipoproteins, has been shown to bind to PPARγ with a relatively high affinity (kd approximately 40 nM), similar to that reported for rosiglitazone [72]. This highlights a new link between PPARγ, low-density lipoproteins and the fate of atherosclerotic plaques. The increased prevalence of obesity, hypertension and insulin resistance in the population exacerbates atherosclerosis and cardiovascular disease outcomes. Many diabetic patients are being treated with insulin sensitizer drugs TZDs, which have been shown to reduce the atherosclerotic lesion size in human patients. Similarly, using mouse models of atherosclerosis lacking apolipoprotein E (apoE) and/or low density lipoprotein receptor (LDLR), several studies have established the anti-atherosclerotic effects of PPARγ agonists [73, 74, 75].

Macrophages are a crucial element in the formation and propagation of an atherosclerotic plaque. The foam cells found in the core of a plaque are lipid-laden macrophages and PPARγ plays an important role in the lipid accumulation in these cells by influencing the lipid uptake as well as efflux [45, 76, 77]. The scavenger recptor CD36, which is responsible for uptake of lipid by the macrophage, is shown to be transcriptionally activated by PPARγ [77] in response to 15-PG_{J2} or TZD treatment. 9- and 13-HODE present in oxidized LDL can also activate PPARγ (kd in a 10-20

μM range) [78]. In a proposed "PPARγ cycle" by Nagy *et al*, oxidized LDL would induce activity of PPARγ resulting in increased expression of CD36 consequently increasing the oxidized LDL uptake by the macrophage [74, 78]. Thus, this cycle can potentially promote formation of foam cells and atherosclerosis. On the other hand, PPARγ activates a pathway of cholesterol efflux from the macrophage [79]. Ligand activation of PPARγ results in increased expression of ABCA-1, a molecule that facilitates cholesterol transfer from cells to high density lipoprotein (HDL), via induction of LXRα. Babaev *et al* demonstrated a significant increase in atherosclerosis when PPARγ null bone marrow was transplanted in low density lipoprotein receptor knockout (LDLR-/-) mice and established the protective effect of PPARγ-mediated induction of ABCA-1 and the cholesterol efflux pathway in the macrophages [80].

The transrepression pathway of PPARγ function has been primarily characterized in the macrophage, where PPARγ is actively involved in regulating their inflammatory properties. In the basal state, inflammatory genes, such as inducible nitric oxide synthase (iNOS) and chemokine CC-chemokine ligand 2 (CCL2) are repressed by the inhibitory complex that includes PPARγ [31]. PPARγ expression in human and mouse monocytes directly inhibits CC chemokine receptor 2 (CCR2) expression and suppresses monocyte chemoattractant protein (MCP-1)-mediated chemotaxis [46]. Also, monocytes that are pretreated with PPARγ agonists have reduced adhesion to endothelial cells [81]. Consistently, macrophages obtained from macrophage-specific PPARγ knockout mice demonstrate increased migration and have a higher CCR2 expression compared to wild type macrophage [80].

In addition to its anti-inflammatory actions, PPARγ plays an important role in macrophage differentiation. Classically activated M1 macrophages produce pro-inflammatory cytokines; whereas alternative activation produces M2 macrophage, which secrete anti-inflammatory cytokines. A balance between the M1 and M2 phenoytpes is crucial in regulating inflammation in atherosclerosis. Macrophage-specific PPARγ activation has been shown to enhance alternative activation and potentially favor the anti-inflammtory actions [82].

In addition to the macrophage, endothelial cells and vascular smooth muscle cells (VSMC) modulate the initiation and propagation of an atherosclerotic plaque. The role of PPARγ-mediated enhancement in NO production by the endothelium not only reduces blood pressure but can also potentially modulate the progression of atherosclerosis by directly reducing reactive oxygen species. Finally, Subramanian *et al* generated VSMC-specific PPARγ knockout and have shown that these mice develop enhanced atherosclerosis upon induction with angiotensin II [83]. Pioglitazone treatment reduced angiotensin II induced atherosclerosis in wild type mice but not in mice lacking PPARγ in VSMC. This emphasizes the importance of PPARγ in VSMC in atherosclerosis protection.

1.6 Mouse Models Used in My Study

Mouse PPARγP465L mutation: P465L mutation in PPARγ involves the proline residue in Helix 12 in the ligand-binding domain of PPARγ in mice [51]. This residue is important for binding to ligands and cofactors resulting in significant effects on PPARγ signaling in the presence of the

mutation. PPARγP465L/+ mice (L/+ mice), are heterozygous for codon 465 mutated from CCC (proline) to CTG (leucine) in mouse embryonic stem cells using the gene targeting technique (Figure 1.5) [84].

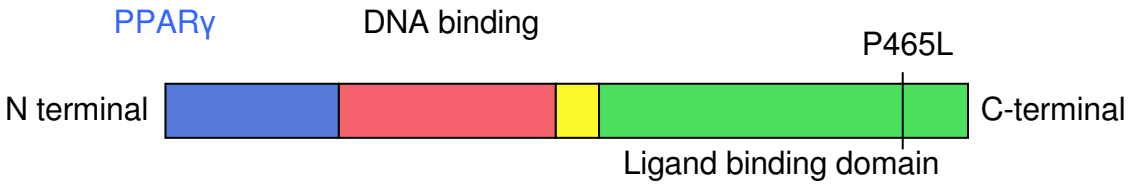

Figure 1.5: Schematic of PPARγ with the location of P465L mutation.

In an in-vitro system using the aP2 promoter, which contains a PPRE, it has been shown that the mutant P465L allele acts in a dominant negative manner [85]. The mutant protein reduces the activity of the wild type protein by more than 50% by blocking its access to the PPREs in downstream genes. It is thus reasonable to conclude that the heterozygous P465L/+ animals have less than 50% functional PPARγ protein. Homozygous mice with P465L mutation die in-utero, establishing that the mutant protein is nonfunctional [84]. This phenotype is very similar to the PPARγ null mice which do not survive [12]. Human patients heterozygous for the corresponding P467L mutation have high plasma glucose and insulin levels, are hypertensive and have an altered fat distribution [51]. L/+ mice heterozygous for the P465L mutation are mildly hypertensive, hyperinsulinemic (Figure 1.6) and have an altered fat distribution; however, they have normal levels of plasma glucose.

PPARγ P465L mutation and pancreatic islets: P465L/+ mice (dark bars) have an increased mean islet area and increased endocrine cell mass, especially on high fat diet compared to wild type animals (Figure 1.7) (Tsai JCI 2004).

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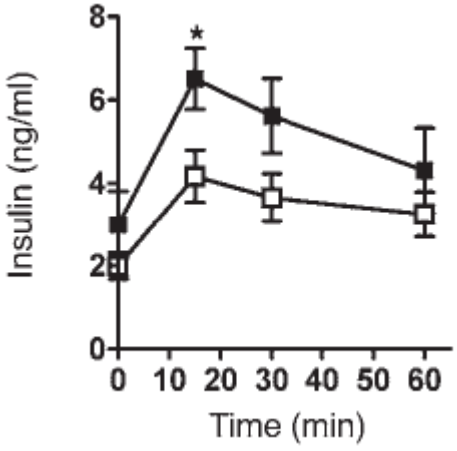

Figure 1.6: Level of plasma insulin during a glucose tolerance curve in L/+ (dark squares) and wild type (open squares) mice on high fat diet. L/+ mice had higher plasma insulin, particularly 15 minutes after the glucose load (Tsai YS, 2004, JCI, ref#84)

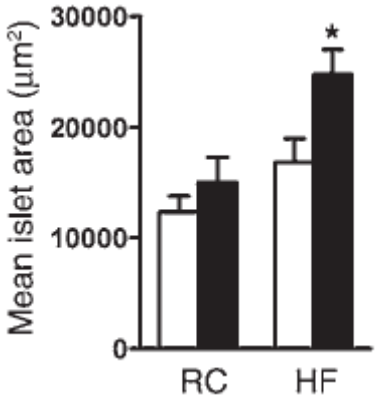

Figure 1.7: Mean islet area. P465L/+ mice (dark bars) have an increased mean islet area, especially on high fat diet compared to wild type animals. RC, regular/ normal chow; HF, high fat (Tsai YS, 2004, JCI, ref#84).

This phenotype is in agreement with other evidence in the literature for the role of PPARγ in islet physiology. The rescued PPARγ null animals were generated by breeding Mox2 Cre mice and floxed PPARγ mice, resulting in the inactivation of PPARγ in the embryo but not in the trophoblast. The rescued PPARγ null mice have bigger pancreatic islets, with significantly higher plasma insulin levels in response to glucose load and impaired insulin sensitivity [86]. Mice with selective knockout of PPARγ in β-cells of pancreatic islets also have an increase in islet cell mass due to β-cell hyperplasia, but did not increase the islet cell mass on high fat feeding [61]. The β-cell selective knockouts, however, have normal plasma insulin levels. These data suggest that at baseline conditions, PPARγ has an inhibitory effect on β-cell proliferation, but it is essential for expansion of islet mass induced by obesity. The phenotype of P465L/+ mice suggests that there is some impairment in the PPARγ signaling and that the increased expansion of islets compared to wild type controls may be due to reduced anti-proliferative action of PPARγ.

Ins2^{C96Y} *or Ins2*$^{Akita/+}$ Mutation

Ins2^{C96Y} or *Ins2*$^{Akita/+}$ mutation is an autosomal dominant mutation in the Ins2 gene in mice resulting from a G to A transition at nucleotide 1907 in exon 3 of one of the Ins2 alleles [87]. Heterozygous *Ins2*$^{Akita/+}$ mice with Akita mutation are hyperglycemic (have increased plasma glucose levels) due to inadequate production of insulin, particularly severe in male mice. Due to the reduced insulin production, Akita mice are considered a model for type 1 diabetes mellitus. However, Hong *et al* recently showed that Akita mice also develop features of type 2 diabetes,

including insulin resistance [88]. In the protein product of this mutation, cysteine is substituted by tyrosine at the seventh amino acid position in the A chain of Insulin 2 molecule [87]. The insulin molecule structurally consists of A and B chains and the above mentioned cysteine is involved in the formation of one of the two intra-molecular disulfide bonds that hold the two chains together. The destruction of this disulfide bond has a significant impact on the conformation of the protein, resulting in misfolding and proteasomal degradation. Mice have another functional insulin gene – Ins1. Thus, the heterozygous Akita mice have 3 normally functional insulin alleles and the insulin mRNA levels are similar to wild type suggesting no transcriptional impairment. However, the amount of proinsulin and insulin proteins is low in the islets of Akita mice, resulting in increased plasma glucose levels. The dominant negative effect of this mutation is shown to be due to generalized degradation of the organelles involved in the intracellular secretory pathways, stress on endoplasmic reticulum induced by the mutant protein and an increase in β-cell apoptosis [89].

In the first part of my thesis, I have used the Akita mutation as a means to reduce circulating plasma insulin levels in the $Pparg^{P465L/+}$ mice to prove the hypothesis that $Pparg^{P465L/+}$ causes insulin resistance.

***Apoe-/-* Mice:**

Apolipoprotein E -/- (*Apoe-/-*) mice are a well established model of atherosclerosis which has been widely used to study the pathophysiology as well as treatment modalities of this disease. *Apoe-/-* mice have spontaneous hypercholesterolemia and hypertriglyceridemia with reduced high-density

lipoprotein (HDL) levels [90]. These mice also develop spontaneous atherosclerotic lesions in their arteries [91]. In young mice, the lesions begin as subendothelial foam cell deposits in the aortic sinus close to the aortic valve area. Over the course of time, accumulation of foam cell deposits and free cholesterol along with smooth muscle cell proliferation leads to the propagation of the atherosclerotic plaque and the lesions spread to other areas of the aorta including the arch. In 8-9 month old mice, complex lesions are demonstrable with well defined fibrous caps.

In the second part of my thesis, I used *Apoe-/-* mice to mimic the human condition of atherosclerosis to study the effect of $Pparg^{P465L/+}$ mutation on atherosclerosis severity and to investigate the hypothesis that $Pparg^{P465L/+}$ will worsen atherosclerotic lesion size.

Most of the scientific evidence presented above was generated in global or tissue specific knockout mice, which are a result of artificial manipulations occurring only in the experimental settings. Thus, it is important to study PPARγ in a context which is possible in human patients. I chose the P465L mutation in mouse PPARγ since the original mutation (P467L) was identified in human patients. Although the mutation itself is a very rare event, it can be considered as a representative of several mutations identified in the ligand binding domain of PPARγ. This study has enabled me to understand the effects of reduction in the level of normal PPARγ protein and the presence of an altered protein on adipose tissue physiology, insulin sensitivity and atherosclerosis. These results can be further extrapolated to human subjects who carry PPARγ polymorphisms.

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C h a p t e r 2

OVERALL GOAL AND HYPOTHESIS

The overall goal of this dissertation is to study the effect of a dominant negative point mutation in Peroxisome Proliferator Activated Receptor γ (PPARγ), PPARγP465L/+, on insulin sensitivity and atherosclerosis.

PPARγ is a ligand-activated transcription factor that belongs to the family of nuclear hormone receptors. It is necessary and sufficient for adipose tissue differentiation and triglyceride accumulation in adipocytes. Thiazolidinediones (TZDs), which are the synthetic ligands of PPARγ, are used as insulin sensitizers to treat millions of pre-diabetic and diabetic patients. The insulin-sensitizing actions of PPARγ are complex, brought about via multiple organs, and are less well characterized than its role in adipocyte differentiation. PPARγ is also shown to play a role in blood pressure regulation and TZDs have been shown to reduce blood pressure in both diabetic and non-diabetic human patients. In addition, PPARγ is also known to affect macrophage differentiation and lipid uptake.

Insulin resistance and hypertension often co-exist and independently and jointly contribute to worsen the outcome of cardiovascular disease. This prompted me to investigate the role of $PPAR\gamma^{P467L/+}$ mutation in these two patho-physiological conditions, both of which are known to be modulated by PPARγ. The $PPAR\gamma^{P467L/+}$ mutation was originally identified in human patients who have severe hyperglycemia and insulin resistance. These patients also have early onset hypertension and lipodystrophy. Mice with the corresponding $Pparg^{P465L/+}$ mutation recapitulate the phenotype of hypertension and lipodystrophy, but are not insulin resistant. They have normal

plasma glucose levels and a small increase in plasma insulin which is exacerbated by high fat diet. Since this mutation was identified by screening hyperglycemic patients with insulin resistance, it is possible that the mutation is merely associated with insulin resistance and part of my work is focused on ruling out this possibility. Thus, the first part of my thesis is an attempt to understand whether the *Pparg*$^{P465L/+}$ (L/+) mutation in the ligand binding domain of PPARγ, can cause insulin resistance.

I hypothesized that the increase in plasma insulin found in P465L/+ mice compensates for the insulin resistance caused by this mutation and acts to maintain normal plasma glucose levels. To test this hypothesis, I used *Ins2*$^{Akita/+}$ or *Ins2*$^{C96Y/+}$ mice. The *Ins2*$^{Akita/+}$ mutation leads to a reduction in circulating insulin levels. Mice carrying this mutation would not have the extra insulin required to maintain normal plasma glucose levels in P465L/+ mice and would potentially uncover the insulin resistance phenotype in the carriers of the P465L/+ mutation. Unlike *Ins2*$^{Akita/+}$ littermates, male *Pparg*$^{P465L/+}$*Ins2*$^{Akita/+}$mice have drastically reduced life-span and enhanced Type I diabetes. Hyperglycemia in *Ins2*$^{Akita/+}$ females is mild. However, *Pparg*$^{P465L/+}$*Ins2*$^{Akita/+}$ females have aggravated hyperglycemia, smaller insulin-producing pancreatic islets and reduced plasma insulin. In an insulin tolerance test, they showed smaller reduction in plasma glucose indicating impaired insulin sensitivity. While gluoconeogenesis is enhanced in *Pparg*$^{P465L/+}$*Ins2*$^{Akita/+}$ female mice compared to *Ins2*$^{Akita/+}$, exogenous insulin equally suppressed gluconeogenesis in primary hepatocytes obtained from these mice, suggesting that *Pparg*$^{P465L/+}$*Ins2*$^{Akita/+}$ livers are insulin sensitive. Comparable expression of genes regulating insulin sensitivity, and glycogen and

triglyceride contents suggest that skeletal muscles are equally insulin sensitive. In contrast, adipose tissue and isolated adipocytes from *Pparg*$^{P465L/+}$*Ins2*$^{Akita/+}$ mice have impaired glucose uptake in response to exogenous insulin. In addition, *Pparg*$^{P465L/+}$*Ins2*$^{Akita/+}$ female mice have smaller fat depots composed of larger adipocytes suggesting impaired lipid storage with subsequent hepatomegaly and hypertriglyceridemia.

PPARγ in endothelium, vascular smooth muscle cell and macrophage is shown to beneficially modulate blood pressure, cellular adhesion and migration and vasoconstriction. In the second part of my thesis, I have tested the hypothesis that increased blood pressure and reduced or altered activity of PPARγ due to the presence of P465L/+ mutation increase atherosclerosis plaques. To test this hypothesis we used mice that are deficient in apolipoprotein E (*Apoe-/-*), which have hypercholesterolemia and spontaneously develop atherosclerotic lesions. ApoE is involved in ligand-dependant clearing of lipids from the plasma and is crucial to maintain normal levels of plasma lipids.

Surprisingly, we did not see an increase in atherosclerotic plaque size in the double mutant mice (Lee) carrying P465L/+ mutation on the apoE null background. The Lee mice had higher blood pressure levels, which can potentially increase atherosclerotic plaque size. As expected, Lee mice had decreased expression of scavenger receptor CD-36 in macrophages. However, unlike *Pparg-/-* macrophages, Lee mice had an increase in *Abca-1* expression, suggesting increased cholesterol efflux. Lee mice had a protective phenotype in the macrophages where VLDL uptake was

significantly reduced compared to *Apoe-/-* control mice, suggesting a reduced formation of foam cells in the Lee mice. It is likely that the increase in blood pressure with its pro-atherogenic potential and the athero-protective phenotype in the macrophage balance each other, resulting in an unchanged plaque.

Chapter 1 gave a detailed scientific background for my study. I describe the structure and mechanism of action of PPARγ followed by the functional role of PPARγ in adipogenesis, blood pressure regulation, macrophage lipid uptake and atherosclerosis. I describe the phenotype of P465L/+ mutation and discuss in detail the choice and characteristics of the other two mouse models ($Ins2^{Akita/+}$ and *ApoE-/-*) used to test my hypotheses. In the next chapters, I will discuss the scientific background on which these studies are based, including a detailed description of the mouse models used, the results, and my interpretation and conclusions based on these data.

Chapter 3 discusses the findings from my first hypothesis that $Pparg^{P465L/+}$ mutation may cause insulin resistance. Utilizing the $Ins2^{Akita/+}$ mouse, I showed that adipose tissue with its dependence on PPARγ and insulin early in its development, is structurally and functionally affected by the P465L/+ mutation and contributes to the insulin resistance phenotype of the double mutant $Pparg^{P465L/+}Ins2^{Akita/+}$ (LA) mice. Liver and skeletal muscles are relatively spared by this mutation. This chapter consists of material published in the manuscript titled "*Pparg-P465L* mutation

worsens hyperglycemia in *Ins2-Akita* female mice via adipose-specific insulin resistance and storage dysfunction" in the Diabetes journal (November, 2010).

Chapter 4 consists of my findings from the Lee double mutant mice

(*Pparg*$^{P465L/+}$*; Apoe-/-*) used to test the hypothesis that the P465L/+ mutation will increase the size of atherosclerotic plaques. We found that the plaque size was not changed in the presence of the P465L/+ mutation on normal chow, high fat diet or with Thiazolidinedione (TZD) treatment. The plaques increased in size when the mice were made diabetic with streptozotocin (STZ) treatment, however there was no genotype difference. In this chapter, I also discuss the phenotype of macrophages obtained from Lee mice and the possibility that hypertension and a protective macrophage phenotype may counteract to explain the absence of an increase in the atherosclerotic plaque.

Chapter 5. Similar to the multifaceted role of PPARγ in adiposity, glucose and lipid homeostasis and blood pressure regulation, apoE is also emerging as a crucial molecule at the cross roads of lipid and glucose metabolism. We discuss the role of apoE in modulating atherosclerosis and metabolic syndrome and look at the contribution of the three human apoE isoforms. This chapter

consists of published material from a review article titled “ApoE Knock-out and Knock-in Mice: Atherosclerosis, Metabolic Syndrome, and Beyond” in the Journal of Lipid Research.

Chapter 6 consists of the overall conclusions of my experiments conducted to prove the two hypotheses and a discussion about the significance of my study.

C h a p t e r 3

PPARG-P465L MUTATION WORSENS HYPERGLYCEMIA IN *INS2-AKITA* FEMALE MICE VIA ADIPOSE-SPECIFIC INSULIN RESISTANCE AND STORAGE DYSFUNCTION.

(This chapter consists of material from a manuscript reprinted with permission from *Diabetes*; 2010 Nov;59(11):2890-7. Epub 2010 Aug 19; titled "*Pparg-P465L* mutation worsens hyperglycemia in *Ins2-Akita* female mice via adipose-specific insulin resistance and storage dysfunction" by Avani A Pendse, Lance A Johnson, Yau-Sheng Tsai and Nobuyo Maeda)

3.1 Summary

The dominant-negative P467L mutation in peroxisome proliferator activated receptor-γ (PPARγ) was identified in insulin resistant patients with hyperglycemia and lipodystrophy. In contrast, mice carrying the corresponding *Pparg-P465L* mutation have normal insulin sensitivity, with mild hyperinsulinemia. We hypothesized that murine *Pparg-P465L* mutation leads to covert insulin resistance, which is masked by hyperinsulinemia and increased pancreatic islet mass, to retain normal plasma glucose. We introduced in $Pparg^{P465L/+}$ mice an *Ins2-Akita* mutation that causes improper protein folding and islet apoptosis to lower plasma insulin. Unlike $Ins2^{Akita/+}$ littermates, male $Pparg^{P465L/+}Ins2^{Akita/+}$mice have drastically reduced life-span with enhanced Type I diabetes. Hyperglycemia in $Ins2^{Akita/+}$ females is mild. However, $Pparg^{P465L/+}Ins2^{Akita/+}$ females have aggravated hyperglycemia, smaller islets and reduced plasma insulin. In an insulin tolerance test, they showed smaller reduction in plasma glucose indicating impaired insulin sensitivity. While gluoconeogenesis is enhanced in $Pparg^{P465L/+}Ins2^{Akita/+}$ female mice compared to $Ins2^{Akita/+}$littermates, exogenous insulin equally suppressed gluconeogenesis in primary hepatocytes isolated from these mice, suggesting that $Pparg^{P465L/+}Ins2^{Akita/+}$ livers are insulin sensitive. Comparable expression of genes regulating insulin sensitivity and glycogen and triglyceride contents suggest that skeletal muscles are equally insulin sensitive. In contrast, adipose tissue and isolated adipocytes from $Pparg^{P465L/+}Ins2^{Akita/+}$ female mice have impaired glucose uptake in response to exogenous insulin. In addition, $Pparg^{P465L/+}Ins2^{Akita/+}$ mice have smaller fat depots composed of larger adipocytes suggesting impaired lipid storage with

subsequent hepatomegaly and hypertriglyceridemia. *PPARg-P465L* mutation worsens hyperglycemia in $Ins2^{Akita/+}$ mice primarily due to adipose-specific insulin resistance and altered storage function. This underscores the important interplay between insulin and PPARγ in adipose tissues in diabetes.

3.2 Introduction

Diabetes mellitus is a major health care challenge in itself and significantly increases cardiovascular disease morbidity and mortality. As a multi-factorial, chronic disease, diabetes emanates from the complex interaction of genetic and environmental influences. Among the many factors presumed or shown to contribute to its pathology, peroxisome proliferator activated receptor-γ (PPARγ) is an important candidate. PPARγ is a nuclear receptor, and is necessary for adipocyte differentiation and triglyceride deposition [1]. Activation of PPARγ has already provided therapeutic potential. One group of its synthetic ligands, the thiazolidinedione drugs, has found applications as anti-diabetic agents with insulin-sensitizing actions [2].

Various point mutations in PPARγ that affect adipose tissue distribution and insulin sensitivity have been identified in humans. For example, the PPARG-P12A polymorphism in humans is associated with reduced body weight and increased insulin sensitivity [3]. Increased PPARγ activity in PPARG-P115Q mutation is associated with severe obesity and mild insulin resistance

[4]. Conversely, two dominant negative mutations resulting in decreased PPARγ activity, PPARG-P467L and PPARG-V290M, were reported in patients with severe insulin resistance [5]. To date, various mouse models have demonstrated the role of PPARγ in varied metabolic processes. Lack of *Pparg* causes embryonic lethality in mice [6,7] and the *Pparg*-null embryos have no perceptible adipose tissue [6,8]. Tissue specific *Pparg* knockouts in liver [9] and skeletal muscle [10] exhibit insulin resistance. In contrast, absence of *Pparg* in beta cells does not affect glucose homeostasis, although it increases β cell mass [11]. Animals entirely lacking *Pparg* are non-viable and tissue-specific knockouts offer a strategy for artificial manipulation which is only possible within experimental settings. Thus, an important step is to extend this work to study the role of PPARγ in a context applicable to human patients.

The dominant-negative heterozygous PPARG-P467L mutation was originally identified in patients with severe insulin resistance, hyperglycemia, lipodystrophy and hypertension [5]. Mice carrying the corresponding *Pparg-P465L* mutation (L/+ or L) recapitulate the hypertension and altered fat distribution phenotype (Tsai). However, L/+ mice exhibit normal plasma glucose and insulin sensitivity [12,13]. These mice have mild hyperinsulinemia and increased pancreatic islet mass, especially on high-fat diet [12]. In this study, we attempted to understand whether the observed insulin resistance phenotype in human patients is indeed attributable to L/+ mutation or a mere association. We hypothesized that mice carrying the L/+ mutation have covert insulin resistance;

however the simultaneous occurrence of islet hyperplasia and hyperinsulinemia compensates for this insulin resistance to retain normal plasma glucose.

Improper folding of insulin due to the C96Y *Ins2*$^{Akita/+}$ mutation causes endoplasmic reticulum stress and β-cell apoptosis leading to reduced plasma insulin [14]. The *Ins2*$^{Akita/+}$ mutation only affects the β-cells in the pancreatic islets and the heterozygous Akita mice develop consistently elevated plasma glucose levels early in post-natal life. Consequently, Akita model is uniquely suited to test our prediction that with reduced insulin production, the *Pparg*$^{P465L/+}$ mice would be unable to compensate for the peripheral insulin resistance. We here show that the Akita females with *Pparg*$^{P465L/+}$ mutation have increased severity of hyperglycemia and insulin resistance restricted to adipose tissue.

3.3 Methods

***Pparg*$^{P465L/+}$ *Ins2*$^{Akita/+}$ double mutant mice:** Heterozygous male *Pparg*$^{P465L/+}$ mice on 129/SvEvTac background [12] were mated with heterozygous female *Ins2*$^{Akita/+}$ mice on C57BL/6J background (Jackson Lab stock # 003548). Experimental mice were F1 littermates: *Pparg*$^{P465L/+}$*Ins2*$^{Akita/+}$ (LA), *Pparg*$^{+/+}$ *Ins2*$^{Akita/+}$(WA), *Pparg*$^{P465L/+}$*Ins2*$^{+/+}$ (L+) and wild type (W+). At 3 and 7 months of age, mice were fasted for four hours for characterization of hyperglycemia and to define the organ specific phenotype respectively. Mice were fed regular chow (LabDiet 5P76; PMI Nutrition International) and were handled with Institutional Animal Care and Use Committees approved procedures. Primers used for L/+ genotyping were CAC-

GAA-TCA-CCA-GCA-ACA-TG and CCC-ATT-CTT-GTC-ATG-ATT-CCC. Primers used for Akita genotyping were CTG-ATG-CCC-TGG-CCT-GCT and TGG-TCC-CAC-ATA-TGC-ACA-TG.

Biochemical determinations: Plasma concentrations of glucose, cholesterol, non-esterified free fatty acids (NEFA) and 3-Hydroxybutyrate (3-HB) were determined by kits from Wako (Richmond, VA, USA). Triglyceride (TG) concentrations were determined using kits from Stanbio (San Antonio, TX, USA). Plasma insulin and leptin were determined by ELISA (Crystal Chem Inc., Chicago, IL, USA). Pooled plasma samples (100 µl) were fractionated by fast protein liquid chromatography using Superose 6 HR10/30 column (GE Healthcare, Piscataway, NJ, USA). Plasma adiponectin was measured by an ELISA using murine adiponectin specific antibody (Sigma, St Louis, MO, USA). Tissue glycogen content was determined as difference of glucose contents before and after digestion with aspergillus niger amyloglycosidase (Sigma #046K8801) as described [15].

Oral Glucose Tolerance Test (OGTT): After 4 hour fast, 7 month old female mice were administered 1.3 mg/g body weight of D-glucose (Columbus Chemical Industries Inc, Columbus,

Wisconsin, USA) by oral gavage. Blood was collected before and at indicated times after glucose administration to determine plasma glucose.

Intraperitoneal Insulin Tolerance Test (IPITT): After 4 hour fast, 7 month old female mice were injected intraperitoneally with insulin (Novolin; 0.5 U/kg body weight; Novo Nordisk Inc, Princeton, NJ, USA). Blood was collected before and at indicated times after insulin injections to determine plasma glucose and NEFA.

Insulin Stimulated Glucose Uptake in Adipose Tissue: Inguinal and gonadal adipose tissues from 2 female mice from each genotype were cut into small pieces (20–40 mg) under aseptic conditions and incubated in high glucose Dulbecco's modified Eagle's medium supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St Louis, Missouri, USA) in the absence or presence of 100 nM insulin (Insulin solution from Bovine Pancreas; Sigma-Aldrich Inc). After 24 hours, glucose reduction in the medium was measured and results normalized to explant weight [16,17].

Gene expression: Total RNA was purified using Automated Nucleic Acid Workstation ABI 6700, and real-time PCR was performed in ABI PRISM 7700 Sequence Detector (Applied Biosystems). β-Actin mRNA was used for normalization. Primers and probes are available on request (Table #3.3).

Morphological analysis: Paraffin sections from adipose tissue and pancreas of female mice ($n \geq 4$) were stained with H&E. Adipocyte size was measured in ~250 cells per mouse using ImageJ software. Mean pancreatic islet area, the average from all islets identified on each section, was determined.

Pyruvate Tolerance Test (PTT): After 14 hour fast, 3 month old female mice were injected intraperitoneally with 2 mg/kg body weight of sodium pyruvate (Sigma, St. Louis, Missouri, USA). Blood was collected before and at indicated times after injection to determine plasma glucose.

Glucose production from primary hepatocytes: Primary hepatocytes were isolated as described [18] and plated on mouse-collagen IV coated plates. Cells were washed twice with PBS to remove

glucose and incubated for 16 hours in 500µl medium containing 10nM dexamethasone, 0.5mM IBMX with and without 2mM sodium pyruvate and 100nM insulin. Glucose concentration was measured in 100µl medium.

2-deoxy-glucose uptake: Hepatocytes were plated at 100,000 cells per well in a 24-well plate and maintained in ~~a~~ hepatocyte culture medium (Xenotech, Lenexa, Kansas U.S.A.) for 24 hours. The cells were washed with PBS and maintained in serum free medium containing 135mM NaCl, 5.4mM KCl, 1.4 mM $CaCl_2$, 1.4mM $MgSO_4$ and 10mM$Na_4P_2O_7$ for 30 minutes [19]. 2-deoxy-D[1-^{3}H]glucose (Perkin-elmer) was added to a final concentration of 1 µCi/ml, and cells were incubated for 10 min [20]. Cells were washed three times with PBS and solubilized in 1 ml 1% SDS. Radioactivity in 350µl aliquots was measured in a scintillation counter. Glucose uptake was normalized to protein content and expressed as milligrams of glucose per gram of protein. In a similar experiment, primary adipocytes were isolated and 2-deoxy-D[1-^{3}H]glucose (Perkin-elmer) uptake was measured in absence or presence of 100nM insulin as previously described [21,22].

Data analysis: Values are reported as mean±SEM. Statistical analyses were conducted using two-way ANOVA with *Pparg* and *Ins2* genotypes as two factors. Student's t test was used for

comparisons between groups, and differences were considered to be statistically significant if $p < 0.05$.

3.4 Results

Increased hyperglycemia in *Ins2*$^{Akita/+}$ mice carrying the *Pparg-P465L* mutation. From our breeding scheme, we obtained F1 mice with four genotypes: wild type (W+), *Pparg*$^{P465L/+}$(L+), *Ins2*$^{Akita/+}$(WA), and *Pparg*$^{P465L/+}$*Ins2*$^{Akita/+}$ (LA) mice. The mice were born in the expected Mendelian ratio; however, all LA male mice died before 150 days demonstrating significantly reduced survival (Fig 3.1A). In contrast, more than 85% WA males survived and all W+ and L+ males survived to this age. Younger LA males at 6 weeks of age had higher fasting plasma glucose (LA 577±31 and WA; 494±40 mg/dl, n = 6, Fig 1B) and triglyceride (TG) compared to WA mice, although these increases did not reach significance (Figure 3.2A). However, LA males had significantly higher fasting plasma ketone body levels (LA; 407± 23 and WA; 271±40 µmol/L, $n \geq 4$, $p < 0.05$, Fig 3.1C) indicating a severe lack of insulin and/or insulin resistance.

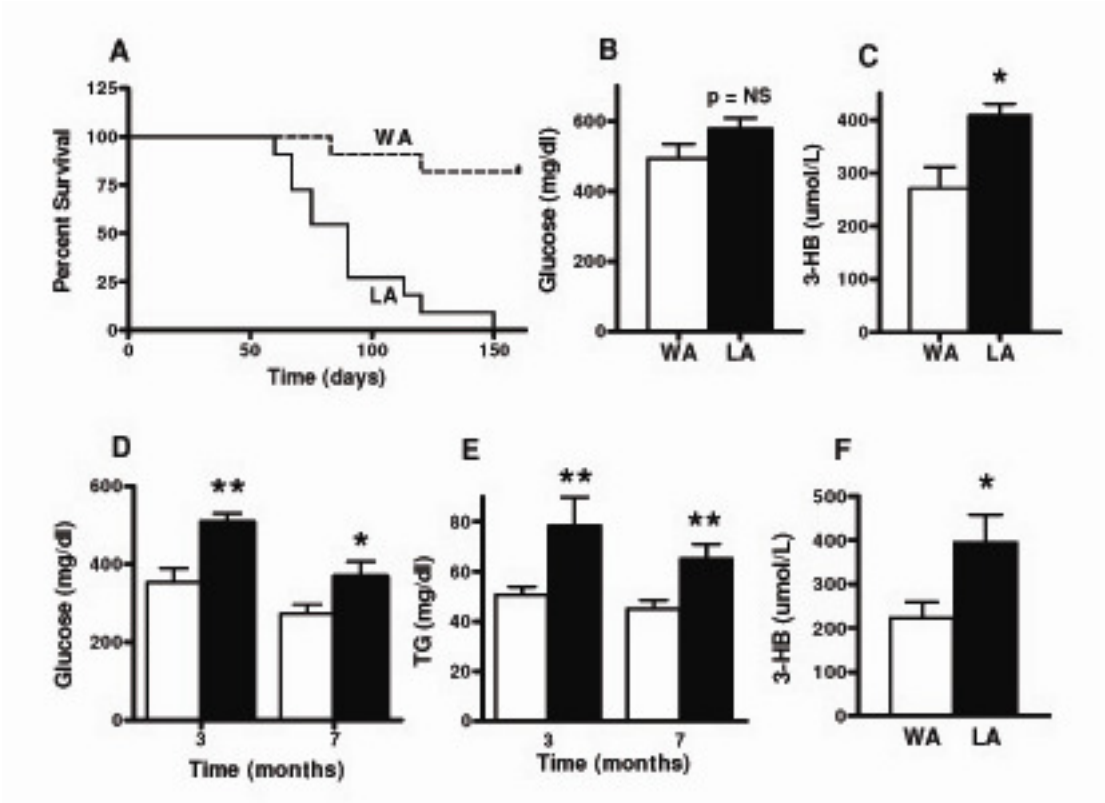

Figure 3.1: Increased hyperglycemia in *Ins2*$^{Akita/+}$ mice carrying the *PPARγ*$^{P465L/+}$ mutation. (A) Reduced survival in male LA (n=12, solid line) mice compared to WA (n=12, dashed line) littermates. (B) Fasting plasma glucose in 6 week old male LA mice (n=10, black bars) compared to male WA littermates (n=12, white bars). (C) Fasting plasma ketone body (3-HB) levels in 6 week old male LA mice (n=4) compared to male WA littermates (n=6). (D,E) Fasting plasma glucose and triglyceride levels in female LA (black bars) and WA (white bars) littermates at 3 and 7 months of age (n≥6). (F) Fasting plasma ketone bodies in 3 month old female LA (n=6) and WA (n=7) littermates. * p< 0.05, ** p< 0.01.

In contrast to LA males, LA females demonstrated normal survival throughout 7 months of the study period. We therefore focused on WA and LA females to understand the effects of *Pparg-P465L* mutation on peripheral insulin sensitivity and diabetes severity. Basic characterization of a set of 3 month old mice is shown in Table 3.1.

The LA double mutant female mice had significantly higher fasting plasma glucose compared to WA littermate control females. This increase in plasma glucose was apparent at 3 months of age (LA; 508±23, n=6 vs WA; 354±36 mg/dl, n=13; $p< 0.01$) and compared to glucose levels in a wild type mouse (~120 mg/dl), hyperglycemia persisted throughout the study period of ~7 months (LA; 369±38, n=6 vs WA; 273±24 mg/dl, n=13; $p< 0.05$ Fig 3.1D). The LA female mice had significantly high fasting plasma TG levels at both 3 months and 7 months of age (LA; 78±12 and 65±6, WA; 51±3 and 45±3 mg/dl, n=6; $p< 0.01$ Fig 3.1E). As expected in a diabetic state, this increase in plasma triglyceride level was mainly due to an increase in the very low density lipoproteins (Figure 3.2). Diabetes, with its low levels of functional insulin, is the most common pathological cause of elevated ketone bodies [23]. Consistently, LA females had significantly higher levels of 3-hydroxybutyrate compared to WA mice after 4 hours of fasting (LA, 395±63, n=8 and WA, 222±37 μmol/L, n=7; $p< 0.05$, Fig. 3.1F).

	+/+;+/+	L/+;+/+	+/+;A/+ (WA)	L/+;A/+ (LA)
Plasma Glucose (mg/dl)	178±37 (11)	171±23 (10)	354±36 (13)	508±23* (6)
Plasma TG (mg/dl)	40±4	38±2	51±12	78±12
Body weight (grams)	23.05±1.14	23.34±0.72	23.41±0.81	22.25±0.8
Food Intake (gm/d)	3.0±0.8	3.8±0.3	3.13±2.3	3.43±0.3
Water Intake (ml/d)	4.0±1.3	3.4±0.3	5.9±2.9	7.9±2
Urine Output (ml/d)	1.3±0.3	1.45±0.2	3.78±2.7	4.84±1.5
Liver/BW (%)	3.64±0.3	4.03±0.17	4.08±0.19	4.27±0.2
Gonadal Fat/BW (%)	1.17±0.13	0.89±0.07	1.24±0.19	0.67±0.07
Inguinal Fat/BW (%)	1.0±0.05	1.2±0.09	0.87±0.08	0.88±0.07
Total Fat/BW (%)	0.56±0.08	0.54±0.05	0.56±0.06	0.4±0.06
Systolic BP (mmHg)	102.3±2	n.d.	104±3	112±2.5*
Liver Glycogen (/gram protein)	0.23±0.06	0.14±0.03	0.19±0.3	0.18±0.03
Liver TG (/gram protein)	0.14±0.02	0.16±0.01	0.13±0.01	0.12±0.01

Table3.1: General characterization of 3 month old female mice.

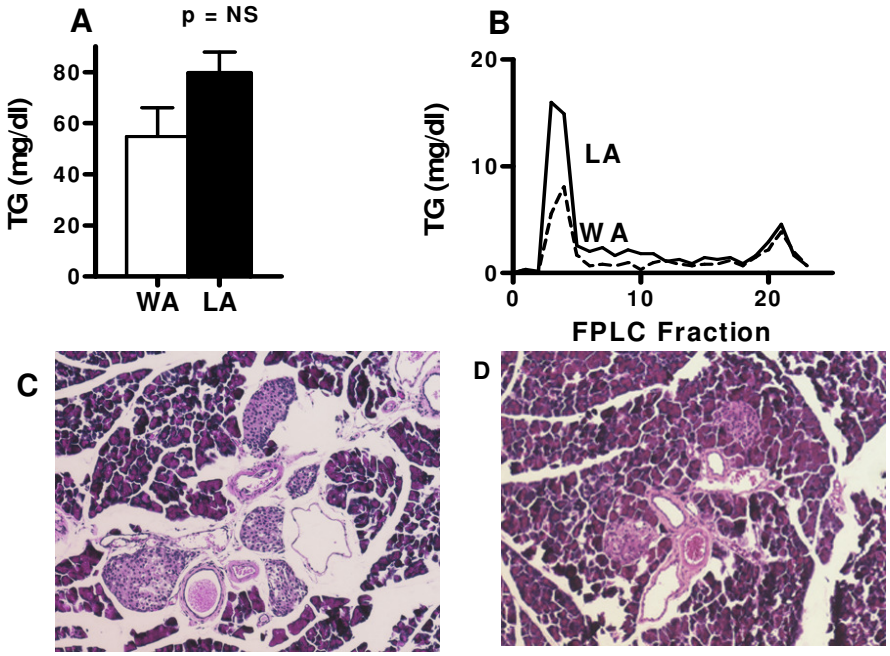

Figure3.2: Plasma lipids and islets in female WA and LA mice. (A) Fasting plasma triglyceride in male LA (n=10, white bars) tended to be higher in male WA (n=12, black bars) littermates. (B) Triglyceride distribution in plasma lipoproteins of 3 month old females fractionated by the fast protein liquid chromatography. LA females (solid line) had increased triglycerides in the very low fractions (fractions 13-15). (C,D) Typical pancreatic islet histology in WA(C) and LA(D) mice. The sections were stained with hematoxylin and eosin. Original magnification 10x.

We next performed an oral glucose tolerance test, where LA females had significantly higher plasma glucose levels at various time points up to 2 hours after administration of glucose load (Fig 3.3A). Fifteen minutes after glucose challenge, the LA female mice had significantly lower plasma insulin levels compared to WA mice (LA, 0.36±0.01, n=4 and WA, 0.49±0.03 ng/ml, n=6; p= 0.01, Fig 3.3B). This lower glucose-stimulated insulin level in LA mice was accompanied by significantly smaller mean pancreatic islet area when compared to age matched WA controls (Fig 3.3C and Figures 3.2C and D).

Thus, the *Pparg-P465L* mutation worsens the hyperglycemia caused by *Ins2-Akita* mutation partly by inadequate secretion of glucose stimulated insulin.

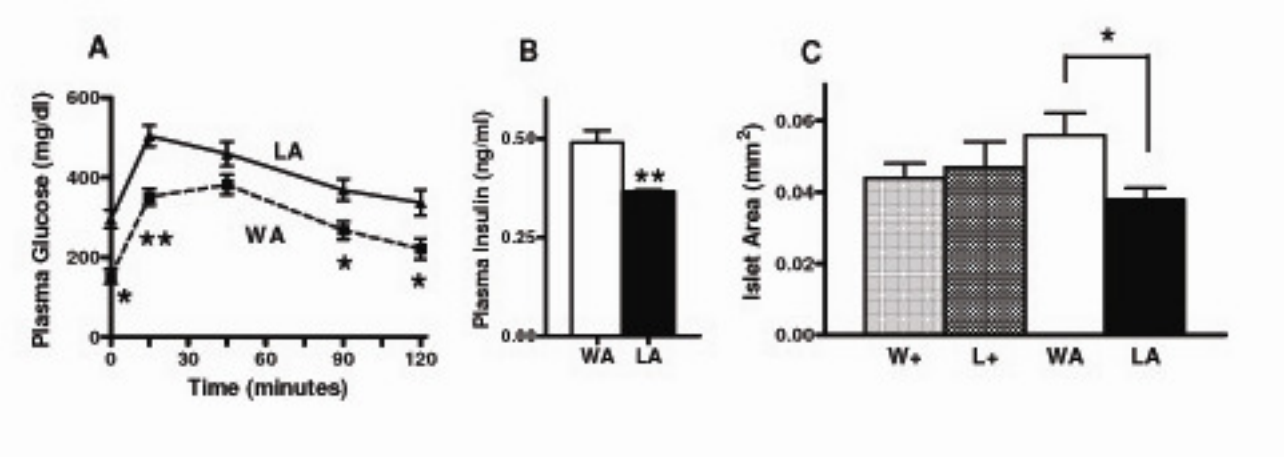

Figure 3.3: Reduced circulating insulin contributes to hyperglycemia in female LA mice. (A) Plasma glucose levels after oral glucose load and (B) Plasma insulin levels at 15 minutes after oral glucose administration in female LA mice (n=5) compared to female WA littermates (n=5). (C) Mean pancreatic islet area in female W+ (n=4), L+ (n=4), LA mice (n=5) and WA littermates (n=6). * P< 0.05, ** P< 0.01. Dashed line and white bars indicate WA, solid line and black bars indicate LA, W+ are speckled bars, L+ are checkered bars.

Adipose Tissue dysfunction contributes to Insulin Resistance in the **LA females:** To test the hypothesis that P465L/+ mutation may cause insulin resistance in mice, we investigated whether insulin resistance contributes to higher plasma glucose levels in female LA mice. In an intraperitoneal insulin tolerance test (IPITT), plasma glucose in WA females dropped to 70±6 % compared to baseline at 15 minutes after an intraperitoneal insulin injection. However, in LA females the glucose levels reduced only to 94±4 % (n=6, $p< 0.01$, Fig 3.4A). Thus, insulin-mediated suppression of plasma glucose was impaired in LA females. In addition to glucose homeostasis, insulin also suppresses non-esterified fatty acis (NEFA) release, largely from the adipose tissue [24]. WA and LA mice have a large difference in their baseline glucose levels, but have comparable fasting NEFA levels. I therefore examined NEFA as an additional parameter to measure the insulin sensitivity in LA mice. In IPITT, NEFA levels in LA mice were significantly higher than WA controls 15 minutes after insulin injection (LA 0.65±0.04, n=5 and WA, 0.51±0.02 mEq/L, n=6; $p< 0.01$, Fig 3.4B). This suggests that LA mice were not able to suppress the NEFA release as effectively as the WA controls. PPARγ is widely recognized as an important regulator of adipose tissue differentiation. Mice carrying the *Pparg-P465L* mutation have normal total adipose tissue mass with altered fat distribution [12]. Insulin is also known to be an important player in adipose tissue physiology. LA mice carrying mutations in both these important genes have a significant reduction in their total adipose tissue mass and have lower body weight (Fig 3.4C, F). They have reduced gonadal (visceral) adipose tissue (similar to L+ mice) but a slight reduction in inguinal (subcutaneous) adipose tissue (unlike L+ mice, which have a significantly

higher inguinal fat mass than W+ mice) (Table 3.1). Thus, simultaneous presence of *Pparg-P465L* and *Ins2-Akita* mutations severely compromises the normal adipose tissue development.

Median size of individual adipocytes from the inguinal depot was significantly larger in the LA mice than those in the WA mice (Figure 3.5) and an assessment of gonadal adipocytes suggested a similar change. Presence of larger cells in a smaller fat depot suggests that the adipose tissue in LA mice is composed of a smaller number of larger adipocytes than that in WA mice.

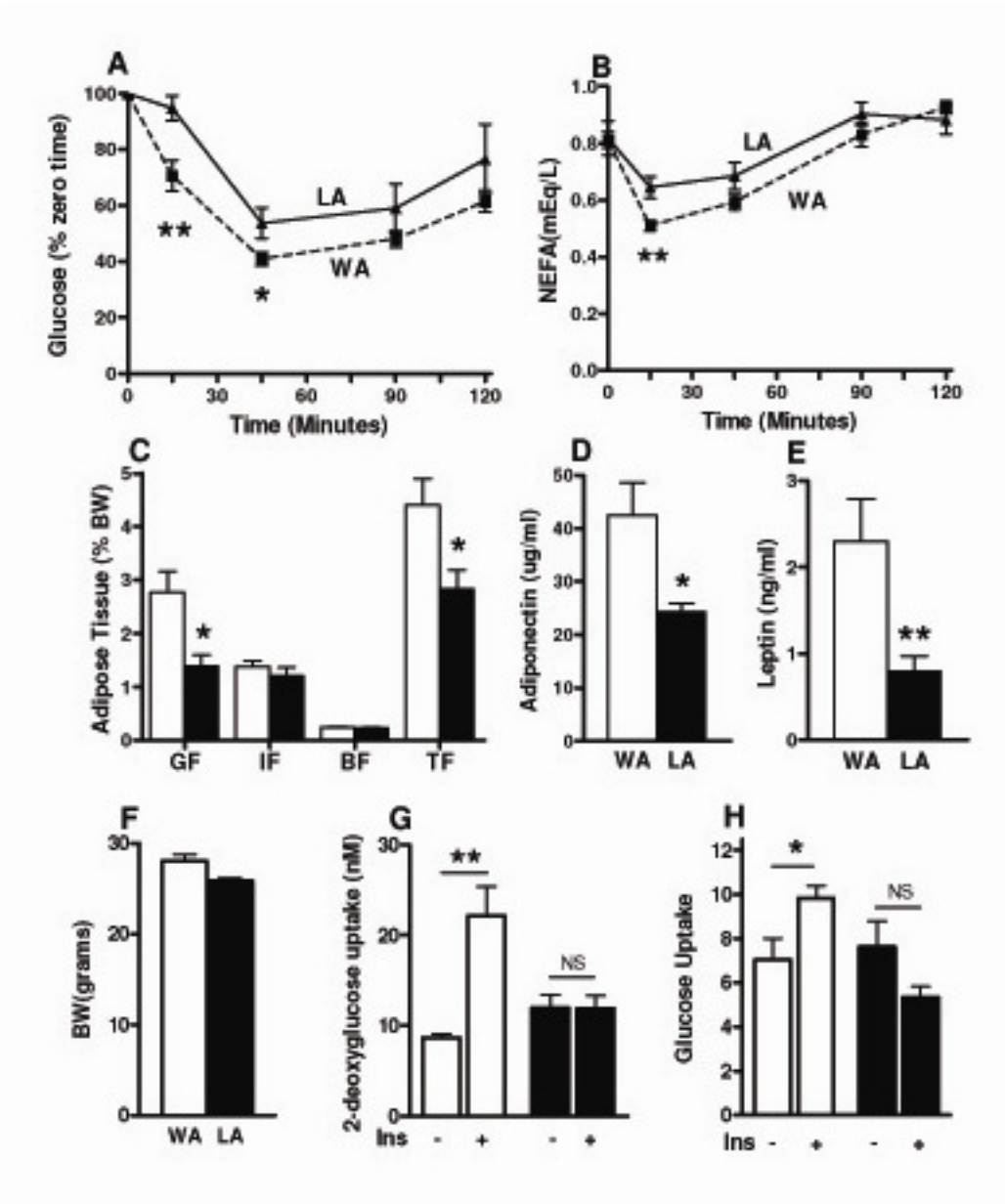

Figure 3.4: Insulin resistance in adipose tissue of LA female mice. (A) Blunted fall in plasma glucose and (B) suppression of NEFA secretion in LA mice (n=5, solid line) compared to WA littermates (n=6, dotted line) in response to intraperitoneally administered insulin 0.5U/Kg body weight. (C) Adipose tissue weight normalized with body weight of 3 mo old female LA

(n=7, black bars) and WA mice (n=7, white bars) GF, perigonadal fat; IF, inguinal fat; BF, brown fat and TF, total fat. (D) Plasma adiponectin and (E) leptin and (F) body weight in LA (n=6) and WA mice (n=7). Glucose uptake in primary adipocytes (G) and adipose tissue explants (H) without and with 100nM insulin. * P< 0.05, ** P< 0.01. NS, not significant.

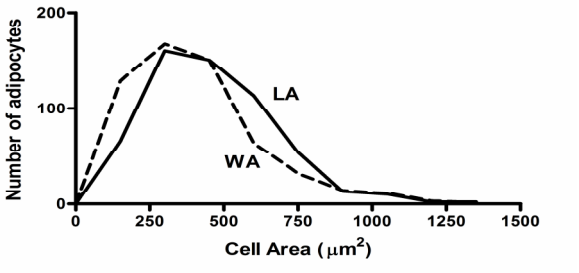

Figure 3.5: Adipocyte size distribution: Size distribution of the adipocytes in the inguinal depots indicates larger adipocytes in female LA mice (solid line) compared to WA littermates (dashed line). Individual cell areas are measured in ~250 cells in paraffin sections of each of the 4 mice per genotype.

The LA mice also had significantly reduced plasma levels of adiponectin (LA; 24.4±1.5, n=3 vs. WA; 42.5±6.2 μg/ml, n=3; $p< 0.05$, Fig 3.4D) and leptin (LA; 0.8±0.1, n=8 vs. WA; 2.3±0.5 ng/ml, n=8; $p< 0.05$, Fig 3.4E). Adiponectin and leptin are primarily produced in adipose tissue and are critical to maintain insulin sensitivity [25,26]. To understand whether reduced amount of adipose tissue and impaired adipokines production extends to impairment in insulin sensitivity, I studied primary adipocytes and adipose tissue explants in-vitro. When mature adipocytes were isolated and treated with 100nM insulin, LA adipocytes had much reduced insulin-stimulated 2-deoxyglucose uptake compared to WA adipocytes (LA; 11.92±1.4nM vs. WA; 22.11±3.23, $p< 0.05$, Fig 3.4G). The basal glucose uptake in the absence of insulin was similar in primary adipocytes obtained from the two genotyopes. Similarly, glucose uptake in adipose tissue explants from WA mice significantly increased upon insulin stimulation. However, glucose uptake in LA explants did not respond to insulin (LA; 5.3±0.5 vs. WA; 9.8±0.6 arbitrary units, $p= 0.0001$, Fig 3.4H). Again, the glucose uptake in the absence of insulin was comparable in the adipose tissue explants from WA and LA female mice. This failure to increase glucose uptake in response to insulin by the primary LA adipocytes and LA explants demonstrate the insulin resistance phenotype in the LA adipose tissue. In separate experiments, both W+ and L+ adipose tissue explants had similar basal glucose uptake and comparable increases in insulin stimulated glucose uptake confirming their normal insulin sensitivity (data not shown).

Metabolic signaling of insulin is mediated by a phosphorylation of a cytoplasmic protein AKT (also known as protein kinase B), a serine/threonine kinase (Ueki, JBC, 1998). We investigated

the phophorylation of AKT as a marker of insulin sensitivity. Incubation with insulin induced a two fold increase in phosphorylation of AKT in primary adipocytes from inguinal and gonadal fat of WA mice. In contrast, insulin stimulated phosphorylation of AKT was significantly blunted in both inguinal and gonadal adipocytes and was not different from the un-stimulated cells from LA mice (Fig 3.7A,B). The total amount of AKT protein was comparable in the two genotypes. Thus, LA female mice have smaller and less functional adipose tissue which contributes to the mild insulin resistance phenotype as observed by IPITT in these mice.

Increased gluconeogenesis contributes to fasting hyperglycemia in LA mice: In a fasted state, liver maintains the plasma glucose at a certain level by glycogenolysis and gluconeogenesis. This glucose generated in the liver is available to tissues such as brain to utilize as an energy source. As LA female mice have significantly high fasting plasma glucose levels, I next studied the contribution of the liver to this phenotype. LA livers revealed a 2.2 fold reduced expression of glucokinase (*GK*), a key glucose uptake enzyme, compared to WA mice ($p= 0.06$, Fig 3.6A). Analysis with two way ANOVA suggests that both *Pparg* genotype ($p< 0.05$) and *Ins2* genotype ($p< 0.05$) have significant effects and additively contributing to the reduced glucokinase expression in LA mice. I observed a 2 fold reduction in the gene expression of sterol regulatory element binding protein (*SREBP-1*) which binds to and increases the transcription of glucokinase gene ($p< 0.05$). Consistent with the gene expression profile, the uptake of 2-deoxyglucose was significantly lower in primary hepatocytes isolated from LA compared to WA females (WA; 4.29±0.7 and LA; 1.51±0.28 mg/g protein, $p<0.01$, Fig 3.6B). In contrast to genes for glucose uptake, there was a

significant 1.5 fold increase in the gene expression for phosphoenolpyruvate carboxykinase (*PEPCK*) ($p < 0.05$), one of the important regulators of gluconeogenesis.

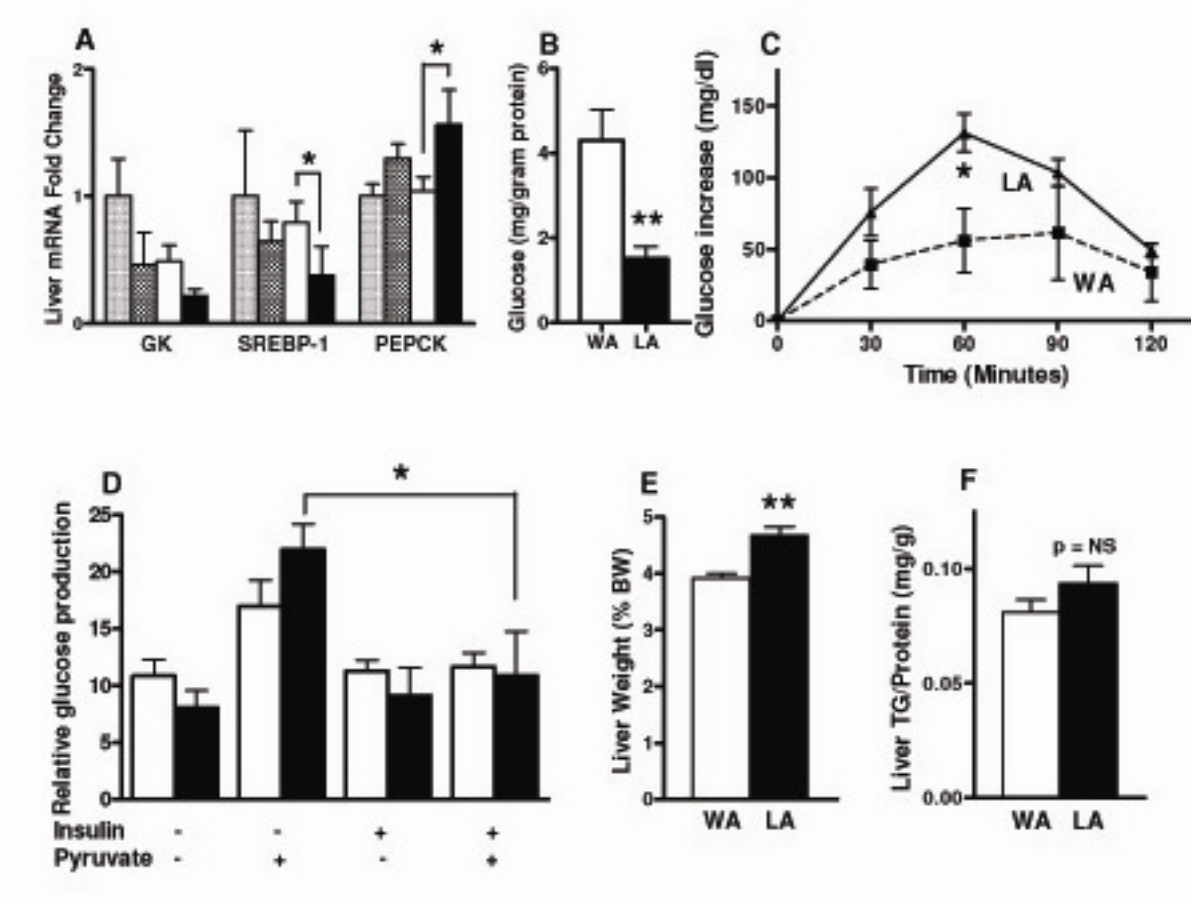

Figure 3.6: Altered liver function due to reduced plasma insulin levels contributes to increased hyperglycemia in LA mice. (A) Average mRNA amounts of genes for GK, SREBP-1 and PEPCK in L+ (checkered) WA (white) and LA (black) mice relative to that in W+ mice (speckled) as 1.0 (n≥ 7). (B) Uptake of 2-deoxy-glucose in primary hepatocytes. (C) Increase in plasma glucose after an intra-peritoneal administration of 2 mg/Kg body weight pyruvate in LA female mice compared to WA littermates (n=4 each). (D) Glucose

production (mg/gram protein) in cultured WA (white bars) and LA (black bars) hepatocytes. Increase in glucose in the medium by the addition of 2mM Sodium pyruvate was suppressed by the presence of 100nM insulin. (E) Increased liver weight and (F) a small increase in liver TG levels in LA mice (n=6) compared to WA mice (n=12). * P< 0.05, ** P< 0.01. NS, not significant.

We next performed a pyruvate tolerance test to assess the rate of gluconeogenesis in WA and LA female mice. LA mice indeed demonstrated a 2.3 fold greater increase in plasma glucose levels compared to WA mice at 60 minutes after an intraperitoneal injection of pyruvate ($p< 0.05$, Fig 3.6C). Thus, impairment in glucose uptake combined with increased hepatic glucose production contributes to the fasting hyperglycemia in LA females compared to WA controls. Corroborating with the in vivo data, glucose production from primary hepatocytes, isolated from LA mice, increased significantly in the presence of pyruvate (2.7 fold) compared to 1.3 fold in WA hepatocytes. (The upregulated gluconeogenesis can be due to a deficiency of circulating insulin or a resistance to insulin described earlier in the LA mice.) This increase in gluconeogenesis was completely suppressed in the presence of exogenous insulin in both genotypes (Fig 3.6D). Furthermore, the Akt phosphorylation in hepatocytes from both WA and LA mice was increased comparably after incubation with 100nM insulin for 10 minutes (Fig 3.7C). Thus, hepatocytes from LA mice are as sensitive to exogenous insulin as WA cells.

At 7 months, the LA female mice also had a small yet significant increase in their liver weight (LA 4.68±0.14 n=6, and WA 3.9±0.08%, n=12, $p< 0.05$, Fig 3.6E). This increase is partially explained by a small increase in liver triglyceride content per gram of protein (Fig 3.6F). Since the liver weight and triglyceride accumulation was not different in LA and WA female mice at 3 months (Table 3.1), the increase in liver weight appears to be an age-dependant phenomenon.

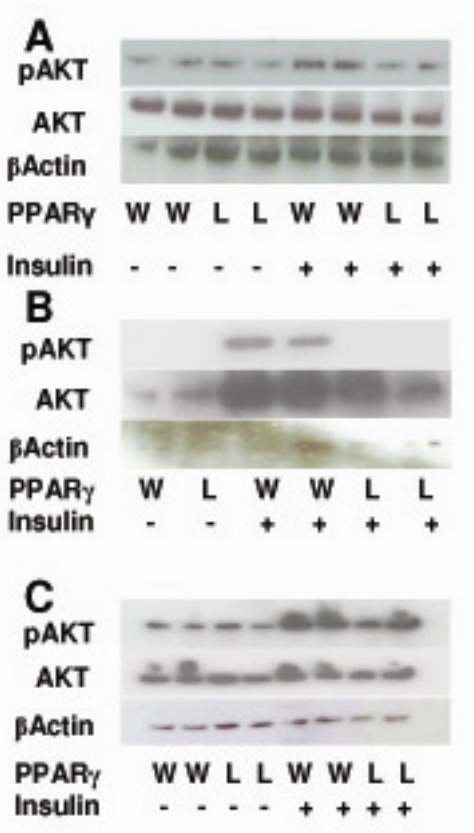

Figure 3.7: Akt phosphorylation status. Western blot for pAKT (upper panels), total AKT (middle panels) and β-actin (lower panels) in adipocytes isolated from inguinal fat (A), gonadal fat (B) and hepatocytes (C). Cells were incubated with 100nM (+) or without (-) insulin for 10 minutes. Antibodies used were phsopho-Akt (Thr308) (Cell Signaling #2965), Akt (Cell Signaling #9272) and β-actin (Cell Signaling # 5125).

***PpargP465L* mutation does not affect glucose handling and insulin sensitivity in the skeletal muscle.** The mRNA level of *Glut-4*, the major insulin-sensitive glucose transporter in skeletal muscle, showed no change in LA mice compared to WA mice. Expression levels of *AdipoR1, IR,* and *IRS-1*, genes important for normal insulin sensitivity were also unchanged (Fig 3.8A and table 3.2).

Insulin resistance is usually associated with increased intracellular accumulation of lipids [27]; however, the skeletal muscle triglyceride levels were unaltered by the presence of *Pparg-P465L* mutation (Fig 3.8B). Glycogen content was increased in mice with the *Akita* mutation but *Pparg-P465L* mutation had no effects (Fig 3.8C). Taken together, these data indicate that glucose handling and insulin sensitivity in skeletal muscle are largely unaffected by *Pparg-P465L* mutation.

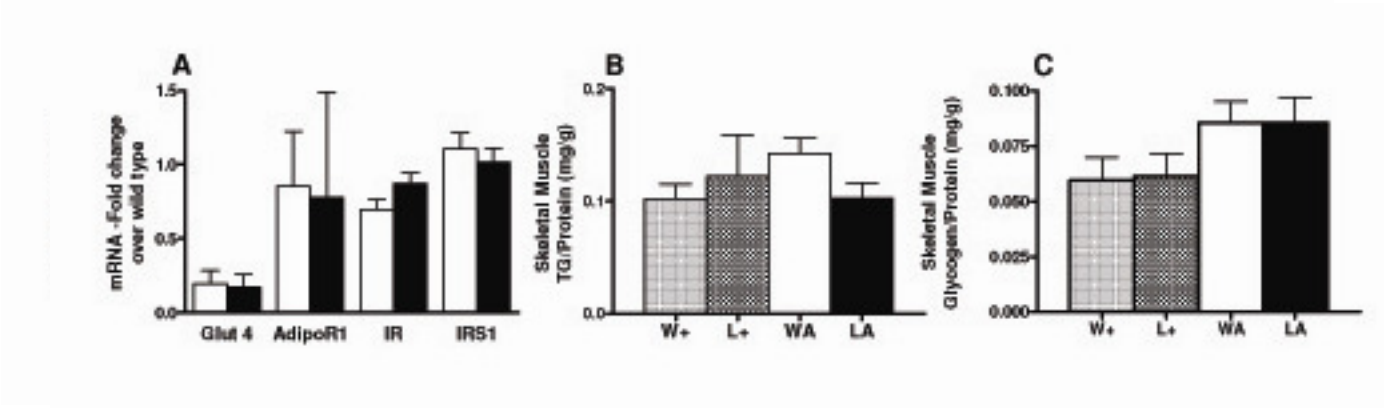

Figure 3.8: Normal glucose handling in skeletal muscle. (A) Gene expression of Glut4, AdipoR1, IR and IRS-1 in female WA (white bars) and LA (black bars) mice. Data are expressed as mean±SE relative to the mean level in wild type animals set as 1.0. (B) Triglyceride content per gram of protein in skeletal muscle in female mice. (C) Skeletal muscle glycogen storage per gram of protein in female mice.

	W+	L+	WA	LA
Liver				
PPARγ	1.0±0.26	0.92±0.16	1.11±0.22	1.15±0.21
PPARα	1.0±0.08	1.39±0.37	1.02±0.1	1.13±0.15
PGC-1α	1.0±0.3	0.77±0.39	1.1±0.18	1.15±0.32
IR	1.0±0.16	1.02±0.23	1.13±0.18	1.15±0.32
IRS-1	1.0±0.7	0.28±0.04	0.41±0.08	0.27±0.05
AdipoR2	1.0±0.37	0.7±0.2	0.97±0.16	1.3±0.6
Skeletal Muscle				
PPARγ	1.0±0.06	0.61±0.03	1.38±0.05	1.23±0.04
PPARα	1.0±0.05	0.65±0.05	0.74±0.03	1.07±0.08
IR	1.0±0.08	1.08±0.13	0.7±0.06	0.87±0.07
IRS-1	1.0±0.08	1.8±0.33	1.11±0.3	1.01±0.09
Glut-4	1.0±0.76	0.84±0.6	0.2±0.08	0.17±0.08
AdipoR1	1.0±0.46	1.41±1.1	0.86±0.4	0.78±0.7

Table 3.2: Gene expression data from 3 month old female mice. n≥4 for all genotypes, mRNA amount was expressed relative to the mean amount in W+ mice as 1.0.

3.5 Discussion

Glucose tolerance and insulin resistance are complex phenomena affected by multiple signaling mechanisms in varied organs. For an individual as a whole, the implications of being hyperglycemic and insulin resistant are serious. In this study we focused on a dominant negative point mutation, *Pparg-P465L,* in the ligand binding domain of PPARγ and established that this mutation can worsen the hyperglycemia caused by the diabetogenic *Ins2-Akita* mutation. The higher plasma glucose in female LA mice compared to WA females can be accounted for by adipose tissue-specific insulin resistance, reduced circulating plasma insulin and increased hepatic gluconeogenesis.

Pparg is expressed in pancreatic islets, where it has a growth inhibitory role. Targeted elimination of *Pparg* in β-cells led to bigger pancreatic islet mass without alterations in glucose homeostasis [11]. Unlike the wild type islets, *Pparg* deficient islets lack the ability to expand in response to high fat diet [11]. In contrast, mice carrying the *Pparg-P465L* mutation also have bigger islets, particularly on a high fat diet [12]. Therefore, while the *Pparg-P465L* mutation is unable to exert a normal growth inhibitory action; it does not interfere with the expansion of islets in response to high fat feeding. The Akita mutation reduces the insulin secretion from the beta cells. Initially, the islets in $Ins2^{Akita/+}$ mice undergo hypertrophy to compensate for the falling plasma insulin levels. Consistently, in our current study of mice with *Ins2-Akita* mutation, the islets in WA female mice

were enlarged. However, the LA females had significantly smaller mean islet area compared to the WA islets. The LA females also had reduced plasma insulin levels 15-minutes after an oral glucose dose compared to the WA littermates. Although the direct effect of the *Pparg-P465L* mutation cannot be excluded, it is likely that the increased insulin demand from the already stressed pancreas accelerates apoptosis of β cells initiated by the *Ins2-Akita* mutation and contributes to the augmented hyperglycemia seen in LA mice. Consistent with our observation, Evans-Mollina et al in 2009 demonstrated the positive effects of PPARγ agonist Pioglitazone on islet function in diabetic mice as measured by higher random insulin levels and improved glucose stimulated insulin release [28]. These improvements were secondary to reduced endoplasmic reticulum stress and improved expression profile of genes involved in glucose sensing and β-cell differentiation.

Our studies have shown that LA mice have increased fasting plasma glucose levels and an insulin resistance phenotype. In fasted states, liver is the main source of plasma glucose where pyruvate, amino acids and glycerol are converted into glucose through gluconeogenesis. This newly synthesized glucose is available as energy for tissues, in particular the brain, which relies primarily on carbohydrate metabolism [29]. Two fold reductions in hepatic *GK* expression in LA mice result in reduced glucose uptake. The glucokinase promoter has a peroxisome proliferator response element and is transcriptionally activated by *PPARγ* agonists [30]. Hepatic *GK* expression is

reduced in diabetic animal models with insulin deficiency [31] and insulin has been shown to be a major activator of *GK* gene transcription [31,32] through the transcription factor *SREBP-1c* [30]. We in turn observed a significant decrease in *SREBP-1c* expression in LA compared to WA livers. Thus, reduced circulating insulin levels and *Pparg-P465L* mutation have an additive effect to reduce *GK* expression. In addition, the upregulation of *PEPCK* in these mice suggests increased gluconeogenesis which could explain the higher fasting plasma glucose in LA mice. A pyruvate tolerance test, where LA mice showed a significantly larger increase in glucose after pyruvate injection, and higher pyruvate stimulated glucose production in the isolated LA hepatocytes than WA hepatocytes in culture, confirmed this possibility. Thus the reduced hepatocyte glucose uptake coupled with upregulated gluconeogenesis contributes to higher plasma glucose in LA compared to WA females. Increased hepatic gluconeogenesis in LA females could possibly result from a deficiency in circulating insulin and/or insulin resistance. However, LA hepatocytes are able to suppress glucose production from pyruvate in response to exogenous insulin, suggesting that the increased hepatic gluconeogenesis observed in LA mice mainly results from a deficiency in circulating insulin but not hepatic insulin resistance.

The insulin resistance phenotype observed in the LA females primarily arises from the adipose tissue. PPARγ and insulin are both widely recognized as essential genes for adipose tissue differentiation and lipid deposition. Thus, we observed that *Pparg-P465L* mutation on *Ins2-Akita*

background significantly reduced adipose tissue mass and adipocytokine levels as measured by plasma leptin and adiponectin. Reduced plasma adiponectin and leptin could contribute to whole body insulin resistance. However, liver and skeletal muscle of LA mice were equally insulin sensitive to those of WA mice indicating that the signaling downstream of these peptides are intact. Possibly the extent of decrease in adiponectin and leptin is not sufficient to alter the insulin sensitivity in liver and muscles. Yet, the LA mice had adipocytes significantly reduced in number but larger in size, which are shown to be associated with reduced insulin sensitivity [33], suggesting the inability of the adipose tissue to recruit new pre-adipocytes. The higher plasma TG, and a slight increase in liver TG content, observed in LA females could also be a consequence of this impaired storage function of the adipose tissue. Similar to our observation, Gray et al reported that leptin deficient ob/ob mice carrying the *Pparg-P465L* mutation had a significant reduction in adipose tissue mass and are insulin resistant [13]. The authors attributed this to an inability of the adipose tissue to expand in the face of increased availability of energy. Our in vitro data using adipose tissue explants and primary adipocytes suggest that LA adipose tissues also have impaired insulin-stimulated glucose uptake, further confirming their adipose tissue dysfunction. While our conclusions were derived using Akita mutation as a source of insulin deficiency, rendering *Pparg-P465L* mutant mice insulin deficient by other means such as Streptozotocin treatment and/or treating our model with various anti-diabetic drugs may provide further insight into the phenotype.

Although our study was focused on *Ins2*$^{Akita/+}$ females, the same mechanisms must be responsible for the increased severity of diabetes in LA males compared with WA males. Diabetes induced by *Ins2-Akita* mutation affects males more severely than females [34] and consequently only males are used as models of type 1 diabetes in general. However, our study shows that *Pparg-P465L* mutation significantly increases hyperglycemia in Akita female and advocates the use of female *Ins2*$^{Akita/+}$ mice as an excellent model to study diabetes, particularly in female specific conditions including polycystic ovary syndrome and gestational diabetes. Our mice also provide a model where reduced insulin and insulin resistance, signatures of type 1 and type 2 diabetes respectively, are simultaneously present.

In conclusion, our study showed that Pparg-P465L mutation worsens the hyperglycemia caused by diabetogenic Ins2-Akita mutation, and have unmasked the insulin resistance phenotype in the adipose tissue of mice carrying the Pparg-P465L mutation on Ins2-Akita background. A simultaneous reduction of PPARγ and insulin, which are both critical in adipocyte differentiation, leads to limited expansion of adipose tissues in these mice. The primary storage defect in adipose tissue triggers hypertriglyceridemia. Adipose tissue insulin resistance also increases pressure on an already stressed pancreas, contributes to a further destruction of beta cells and reduction in circulating plasma insulin. This, in turn, causes the liver to upregulate gluconeogensis resulting in the enhanced hyperglycemia.

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C h a p t e r 4

MILD REDUCTION OF VLDL UPTAKE IN *PPARG*$^{P465L/+}$ MACROPHAGES IS NOT SUFFICIENT TO CHANGE THE ATHEROSCLEROTIC LESION SIZE IN *APOE* KNOCKOUT MICE.

4.1 Summary

The increasing prevalence of obesity and insulin resistance and its negative impact on the outcome of atherosclerotic cardiovascular disease is of major concern. Several mouse studies have established the anti-atherosclerotic effects of PPARγ and of PPARγ agonists. The PPARγP467L (L/+) mutation, originally identified in human patients, affect adipose tissue distribution, insulin sensitivity and blood pressure. In this study, we hypothesized that the L/+ mutation that causes hypertension, hyperinsulinemia, partial lipodystrophy and reduced macrophage PPARγ activity will worsen atherosclerosis in *Apoe-/-* mice. Despite a persistent increase in blood pressure and lipodystrophy, presence of PPARγP465L/+ mutation did not affect atherosclerotic plaque size in *Apoe-/-* mice. Plaque sizes were also comparable in the two genotypes after TZD treatment or Streptozotocin-induced hyperglycemia. Gene expression studies revealed a significant increase in ABCA-1 mRNA and a small decrease in CD-36 mRNA in *Apoe-/-* macrophages with L/+ mutation compared to littermates. Consistent with this observation, cultured *Apoe-/-* macrophages with L/+ mutation, had a small yet significant reduction in DiI-labeled-VLDL uptake from the media by ~11%, compared to *Apoe-/-* macrophages. Transfer of bone marrow from mice with L/+ mutation to *Apoe-/-* recipients resulted in a small decrease in plaque size compared to the recipients that received macrophages without the L/+ mutation. It is likely that balance between increased blood pressure with its pro-atherogenic potential and macrophage athero-protective phenotype results in an unchanged plaque.

4.2 Introduction

Most individuals with cardiovascular disease (CVD) have multiple risk factors including abdominal obesity, dyslipidemia, hypertension, insulin resistance, proinflammtory and prothrombotic state, which together constitute the Metabolic Syndrome (MetS) [1]. The increased prevalence of obesity and its contribution to the pathogenesis of cardiovascular disease is of major concern. Although insulin resistance is a significant component of MetS, diabetes by itself can make patients more susceptible to atherosclerotic disease and also increases the severity of pre-existing cardiovascular disease. Many diabetic patients are being treated with Peroxisome Proliferator Activated Receptor (PPARγ) agonist Thiazolidinedione drugs which act as insulin sensitizers. Using mouse models of atherosclerosis lacking apolipoprotein E (apoE) or low density lipoprotein receptor (LDLR), several studies have established the anti-atherosclerotic effects of PPARγ agonists [2, 3, 4]. PPARγ is a nuclear receptor, and amongst its myriad functions, is necessary for adipocyte differentiation and triglyceride deposition [5]. PPARγ is expressed in activated monocytes and tissue macrophages and has been demonstrated in the foam cells of an atherosclerotic plaque [6, 7]. PPARγ has emerged as an important regulator of scavenger receptor CD-36 that can affect lipid accumulation in the macrophages [8]. On the other hand, a PPARγ-LXR-ABCA-1 axis has been implicated in lipid efflux from the macrophages [9]. Consistent with this knowledge, high fat fed LDLR-deficient mice reconstituted with bone marrow from a conditional knockout of macrophage PPARγ had increased atherosclerosis compared to control LDLR-deficient mice reconstituted with wild type macrophages [10].

In addition to the macrophage, PPARγ is expressed in endothelial [11] and vascular smooth muscle cells [12] and plays a role in blood pressure regulation. A two fold genetic increase or decrease in PPARγ expression decreases or increases blood pressure by ~3 mmHg respectively [13]. Endothelial PPARγ appears to be essential for the hypotensive effects of Rosiglitazone [14] and Rosiglitazone has been shown to improve the impaired acetylcholine-induced relaxation of carotid arteries in hypertensive mice over-expressing both human rennin and angiotensin transgene [15]. However, PPARγ deficient mice rescued from embryonic lethality have hypotension, suggesting a complex role for PPARγ in blood pressure regulation [16].

Various point mutations in PPARγ that affect adipose tissue distribution and insulin sensitivity have been identified in humans. Among these, the dominant-negative heterozygous PPARG-P467L mutation, which results in decreased PPARγ activity, was originally identified in patients with severe insulin resistance, hyperglycemia, lipodystrophy and hypertension [17]. Mice heterozygous for the corresponding *Pparg*$^{P465L/+}$ mutation (L/+ or L) also have ~7mmHg higher blood pressure than normal and have mild hyperinsulinemia and increased pancreatic islet mass, especially on high-fat diet [18]. They exhibit normal plasma glucose and insulin sensitivity [18, 19], unless they are stressed by severe obesity [19] or by suppression of insulin production (Pendse, Chapter 3). In this study, we hypothesized that the L/+ mutation that dominant negatively

reduces PPARγ activity and causes hypertension, hyperinsulinemia will worsen atherosclerosis in *Apoe-/-* mice. Although the L/+ mutation itself is a rare occurrence, several point mutations that have been identified in the ligand binding domain of PPARγ share somewhat similar phenotype that prominently includes reduced sensitivity to insulin. Here, we attempt to understand the impact of altered PPARγ function on atherosclerosis, in a context possible in the human population. We found that *PPARγ*$^{P465L/+}$*;Apoe*$^{-/-}$ (Lee) macrophages, in culture, had a significant reduction by about 11% in their DiI labeled VLDL uptake from the media compared to macrophages isolated from the *Apoe*$^{-/-}$ (Wee) controls. However, overall atherosclerosis lesion size in the two genotypes is not different, suggesting that a reduction/ alteration in PPARγ function (due to P465L mutation) does not have a significant effect on plaque size in *Apoe* deficient mice.

4.3 Methods

Generation of Mutant Mice and Diet: Mice deficient in Apoe and harboring a *Pparg*$^{P465L/+}$ mutation (Lee) were generated by crossing *Apoe-/-* mice with heterozygous *Pparg*$^{P465L/+}$ mice. Both the mutations are on the identical 129/SvEvTac background. Littermate *Apoe-/-* mice with (Lee) or without (Wee) were used for experiments. The mice were fed normal rodent chow (Lab Diet 5P76; PMI Nutrition International, Richmond, Indiana, USA), or a high fat western type diet containing 21% (w/w) fat and 0.15% (w/w) cholesterol (TD88137; Harlan Teklad, Madison, WI, USA). The mice were maintained in a temperature controlled environment at a 12 hour light and dark cycle. They had free access to food and water except when fasting blood samples were

obtained. The animals were handled following procedures approved by the Institutional Animal Care and Use Committee at The University of North Carolina at Chapel Hill.

Mouse Genotype Analysis: DNA samples were obtained from mouse tails and amplified in PCR reactions using Applied Biosystmes 2720 Thermal Cycler. Primers used for ApoE genotyping are 5'-primer (Intron 3) AGA ACT GAC GTG AGT GTC CA, 3'-primer (Intron 3) GTT CCC AGA AGT TGA GAA GC and Neo (3' end) CTT CCT CGT GCT TTA CGG TA. Primers used for PPARγ genotyping were 5'-primer CAC GAA TCA CCA GCA ACA TG and 3'-primer CCC ATT CTT GTC ATG ATT CCC.

Measurement of Plasma Glucose and Lipids: Animals were fasted for 4 hours and plasma glucose and cholesterol were measured in colorimetric assays from Wako Chemicals USA Inc (Richmond, Virginia). Plasma Triglyceride levels were determined using an enzymatic assay from Stanbio Lab (Boerne, Texas USA).

Blood pressure (BP) measurements: BP and pulse rate were measured by the tail-cuff method and were calculated from the mean of the 30 daily measurements each day for 6 consecutive days [20].

Determination of Atherosclerotic Lesion Size: Mice were sacrificed using an overdose of Avertin and perfused with Heparinized PBS followed by 4% paraformaldehyde. The heart with the aorta was then cut out, embedded in paraffin blocks, aortic root serially sectioned and stained with Sudan IV and Hematoxylin. Four sections, from anatomically defined positions in the aorta, were used for quantitative assessments of the atherosclerotic plaques and their mean was used as a measure of lesion area [21].

Triglyceride secretion assay: In order to estimate liver TG secretion, a 10% solution of Triton WR-1339 (Tyloxapol, Sigma-Aldrich) in 0.9% saline was injected into the tail vein at a dose of 0.7 mg/g body weight (total injected volume was ~ 200µl). Blood was collected at time 0 and at specified times after tyloxapol injection and plasma TG concentrations (mg/dl) were then measured and normalized to body weight [22]. The TG values were plotted against time for each genotype and a linear curve fit was performed. The slopes from the curve fit equations were used to determine the TG secretion from the liver in the two genotypes.

Fat tolerance test: At 5 months of age, male mice of each genotype fed normal chow were fasted for 4h and the animals were challenged with 250uL of olive oil. Plasma was collected for triglyceride measurement at 0, 1, 2, 4 and 6 hour after oral gavage [23].

Isolation of peritoneal macrophages and VLDL uptake: Mice were given intraperitoneal injections of sterile 4% thioglycollate (BD Biosciences) in PBS. Four days later, macrophages were collected by peritoneal lavage into PBS after sacrifice by cervical dislocation [24]. Macrophages were either directly used for gene expression analyses or washed with Ham's nutrient mixture F-10 medium (F-10), spun at 1000 × *g* for 5 min, and plated in 12-well plates at a density of 6×10^5 cells/well in F-10 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were washed 2 h later to remove non-adherent cells and cultured in medium without fetal bovine serum for 24 h before measuring VLDL uptake. Mouse VLDL fractions were isolated from pooled plasma of *Apoe*$^{-/-}$ mice, by ultracentrifugation at $d < 1.006$ and were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI C_{18}; Molecular Probes, Inc., Eugene, OR) [25]. Macrophages in culture were incubated with medium containing 1 μg/ml of DiI-labeled mouse VLDL. After 2 h, cells were washed with fresh medium, lysed and fluorescence was observed with an IX70 inverted microscope (Olympus) equipped with a filter set for Texas red (exciter 560/55,

dichroic 595, emitter 645/75; Chroma Technology Corp.) [24]. Fluorescence pixel intensity of each cell was recorded with a SPOT RT Slider digital camera and analyzed with SPOT version 4.0.9 (Diagnostic Instruments) and ImageJ 1.33u software (National Institutes of Health).

Bone Marrow Transfer: Bone marrow cells were collected from the femurs and tibias of donor Lee and Wee male mice by flushing with F-10 medium. Recipient female *Apoe-/-* mice (Wee), 6–8 weeks of age, were lethally irradiated (9.5 grays) and injected with 2×10^6 bone marrow cells in 0.2 ml of medium through tail veins. For 2 weeks after transplantation, mice were given drinking water acidified to pH 2.0 by HCl and containing 100 mg/liter neomycin [24].

Gene expression: Total RNA was purified using Automated Nucleic Acid Workstation ABI 6700, and real-time PCR was performed in ABI PRISM 7700 Sequence Detector (Applied Biosystems). β-Actin mRNA was used for normalization.

ABCA1 Forward CAG ATC TAT AGG AGG AAG CG

Reverse GTT AAC TCC CAG GAG TCC AA

Probe f TGC ATC GGC ATC CCT CCC G q

CD36 Forward GCC AAG CTA TTG CGA CAT GA

Reverse AGA ATC TCA ATG TCC GAG ACT

Probe f CAC AGA CGC AGC CTC CTT TCC ACC q

CCR2 Forward GAT GAT GGT GAG CCT TGT CA

Reverse ACC AAA GAT GAA TAC CAG GGA

Probe f ATT GGA GCT TGG ATC CTG CCT CCA CT q

NF-kB Forward AAG TGA TCC AGG CAG CCT TC

Reverse CTA TGT GCT GCC TCG TGG AG

Probe f TCA GGT CCA CTG TCT GCC TCT CTC GTC q

Data analysis: Values are reported as mean±SEM. Student's t test was used for comparisons between genotypes, and differences were considered to be statistically significant if $p < 0.05$.

4.4 Results

Phenotype of Lee and Wee mice: Lee and Wee mice were born in the expected mendelian ratio. They appeared healthy and gained weight normally. General

phenotypes of normal chow and high fat diet fed Wee and Lee mice are shown in table 4.1.

Blood pressure in 4 month old male Lee mice was significantly elevated compared to Wee littermate controls by about 8 mmHg.

	NC (Age = 5 mo)			**HFD** (Age = 4 mo with 2 mo on HFD)		
	Wee	Lee	P	Wee	Lee	P
BP (mmHg) Male	112±2 (9)	120±2 (7)	0.03	105±2 (21)	114±3 (14)	0.04
Lesion ($10^3\mu m^2$) Male+Female	68±9 (29)	59±10 (24)	NS	89±11 (25)	81±12 (19)	NS
Glucose (mg/dl) Male	184±10 (10)	180±11 (8)	NS	196±6 (23)	183±8 (18)	NS
Chol (mg/dl) Male+Female	670±87 (13)	607±71 (16)	NS	2810±107 (22)	2797±104 (17)	NS
TG (mg/dl) Male+Female	106±12 (13)	92±6 (16)	NS	286±31 (22)	160±15 (17)	0.002
IF/GF ratio Male+Female	0.69±0.04 (20)	1.4±0.1 (22)	0.0001	0.74±0.09 (14)	1.21±0.08 (9)	0.002

Table 4.1: Phenotypes of normal chow and high fat diet fed male Wee and Lee mice. GF, Gonadal Fat; HFD, high fat diet; IF, Inguinal Fat; NC, normal chow.

The difference in blood pressure between the two genotypes was retained with high fat diet feeding for 2 months (from 2 months of age to 4 months of age). We noticed no gender differences between parameters measured in the two geneotypes. The plasma glucose levels were similar in the two groups; the plasma TG levels were lower in the normal chow fed Lee animals, although the difference was not statistically significant. Plasma TG levels increased in both genotypes in response to high fat feeding and Lee mice had significantly lower TG levels in plasma compared to Wee controls. Plasma cholesterol was not different between the two genotypes on normal chow diet. Cholesterol levels increased in both genotypes upon high fat diet feeding; however, there was no genotype difference.

Although the mice of the two genotypes had similar body weights and similar total body fat content, there was a difference in their fat distribution pattern. Lee mice had a smaller visceral gonadal adipose tissue but a larger subcutaneous inguinal adipose tissue when compared to the corresponding fat depots in the Wee mice (gonadal fat/body weight: Wee, 1.76±0.16; Lee, 1.08±0.14 and inguinal fat: Wee, 1.18±0.14; Lee, 1.49±0.13; $p<0.05$). Thus, Lee mice had a significantly higher inguinal to gonadal ratio (IF/GF ratio) when compared to the Wee littermate controls. High fat feeding increased the total amount of adipose tissue in both genotypes (gonadal fat/body weight: Wee, 3.67±0.25; Lee, 3.05±0.28 and inguinal fat: Wee, 2.52±0.35; Lee, 3.09±0.39; $p<0.05$). However, the fat distribution, represented by I/G ratio was maintained similar to that on a regular chow diet. We next measured the average size of the atherosclerotic plaques in

serial sections of the aortic root in 5 month old regular chow fed mice and 6 month old mice fed high fat diet for 2 months (Figure 4.1).

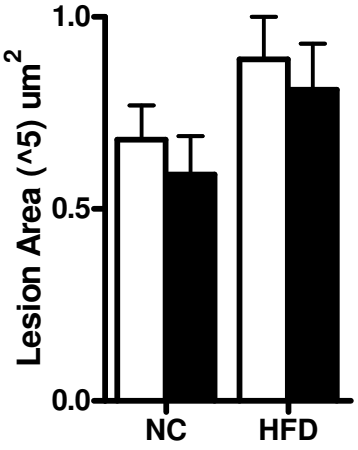

Figure 4.1: Atherosclerotic plaque size in normal chow and high fat diet fed Wee and Lee mice. NC, normal chow; HFD, high fat diet. Sample size for Wee, NC = 29; Lee, NC = 24, Lee, NC = 25 and Lee, HFD = 19. Data obtained from both male and female mice. White bars represent Wee mice, black bars represent Lee mice.

The lesion size in Wee and Lee mice did not differ from each other in either dietary condition (Wee NC, 0.68±0.09; Lee NC, 0.59±0.1; Wee HF, 0.89±0.11; Lee HF 0.81±0.12 x 10^5 μm^2; table 4.1). We did not notice any difference in the plaque composition based on histological assessment.

Due to the trend towards lower plasma TG in Lee mice, we investigated the liver in regular chow fed Lee and Wee mice. In 5 month old males, the liver weight was significantly smaller in Lee mice than that in Wee controls (Wee, 1.243±0.045, n= 5; Lee, 1.038±0.073 mg, n=5; $p<0.05$; Fig 4.2A). Despite the lower liver weight, the Lee livers had a higher TG (Wee, 2.76±0.31, n=5; Lee, 3.57±0.53, n=3 mg/g protein; p=NS; Fig 4.2C) and cholesterol per gram of protein (Wee, 1.04±0.027, n=5; Lee, 1.51±0.039 mg/g of protein, n=3; $p<0.05$; Fig 4.2C), although only the difference in cholesterol content was statistically significant.

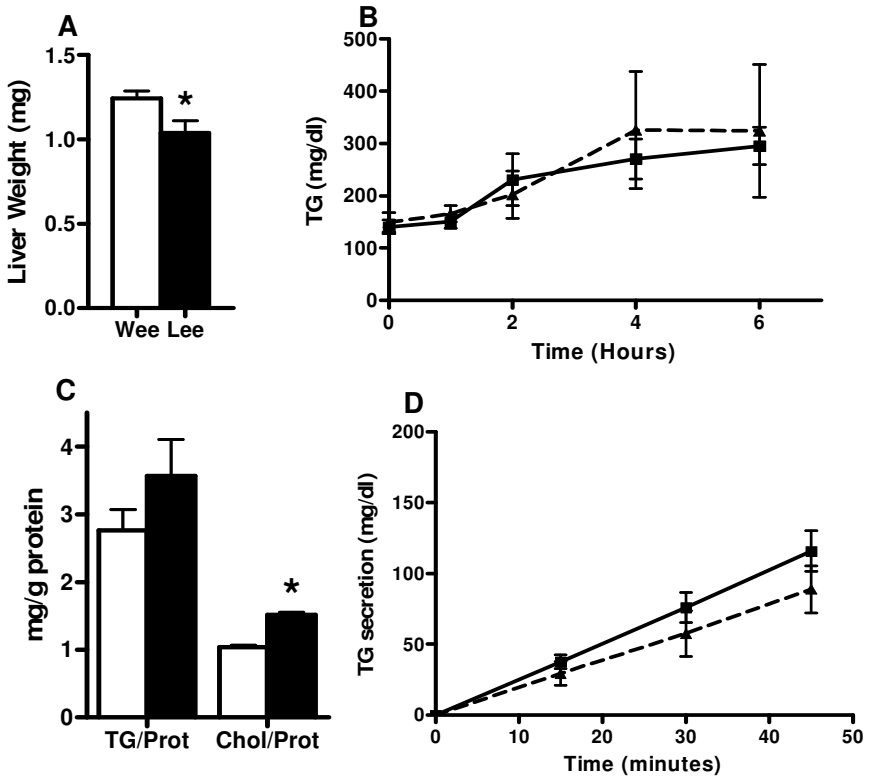

Figure 4.2: Liver phenotypes in Lee and Wee mice. Liver weight normalized to body weight (A), TG levels during lipid tolerance test (B), Liver triglyceride and cholesterol content normalized to protein (C) and liver triglyceride secretion after tyloxapol injection to prevent lipoprotein uptake (D) in 5 month old normal chow fed male mice. Dashed lines and white bars represent Wee mice, solid lines and black bars represent Lee mice.

To examine the post-prandial lipid handling, which can affect the outcome of an atherosclerotic lesion, we performed a lipid tolerance test in 5 month old male mice. TG levels in plasma samples collected after a 4 hour fast following an olive oil gavage were similar in the Lee and Wee mice (Fig 4.2B). These TG values are a function of lipid absorption, rate of clearance and liver TG synthesis and secretion. Since the TG and cholesterol content is higher in the Lee livers, we specifically looked at the secretion of TG from the livers. In an *in vivo* experiment where the lipoprotein particles coming from the livers of fasted mice were coated with Tyloxapol to prevent their uptake and clearance from plasma, we detected no difference in liver TG secretion between Lee and Wee mice (Fig 4.2D).

Despite a consistent, small yet significant increase in the blood pressure of Lee mice, the presence of PPARγP465L/+ mutation does not affect the atherosclerotic plaque size in *apoe-/-* mice. It is likely that the pro-atherogenic effect of the higher blood pressure may be counteracted by a presence of lower plasma TG levels and a favorable adipose tissue distribution pattern characterized by an increase in subcutaneous fat.

Macrophage function and bone marrow transfer: PPARγ is expressed at high levels in the macrophages and macrophage derived foam cells in atherosclerotic plaques [6, 7]. To investigate whether P465L/+ mutation in the ligand binding domain of PPARγ affects macrophage function in *apoe-/-* mice, we isolated thioglycollate-stimulated peritoneal macrophages from Lee and Wee

mice. Gene expression studies revealed a significant increase in ABCA-1 and a small decrease in CD-36 in Lee macrophages compared to Wee littermates (Fig 4.3A).

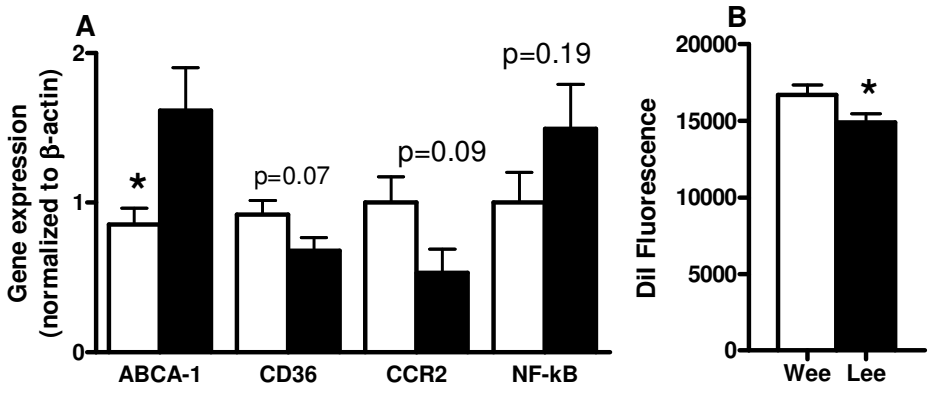

Figure 4.3: Macrophage gene expression and lipid uptake. (A) Gene expression data normalized to β-actin from primary macrophages isolated after thioglycollate injection. (B) DiI labeled VLDL uptake from the media by macrophages. ABCA-1, ATP binding cassette transporter protein; CD36, scavenger receptor-36; CCR2, CC chemokine receptor-2; NF-κB, nuclear factor- κB. Sample size for macrophages from Wee = 9 wells from 3 mice, Lee = 10 wells from 3 mice. White bars represent Wee mice, black bars represent Lee mice.

Since ABCA-1 stimulates cholesterol efflux, and CD-36 facilitates the uptake of oxidized LDL, this gene expression data suggests reduced lipid accumulation in Lee macrophages. Other genes including CCR2 and NF-κB were not significantly different between the two genotypes, suggesting that the inflammatory response is not altered in Lee macrophages. In agreement with the gene expression data, we found that Lee macrophages, had a significant reduction by about 11% in their DiI labeled VLDL uptake from the media in culture, compared to macrophages isolated from the Wee controls (Fig 4.3B). Thus, the presence of L/+ mutation reduced macrophage lipid accumulation via modulation of ABCA-1 and CD36 gene expression.

We next tested whether the reduced lipid content in the Lee macrophages would reduce the atherosclerotic lesion size in ApoE-/- mice. A bone marrow transfer experiment was performed by harvesting the bone marrow from Lee and Wee male mice and transplanting it into the Wee female recipients. Two months after the transfer, the plaque size in mice that received Wee bone marrow was slightly but not significantly larger than those received the Lee marrow (Fig 4.4).

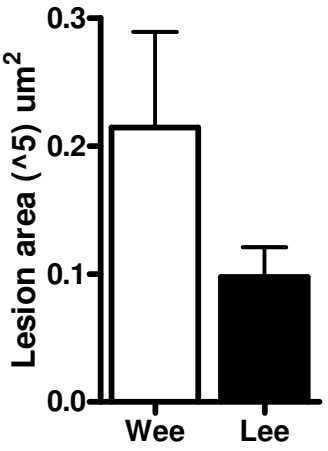

Figure 4.4: Atherosclerotic plaque size in chow fed female mice reconstituted with Wee and Lee bone marrow. P value = 0.19. Sample size for Wee = 9 and Lee = 10. White bars represent Wee mice reconstituted with Wee bone marrow, black bars represent Wee mice reconstituted with Lee bone marrow.

4.5 Discussion

Apoe$^{-/-}$ mice with PPARγP465L/+ mutation have a significantly higher blood pressure, by ~7 mmHg, compared to *Apoe*$^{-/-}$ controls. Using mice deficient in endothelial nitric oxide synthase, higher blood pressure has been shown to increase atherosclerotic lesions in the aorta of apoE-/- mice [26, 27]. Thus, we hypothesized that the higher blood pressure due to PPARγP465L/+ mutation will have a pro-atherogenic effect in apoE-/- mice. Despite higher blood pressure, the plaque sizes in regular chow and high fat fed Lee mice did not differ from the Wee littermate controls. Induction of hyperglycemia with streptozotocin and thiazolidinedione treatment of the Wee and Lee mice did not reveal any genotype differences (data not shown). The blood pressure in eNOS-/-;apoE-/- or NPRA-/-,apoE-/- double knowckout mice is much higher, ~20mmHg, compared to the increase of ~ 6 mmHg caused by the PPARγP465L/+ mutation [26, 28]. It is likely that in the presence of strongly atherogenic apoE deficient state, a small increase in blood pressure in not sufficient to worsen atherosclerosis. While drugs inhibiting both rennin-angiotensin system and diuretics effectively lowered atherosclerosis, treatment of eNOS-/-;apoE-/- mice with Hydralazine at a dose that lowers blood pressure levels to those seen in apoE-/- mice did not improve the outcome of atherosclerosis [26, 29]. Hypertension by itself may not influence atherosclerosis development significantly without a contribution of macrophage dysfunction and inflammation.

In our experiments using the Lee double mutant mice, generated from a cross between L/+ and *Apoe*$^{-/-}$ mice, it is not possible to differentiate between the contribution of the mutant PPARγ in macrophage and foam cells versus its global direct and indirect effects on plasma lipid levels, blood pressure and adipose tissue function. It is likely that the roles of PPARγ in macrophage versus the vessel wall have opposing effects, resulting in an unchanged atherosclerotic lesion in Lee mice. Thus, we specifically examined the role of PPARγP465L/+ mutation in peritoneal macrophages. PPARγ is expressed at high levels in the macrophages and macrophage derived foam cells in atherosclerotic plaques [6, 7]. It plays a complex and multi-faceted role in macrophages affecting the activation and recruitment of foam cells to the plaques, cholesterol homeostasis and inflammation. PPARγ agonists have been shown to inhibit atherosclerosis in LDL receptor deficient and apoE knockout mice [2, 3, 4]. Babaev at al showed that a knockout of macrophage PPARγ increased atherosclerosis in C57BL/6 and LDL receptor deficient mice [10]. The conditional disruption of PPARγ in murine macrophages regulates cholesterol efflux via reduction in ABCA-1 transcription. ABCA-1, a member of the ATP-binding cassette (ABC)-transporter family, is crucial for the high density lipoprotein (HDL) and apolipoprotein A1 (apoA1) mediated cholesterol efflux from macrophages [30]. PPARγ has been shown to induce macrophage ABCA-1 expression, via the mediation of LXR [9]. Additionally, TZDs up-regulate ABCA-1 expression, a phenomenon which is not seen in macrophages deficient in PPARγ. Thus, ABCA-1 mediated cholesterol efflux from the macrophage may contribute to the anti-atherogenic effect of PPARγ as well as its ligands. In contrast with the above findings, we found that the apoe-

/- macrophage with the dominant negative P465L/+ mutation had a higher expression of ABCA-1 compared to apoe-/- macrophage with wild type PPARγ. It is possible that this unexpected effect of L/+ mutation on ABCA-1 expression is attributable to altered function of the mutant PPARγ allele, rather than due to dominant negative reduction in the wild type allele in presence of L/+ mutation.

PPARγ is also shown to be an important regulator of the scavenger receptor CD36, which is associated with lipid accumulation in the macrophage [6, 8]. Although treatment of wild type but not PPARγ deficient macrophages with rosiglitazone resulted in a robust upregulation of CD36 expression, the basal gene expression level for CD36 were fairly similar between wild type and PPARγ deficient macrophages. However, in our Lee mice with the PPARγP465L/+ mutation, the gene expression of CD36 in thioglycollate-induced macrophages is lower by ~25% than Wee macrophage, with the difference approaching statistical significance. It is likely that these differences in basal gene expression contribute to the reduced lipid accumulation in Lee macrophages, which was confirmed by a small yet significant reduction in their DiI labeled VLDL uptake from the media compared to macrophages isolated from the Wee controls. In spite of this athero-protective phenotype in the Lee macrophages, the bone marrow transfer experiment, set up to study the effect of macrophage PPARγ mutation, did not reveal a protective effect on plaque size *in vivo*.

Thus, the reduced PPARγ activity and/or an altered function of the mutant protein in mice harboring the PPARγP465L/+ mutation affects the expression of target genes differentially compared to its complete absence. Thus variation in the amount or activity of PPARγ protein, which may naturally exists in the human population or due to the effect of TZD treatment, can modulate the lipid accumulation in the macrophage potentially influencing atherosclerosis.

The pro-atherogenic effect of the higher blood pressure in Lee mice is likely to be counteracted by the anti-atherogenic effect caused by a protective phenotype in the Lee macrophages. However, when the hypertensive effect of the PPARγP465L/+ mutation was eliminated in the bone marrow transfer experiments, plaques in mice transfected with L/+ bone marrow were only somewhat smaller than the wild type controls. The protective phenotype suggested by the in vitro experiments in Lee macrophages did not bring about an overt reduction in the atherosclerosis lesions in the bone marrow transfer experiments.

In addition to adipose tissue, where PPARγ is essential for the development and optimum function, deficiency of macrophage PPARγ reduces apoE gene expression and its protein level in the plasma [31]. ApoE has been recognized to regulate cholesterol efflux from macrophages [32]. Thus,

apoE null macrophages have reduced cholesterol efflux to HDL3 and apolipoprotein A1 explaining the observation by Fazio et al that transplantation of bone marrow derived from apoE deficient mice into C57BL/6 mice leads to a significant increase in atherosclerotic lesion size [33, 34]. The possibility that PPARγ mutation may differentially alter the phenotype of some other model of atherosclerosis, such as LDLR-/- mice which retain endogenous apoE, can not be denied.

It is important to acknowledge that PPARγ is identified as a crucial athero-protective molecule in the experimental setting of its complete lack in a specific tissue while present in the rest of the body. Unlike the tissue specific knock-out mice, a reduction in functional PPARγ due to P465L mutation in the whole body resembles a phenotype due to human mutations and polymorphisms more closely. This model takes into consideration, the potential cross talk between different cell and organ systems, which seems to dampen the potential deleterious effects in a single cell type/ organ, as expected from tissue specific knockouts.

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C h a p t e r 5

APOLIPOPROTEIN E KNOCK-OUT AND KNOCK-IN MICE: ATHEROSCLEROSIS, METABOLIC SYNDROME AND BEYOND.

(This chapter consists of material from a manuscript reprinted with permission from JLR; 2009 Apr;50 Suppl:S178-82. Epub 2008 Dec 5; titled "Apolipoprotein E knock-out and knock-in mice: atherosclerosis, metabolic syndrome and beyond" by Pendse AA, Arbones-Mainar JM, Johnson LA, Altenburg MK and Maeda N)

5.1 Summary

Given the multiple differences between mice and men, it was once thought that mice could not be used to model atherosclerosis, principally a human disease. Apolipoprotein E deficient (apoEKO) mice have convincingly changed this view, and the ability to model human-like plaques in these mice has provided scientists a platform to study multiple facets of atherogenesis and to explore potential therapeutic interventions. In addition to its well-established role in lipoprotein metabolism, recent observations of reduced adiposity and improved glucose homeostasis in apoEKO mice suggest that apoE may also play a key role in energy metabolism in peripheral organs including adipose tissue. Finally, along with apoEKO mice, knock-in mice expressing human apoE-isoforms in place of endogenous mouse apoE have provided insights into how quantitative and qualitative genetic alterations interact with the environment in the pathogenesis of complex human diseases.

Apolipoprotein E (apoE) plays a central role in lipoprotein metabolism and is required for the efficient clearance of diet-derived chylomicrons and liver-derived very low density lipoprotein (VLDL) remnants by the liver (1). Consequently, mice lacking apoE (apoEKO) provided the first practical model of hyperlipidemia and atherosclerosis. In this review, we revisit the primary features of lipoprotein metabolism and atherosclerosis in apoEKO mice and the contributions of human apoE isoforms using the apoE knock-in mice. We then extend our discussion to more recent observations that suggest an important role for apoE in peripheral energy metabolism and consequently in metabolic syndrome and its components, mainly obesity and diabetes.

Lipoprotein metabolism in apoEKO mice: Plasma cholesterol in wild type mice on a regular chow diet is approximately 80 mg/dl, primarily carried in high density lipoprotein (HDL) particles. Mice have a small amount of low density lipoprotein (LDL) and other atherogenic lipoproteins, such as VLDL remnants. This high HDL to LDL ratio is maintained even when mice are fed diets similar to those consumed by humans in Western society. This is in marked contrast to humans who carry the majority of their plasma cholesterol in the LDL (110 mg/dl) (2). It is well established in humans that a low ratio of HDL to LDL cholesterol confers a high risk of atherosclerosis and subsequent cardiovascular disease (3). Thus, the natural athero-protective lipoprotein profile in mice could account for the absence of these pathologic conditions.

In spite of the different plasma lipid profiles, cholesterol transport and metabolism are sufficiently similar in the two species to suggest that inducing suitable disturbances in plasma lipoprotein metabolism would also lead to atherosclerosis in mice. Gene targeting in embryonic stem cells developed during the 1980s (4-6) opened the door to test this concept, and mice homozygous for a defective apolipoprotein E gene were produced by us and independently by Plump *et al.* in 1992 (7-9).

Although extremely rare, humans lacking apoE are reported to have elevated remnant cholesterol in plasma (10). Similar to these individuals, apoEKO mice accumulate cholesterol-rich remnant particles with plasma cholesterol levels reaching 400 mg/dl, even when fed a regular low-fat, low-cholesterol diet.

Atherosclerosis in apoEKO mice: Although atherosclerosis is not a distinguishing feature described in apoE-deficient humans (10), apoE-deficiency alone proved to be sufficient for aortic atherosclerotic plaques to develop in mice. In addition, diets high in fat and cholesterol markedly accelerate plaque development in these mice. Thus, apoEKO mice, and subsequently mice lacking the LDL receptor (LDLR) that develop severe atherosclerosis on a Western-type diet (11), have demonstrated that hyperlipidemia is an essential prerequisite for the development of atherosclerotic lesions.

The lesion development and plaque composition in apoEKO mice is also similar to that in humans, establishing it as an excellent animal model for studying the pathogenesis of atherosclerosis. A small collection of foam cells that are tightly adhered to the aortic valve begin to appear in mice at about two months of age. With time the complexity of the lesion increases to have fibrous caps, a necrotic core of foam cell debris, cholesterol crystals and calcifications. Large advanced plaques are often associated with the thickening of medial and adventitial tissue, accompanied by chronic inflammation. Lesions with spontaneous hemorrhage and rupture, the features associated with clinical symptoms of human atherosclerosis, have been observed in some studies of older, cholesterol-fed mice (12, 13). However, the occurrence of these events in

apoEKO mice is not sufficiently reliable, leaving room for improvement in studying the plaque rupture process.

ApoEKO mice have been extensively used for several years to study the impact of various genetic and environmental risk factors on atherosclerotic susceptibility and resistance and to evaluate the effects of various therapeutic means. These studies have been comprehensively reviewed elsewhere (14).

Mice with human apoE isoforms: While the apoEKO mouse has been established as an excellent model of atherosclerosis, the lack of apoE is extremely rare in the human population.

However, apoE is polymorphic in humans, and plasma LDL cholesterol levels and atherosclerosis risk are both strongly associated with the three common apoE isoforms in the order of apoE4 > apoE3 > apoE2. This association is rather counterintuitive because apoE4 (Arg-112, Arg-158) binds to LDLR with a slightly higher affinity than apoE3 (Cys-112, Arg-158), while apoE2 (Cys-112, Cys-158) binds to the receptor with much reduced affinity (15).

Unlike in humans, the plasma lipoprotein profiles in apoE knock-in mice expressing the human apoE proteins in place of mouse apoE are reflective of their different LDLR affinities. Thus, mice with apoE3 and apoE4 are normolipidemic and do not develop atherosclerosis even on a Western-type diet (15). In contrast, mice with human apoE2, which binds to LDLR with less affinity, accumulate plasma remnants with high cholesterol and triglycerides (TG), and develop atherosclerosis (16).

Surprisingly, however, mice with human apoE2, E3 or E4 recapitulate the lipoprotein profiles of their human counterparts when they also express a high amount of the human LDLR and are fed a Western-type diet (17). Thus, an increased expression of LDLR in mice with human apoE4 causes an accumulation of cholesterol-rich, apoE-poor remnants in plasma, a reduction of HDL, and severe atherosclerosis. In marked contrast, the same increase in LDLR in apoE2 mice ameliorates their hyperlipidemia and diet-induced atherosclerosis. These results raise the possibility that apoE4, by binding strongly to excess LDLR, is prevented from transferring to nascent lipoproteins, a step necessary for their subsequent clearance. This in turn leads to an increase in the plasma

concentration of apoE-poor remnants. Indeed, we found that primary hepatocytes from apoE4 mice secrete less apoE into the medium than hepatocytes from apoE2 mice. Increased LDLR expression leads to a localization of apoE4 on the hepatocyte surface and enhances sequestration of apoE-deficient VLDL remnants injected into apoE4 mice. However, these surface-bound VLDL were poorly internalized compared to apoE2 mice (18).

ApoE isoform-dependent changes in cholesterol uptake and efflux from macrophages have been reported (19, 20). Cholesterol delivery to macrophages in culture increases as LDLR expression increases, and the effect was more prominent in apoE4 macrophages than those with apoE3 (21). Conversely, increased LDLR expression reduces cholesterol efflux from macrophages expressing apoE4 but not apoE3 (22). Consequently, in mice with human apoE4 that lack the LDLR (LDLRKO), the replacement of bone marrow cells with cells expressing LDLR increased atherosclerosis in a dose dependent manner compared to mice transplanted with LDLRKO cells. In contrast, atherosclerosis in LDLRKO mice expressing human apoE3 was not affected by the bone marrows with varying levels of LDLR expression (22). Although further tests are required to extrapolate these findings in mice to humans, interactions between apoE-isoforms and LDLR in macrophages likely contribute to the association of apoE4 with an increased cardiovascular risk in humans.

ApoEKO mice and adipose tissue biology: In addition to its primary site of synthesis in the liver, apoE is also synthesized in peripheral tissues including adipose tissue (23, 24). Recent studies indicate that apoE may be a crucial player in peripheral lipid uptake and energy homeostasis and consequently in the development of metabolic syndrome (MetS). MetS is a combination of several conditions including obesity, hyperglycemia, hyperinsulinemia, dyslipidemia, hypertension, and a prothrombotic, proinflammatory state (25). Obesity is a prominent aspect of MetS, and adipose tissue is now considered to be an important regulator of energy metabolism. Expression of apoE in adipocytes decreases in response to obesity and TNF-α, but increases with fasting and weight loss (26, 27). ApoE expression is regulated by nuclear receptors such as liver X receptor and Peroxisome Proliferator Activator Receptor γ (PPARγ), which is vital for adipocyte differentiation (28).

ApoEKO mice are leaner than wild-type mice (29-31). Absence of apoE also reduces body weight and some of their obesity-associated metabolic complications including impaired glucose tolerance and insulin resistance in obese models such as ob/ob and Ay/+ mice (30, 32, 33). Impaired delivery of liver-derived VLDL to adipocytes could partly account for the suppressed body weight gain and fat accumulation in apoEKO mice. In the adipocyte, apoE interacts with the VLDL receptor (VLDLR), which facilitates hydrolysis of TG by lipoprotein lipase (LPL) (34). Indeed, mice lacking the VLDLR are protected from obesity (35). Chiba *et al*. (30) showed that wild type VLDL, but not VLDL from apoEKO mice, induces differentiation of bone marrow stromal cells and 3T3-L1 cells into adipocytes. However, LPL inhibition did not alter the adipogenic activity of

VLDL, suggesting that apoE-mediated VLDL uptake, but not hydrolysis of VLDL, plays a major role in adipogenesis.

Modulation of adiposity and tissue insulin sensitivity by adipose-derived apoE is suggested by the work of Huang *et al.,* who showed that apoE synthesized by adipocytes promotes TG uptake in culture, and that the lack of endogenous apoE led to a marked defect in TG uptake from exogenous VLDL even when the VLDL contained apoE. The uptake was restored by adenoviral expression of apoE (36). The authors further showed that a PPARγ agonist increased apoE expression and TG accumulation in wild type adipocytes, but the same PPARγ stimulation produced significantly less TG synthesis and TG accumulation in apoEKO adipocytes. Thus, adipose-derived apoE may play a role in intracellular lipid storage in an autocrine and/or paracrine fashion. Further studies are necessary to determine the relative roles of lipoprotein-associated, circulating apoE and apoE synthesized by adipose tissue in metabolic homeostasis.

Human apoE isoforms and adiposity: Epidemiological studies have suggested that the apoE polymorphism modifies a long recognized association between increased body fat, particularly abdominal fat, and increased plasma VLDL in humans (37). For example, in the Heritage Family Study population, a pleiotropic Quantitative Trait Locus for TG and adiposity was found on ch19q13 where *APOE* is located (38). ApoE-isoforms were associated with body mass index in

the order of apoE4<apoE3<apoE2 in 15,000 individuals from the Atherosclerosis Risk in Communities (ARIC) study (39).

Similar to their human counterparts, mice expressing human apoE3 gain more body weight and adipose tissue mass than mice with apoE4 when fed a Western type diet. Despite being leaner, apoE4 mice begin to show impairment of glucose tolerance earlier than apoE3 mice, mainly because adipocytes expressing apoE4 fail to buffer postprandial lipids and glucose completely (40). Adenoviral expression of apoE3 in cultured apoE-null adipocytes induced mRNA expression for adiponectin in a dose dependent manner, but the induction was significantly blunted in cells expressing apoE4. ApoE4 expression increased mRNA for Glut1, but not Glut4, in adipocytes, suggesting that apoE4 may be interfering with insulin-regulated pathways. These apoE isoform-dependent effects on body fat are a reminder that in addition to total fat mass, the functionality of fat cells is also an important determinant of disease risk.

ApoE in diabetes and beyond: Cardiovascular incidents as a consequence of atherosclerosis are significantly increased in diabetic patients**.** Multiple diabetic atherosclerosis studies have employed apoEKO mice to induce diabetes with streptozotocin (STZ) injection, which ablates the insulin producing beta-cells in the pancreas. The resulting type 1 diabetes accelerated atherosclerosis development in apoEKO mice in association with an increase in plasma cholesterol levels (41). However, the diabetes-induced enhancement of atherosclerosis is attenuated, without a

significant change in plasma lipid levels, by the administration of the soluble receptor for advanced glycation end products (42), rosiglitazone (43), or lipoic acid (44), among others. Thus, the increased oxidative stress and inflammation consequent to the high plasma glucose are major contributors to accelerated atherosclerosis in diabetes.

Type 2 diabetes is more common than type 1 diabetes in humans. In contrast to the consistently observed increase in atherosclerosis by the STZ-induced type 1 diabetes, data are conflicting regarding the effects of type 2 diabetes on atherosclerosis development in apoEKO mice. For example, insulin resistance due to the lack of insulin receptor substrate 2 (*Irs2*) gene was shown to accelerate atherosclerosis development in apoEKO mice (45). In contrast, a reduction of atherosclerosis has been reported in apoEKO mice treated with gold thioglucose to destroy the hypothalamic satiety center and to produce type 2 diabetes (46). Similarly, while leptin receptor deficiency (db/db) induces key features of type 2 diabetes in apoEKO mice including hyperglycemia, hyperinsulinemia, dyslipidemia, and accelerated atherosclerosis (47), deficiency in the leptin receptor protects LDLRKO mice from atherosclerosis (48). Conflicting results have been reported in apoEKO mice with leptin deficiency (ob/ob); an increased atherosclerosis in chow-fed mice by Gruen *et al.* (49) and smaller plaques in mice fed an atherogenic diet by Chiba *et al.* (50). While different experimental conditions such as diet and methods employed for atherosclerosis evaluation may be contributing to these different outcomes, reconciliation of these differences is of great interest because hyperleptinemia and insulin resistance frequently occur together in MetS patients.

Figure 5.1 illustrates the metabolic roles of apoE in vivo. ApoE-mediated lipoprotein uptake in liver and adipose tissue lowers plasma lipids (left panel). In contrast, impaired lipoprotein uptake by the liver, in the absence of apoE, leads to an increase in VLDL and chylomicron remnants in the plasma as well as increased formation of foam cells in the vessel wall and atherosclerosis (right panel). On the other hand, apoE deficiency also reduces adiposity and improves insulin sensitivity, which may have an athero-protective potential. In the studies of diabetic atherosclerosis such as those described above, apoEKO mice have provided an effective sensitizer for atherosclerosis, but the possibility that apoE may also have direct roles in the pathogenesis of diabetes and its complications has not been addressed. The potential roles of apoE isoforms in MetS and the development and progression of diabetic complications highlight the future utility of the apoE-knock-in mice in elucidating the mechanisms by which apoE exerts isoform-specific effects.

Our current understanding of apoE in energy metabolism beyond its role in lipoprotein metabolism is still far from complete. The impairments in metabolic disorders have a potential to modulate atherosclerosis and cardiovascular disease progression. Similar to their contributions toward the understanding of lipoprotein metabolism and pathogenesis of atherosclerosis, apoEKO mice and mice with humanized apoE will be invaluable in elucidating these roles in the future studies.

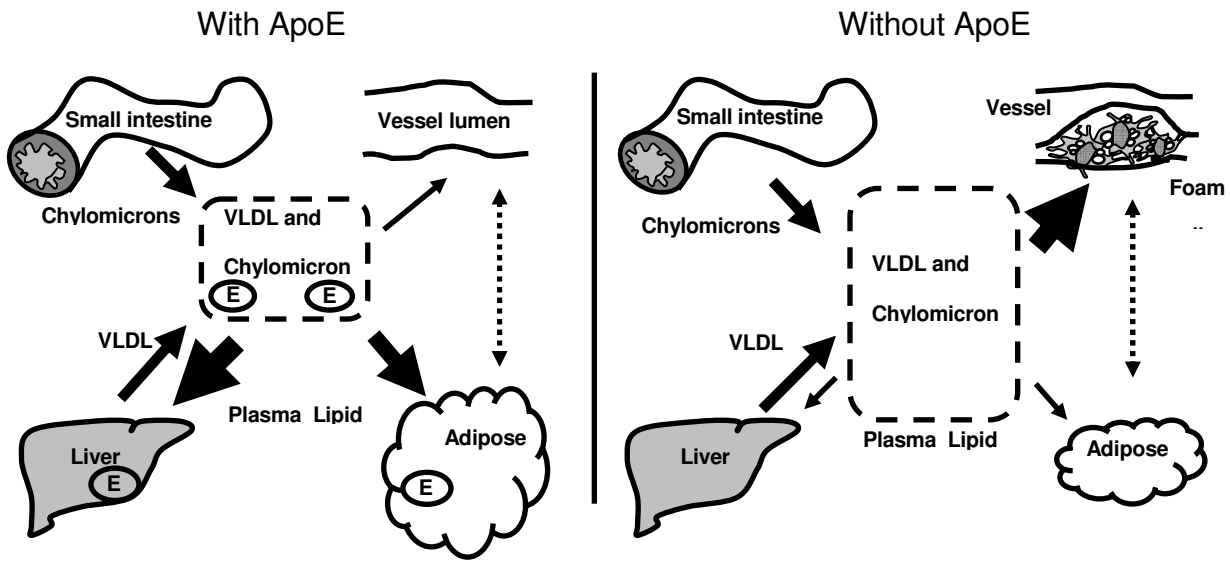

Fig 5.1: Physiological role of ApoE. ApoE-mediated triglyceride uptake by liver and adipose tissue contributes to maintaining normal plasma lipid levels (left panel). Impaired triglyceride uptake in liver and adipose tissue in the absence of apoE contributes to the accumulation of VLDL and chylomicron remnants in the plasma and foam cell accumulation in the vessel wall (right panel). The arrows represent changes in lipid accumulation in different tissues in the presence and absence of apoE. ApoE is increasingly becoming important in peripheral energy metabolism and may have an effect on plaques in addition to its established role in lipoprotein trafficking.

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Chapter 6

CONCLUSIONS AND SIGNIFICANCE

PPARγ is well established as an important gene for adipocyte differentiation and triglyceride accumulation; and insulin sensitivity. The knockout mice lacking PPARγ in specific tissues have enabled us to understand some of the basic functions of this gene. Mutations in PPARγ occur in people and are a useful way to learn about PPARγ, beyond its established functions. Thus, I sought to study the human P467L mutation in the ligand binding domain of PPARγ. Using a mouse model carrying the corresponding P465L mutation, I have shown that the heterozygous P465L mutation can lead to insulin resistance.

In my experiments, I have shown that the suboptimal levels of insulin (due to $Ins2^{Akita/+}$ mutation) in the presence of P465L mutation affect the development of adipose tissue structure and function. In mice lacking PPARγ in adipose tissue, a similar but severe phenotype of lipodystrophy was observed. My data suggests that functional consequences of alterations in PPARγ levels (due to various point mutations and/ or polymorphisms) can be modulated by the reduction in the availability of insulin. This interaction between PPARγ and insulin is especially important early in the development during adipose tissue differentiation.

In my double mutant LA mice, P465L mutation led to a decrease in insulin levels that were already compromised by the akita mutation. Thus, there is a direct effect in the β-cells where P465L

mutation worsened the impact of Akita mutation and this interaction indirectly affected the adipose tissue and liver. PPARγ in liver is essential for the optimum level of TG accumulation in hepatocytes. Liver-specific PPARγ knockout mice have hyperlipidemia and reduced insulin sensitivity. In my model of P465L mice, the liver is not directly affected by the PPARγ mutation. In spite of the reduction in functional PPARγ, the lack of an obvious liver phenotype suggests that the dependence of liver function on PPARγ availability is much lower compared to that of the adipose tissue. Reduced circulating insulin levels in addition to functional impairment in the adipose tissue have indirect effects on the liver, leading to increased gluconeogenesis which contributes to the fasting hyperglycemia in LA females.

Ultimately, it is also important to understand that the skeletal muscle in LA mice can maintain a fairly normal homeostasis in the face of a scarcity in available plasma insulin as well as reduction in functional PPARγ. The relatively lower levels of PPARγ in skeletal muscle compared to adipose tissue may reflect a lesser requirement of PPARγ for the skeletal muscle insulin responsiveness.

Overall, my project confirms the findings of the tissue specific knockout models of PPARγ. Most importantly, it has allowed me to extrapolate these concepts to the phenotypes in human patients and clearly establish that P465L mutation can lead to insulin resistance.

A similar link has not emerged in the second part of my thesis regarding the role of PPARγ in atherosclerosis. Considering the knowledge generated by using PPARγ knockout mice in endothelium, macrophages and vascular smooth muscle cells, it can be concluded that intact PPARγ function in these organs is important to reduce the severity of atherosclerotic plaques. Based on this, we hypothesized that P465L mutation (with reduced functional PPARγ and higher blood pressure) will increase the size of atherosclerotic plaques. Since a global knockout mouse of PPARγ does not survive, an attempt to study the effect of the lack of PPARγ on atherosclerotic plaques is not possible. In our model of P465L mice bred to *Apoe-/-* mice, the plaque size did not change. Our initial experiments with the Lee macrophages suggest a athero-protective phenotype; where as the higher blood pressure due to P465L mutation is likely to increase the atherosclerotic plaque size. This leads me to speculatively conclude that the potential pro-atherosclerotic effect/s of higher blood pressure and VSMC dysfunction versus anti-atherosclerotic effects suggested by macrophage gene expression and reduced VLDL uptake likely act as opposing influences resulting in no change in the atherosclerotic plaque size.

It is important to acknowledge that PPARγ is identified as a crucial athero-protective molecule in the experimental setting of its complete lack in a specific tissue while present in the rest of the body. These models do not accommodate a potential cross talk between different cell and organ systems. Compared to this setting, a reduction in functional PPARγ due to P465L mutation in the whole body is a very different scenario and resembles a phenotype due to human mutations and polymorphisms more closely. In the event of a global reduction in PPARγ, the impact on different organs seems to dampen the unopposed effect caused by a single cell type/ organ. In addition to the tissue specific knockouts, which are invaluable tools to learn protein functions, my hypothesis tested PPARγ function in a clinically relevant, humanized mouse model. Without undermining the role of PPARγ in individual cell types crucial in the development of atherosclerosis, the Lee mouse model suggests that the reduction/ alteration in PPARγ function (due to P465L mutation), does not have a significant effect on plaque size in *Apoe* deficient mice.

In the first part of my thesis, the lessons learnt from the tissue specific knockout mice and humanized P465L mice were similar and my findings have consolidated the understanding of PPARγ function. In the second part, however, the reconciliation of knowledge acquired from the tissue specific knockout mice and the humanized P465L mice is not exactly straight-forward. However, it highlights the inter-dependence of organ systems in a systemic, chronic disease state to

ward off the negative impact (in my case the P465L mutation) and maintain a reasonable level of physiological function.

Recently, the concept of selective PPAR modulators, similar to the estrogen receptor modulators, has emerged. It is important to devise partial agonists for PPARγ, which will have beneficial effect in a particular tissue without causing adverse effects via its actions in other tissues. Recently, there have been reports about investigation into such compounds. The future of PPARγ research and harnessing its power for human well being may lie in these selective PPAR modulators.