NLRP3 inflammasome immunometabolism and statins

The NLRP3 inflammasome is involved in statin-induced insulin resistance

By:

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DESCRIPTIVE NOTE

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Lay abstract

Statins are one of the most widely prescribed drugs and used in the treatment of cardiovascular disease. Statins lower LDL often called bad cholesterol. However, some patients experience side effects such as higher blood glucose and increased risk of type 2 diabetes. Type 2 diabetes is generally preceded by insulin resistance and this period of "prediabetes" is reversible. Inflammation is one factor involved in insulin resistance and in the development of type 2 diabetes. In this thesis, we discover that statins activate an inflammatory response known as the NLRP3 inflammasome, which leads to insulin resistance, particularly in fat tissue. Activation of this inflammasome by a statin, did not require lower cholesterol, but increased the inflammatory cytokine IL-1β, which was the key factor leading to insulin resistance in fat tissue. Inhibition of the NLRP3 inflammasome or IL-1β may prevent insulin resistance and risk of type 2 diabetes in people taking statins.

Abstract

Statins are one of the most widely prescribed drug classes because they lower circulating low density lipoprotein-cholesterol (LDL) and reduce the risk of cardiovascular events. Statin-mediated inhibition of HMG-CoA reductase also lowers substrates required for protein prenylation. This cholesterol-independent effect of statins can alter immune function. Lower protein prenylation can increase IL-1β. This pro-inflammatory cytokine can promote insulin resistance, which may be a factor in the recent evidence linking statins to increased incidence of diabetes. IL-1β is unique compared to most cytokines because it can be regulated by the NLRP3 inflammasome. In this thesis, we discovered that statins promote NLRP3-dependent insulin resistance in adipose tissue. We next hypothesized that statin-induced lowering of protein prenylation activated the NLRP3/Caspase-1 inflammasome, which would cause IL-1β-dependent insulin resistance in adipose tissue. We showed that atorvastatin impaired insulin signalling in adipose tissue from WT, but not IL-1β$^{-/-}$ mice. Treatment with a caspase-1 inhibitor prevented atorvastatin-inhibition of insulin signalling. The isoprenoid, Geranylgeranyl pyrophosphate (GGPP) also prevented atorvastatin-induced defects in insulin signalling. Interestingly, atorvastatin-inhibition of insulin action was associated with decreased insulin-stimulated lipogenesis in both white adipose tissue and 3T3-L1 adipocytes. The findings in this thesis suggest a statin-induced reduction in isoprenoids, required for protein prenylation production, impairs insulin action via IL-1β derived from activation of NLRP3/Caspase-1 inflammasome in adipose tissue.

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LIST OF ABBREVIATIONS

2DG	2-[1,2-^{3}H (N)]-Deoxy-D-glucose (2DG)
AS160	Akt substrate of 160 kDa
β	Beta
BMI	Body mass index
CARD	caspase-activation and recruitment domain
DAG	diacylglycerol
DM	diabetes mellitus
DNA	deoxyribonucleic acid
DPI	Diphenyleneiodonium
ELISA	enzyme-linked immunosorbent assay
FOXO1	forkhead box protein O1
GLUT#	glucose transporter #
GSK3	glycogen synthase kinase 3
GTT	glucose tolerance test
HbA1C	Glycated hemoglobin
HFD	high-fat diet
HMG-CoAR	3-Hydroxy-3-methyl-glutaryl-CoA Reductase
IL#	interleukin #
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
HPLC/MS	high performance liquid chromatography- mass spectroscopy
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
mRNA	messenger Ribonucleic acid
mtROS	mitochondrial reactive oxygen species
NAFLD	non-alcoholic fatty liver disease
NF-κB	nuclear factor kappa-B
NLR	nucleotide oligomerization domain-like receptor
NLRP3	nucleotide oligomerization domain-like receptor, pyrin domain-containing 3
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PRR	pattern recognition receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
TAG	triacylglycerol

T2D	type 2 diabetes
TLR	toll-like receptor
TNF	tumor necrosis factor
USD	US Dollars
WT	wild type

Declaration of academic achievement

The work in this PhD thesis was published in the journal *Diabetes*, where I was the first author on both papers. Text within the introduction and discussion of this PhD thesis is also based on a first authored review paper in the journal *Adipocyte*. This PhD thesis also contains some unpublished work. The results in section 3.1.7 were completed in collaboration with Dr. Justin Crane. The results in section 3.1.1 pertaining to IL-1β release from macrophages with the inhibitors was completed with the assistance of Dr. Schertzer.

First-author peer reviewed contributions

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Rebalka IA, Cao AW, Raleigh MJ, Henriksbo BD, *et al.* Statin therapy negatively impacts skeletal muscle regeneration and cutaneous wound repair in type 1 diabetic mice. *Front. Physiol.* **8**, 1–9 (2017).

Cavallari JF, Fullerton MD, Duggan BM, Foley KP, Denou E, Smith BK, Desjardins EM, Henriksbo BD, *et al.* Muramyl Dipeptide-Based Postbiotics Mitigate Obesity-Induced Insulin Resistance via IRF4. *Cell Metab.* **25**, 1063-1074.e3 (2017).

Chapter One: Introduction

1.1 Obesity and Metabolic disease

Obesity is a global health concern, affecting over 650 million adults world-wide[1]. Current projections estimate nearly half our population will be classified as overweight by 2030, costing global health care over $2 trillion (USD)[2]. An individual is clinically defined as obese if their height to weight ratio, referred to as body mass index (BMI), exceeds 30[3]. Obesity is characterized by accumulation and expansion of adipose tissue. Adipose tissue expansion often cannot capture sustained nutrient overload and will reach a point of maladaptation during obesity resulting in ectopic lipid deposition in muscle and liver. Obesity and ectopic lipid deposition are factors that can contribute to insulin resistance (refer to section 1.3.2).

Obesity is associated with several comorbidities including cardiovascular disease, type 2 diabetes, cancer and non-alcoholic fatty liver disease (NAFLD)[4]. These obesity-associated complications and comorbidities, paired with the progressive increase in global obesity burden, highlight the importance in determining how various factors converge to influence obesity-related outcomes. This foundational knowledge may reveal where to combat obesity or obesity-related comorbidities. Although the full etiology of obesity is unknown, it is clear that it is multifactorial with evidence suggesting contributions from: genetics, excess energy intake, sedentary lifestyle, high intake of

processed foods, drug and environment obesogens, gut microbiome and circadian rhythm disruption[4]. Understanding the mechanisms can explain how these factors interact to contribute to the onset of obesity and various aspects of metabolic disease, and can lead the development of personalized lifestyle recommendations, social policy reform and development of pharmacotherapies or refinement of existing therapies used in metabolic disease patients.

1.2 Type 2 Diabetes

It was estimated that over 400 million people in the world had diabetes in 2014 with a global adult prevalence of 8.5% (WHO, October 2018)[5]. Individuals with glycated haemoglobin (A1C) levels greater than 6.5 or an overnight fasting blood glucose greater than 7 mmol/L are diagnosed as diabetic[6]. Type 2 diabetes (T2D) is characterized by failure to sufficiently produce the required amount of insulin to effectively maintain a state of euglycemia. The two predominant factors influencing the maintenance of euglycemia are insulin production from the pancreatic β-cell and insulin sensitivity of many tissues. Insulin sensitivity and insulin secretion are intimately linked. In general, decreased insulin sensitivity (i.e. increased insulin resistance) precedes overt T2D. An increase in insulin resistance in prediabetic patients can stimulate or coincide with increase insulin production to effectively lower blood glucose to maintain euglycemia. It is

also possible that increased insulin secretion coincides and/or contributes to insulin resistance and increased adiposity during the progression from prediabetes to T2D[7]. In some individuals, eventual failure to sufficiently maintain or increase insulin secretion, often termed of β-cell failure, can precipitate hyperglycemia and overt T2D[7].

Current treatments for prediabetes and T2D aim to restore blood glucose levels through various mechanisms including increasing insulin sensitivity, increasing insulin secretion from the pancreas, decreasing glucose uptake following a meal, and decreasing glucose reabsorption from the kidney[6].

1.3 Insulin

Insulin is an anabolic hormone peptide produced by the β-cell of the pancreas, and is a key regulator of carbohydrate, lipid, and protein metabolism. Insulin exerts many functions such as (i) promoting glucose uptake into peripheral tissue (ii) increasing glycogen synthesis in muscle and liver and (iii) increasing lipogenesis in adipose and liver[8]. Insulin inhibits hepatic gluconeogenesis and glycogenolysis, processes that provide glucose into circulation. Further, energy liberation from adipose tissue in the form of lipolysis, which is the breakdown of triglycerides for release, is also inhibited by elevated levels of insulin[9]. The inhibition of nutrient mobilization and stimulation of energy storage by insulin is

characterized by a shift in fuel partitioning following a meal. Ingestion of food, particularly those higher in carbohydrate content, stimulate the release of insulin into circulation, as β-cells are sensitive to elevations in blood glucose[10]. Hormones produced by the gut known as incretins, in particular Glucagon-like peptide-1 (GLP-1), are secreted following meal ingestion, and stimulate an initial release of insulin from the pancreas[11].

1.3.1 Insulin signalling

Insulin achieves its function through binding to the insulin receptor and initiating activation of a complex signalling cascade. Insulin binds to the α-subunit of the insulin receptor inducing dimerization and auto-phosphorylation of tyrosine residues of the β-subunit[8]. Tyrosine phosphorylation of the insulin receptor facilitates docking and activation of insulin receptor substrate 1 and 2 (IRS-1,2). The isoforms of IRS have tissue specific functions with IRS-1 predominantly in the muscle and adipose and IRS-2 in the liver. IRS-3 is predominantly found in adipocytes and the brain but is not essential for glucose homeostasis or uptake[12,13]. Activation of IRS-1 causes recruitment of phosphoinositol-3 kinase (PI3K) which generates phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and activates 3-phosphoinositide-dependent protein kinase 1 (PDK1)[14]. Important negative feedback occurs here with phosphatase and tensin homolog (PTEN) converting PIP_3 back to phosphatidylinositol 4,5-

bisphosphate (PIP_2) to attenuate insulin signalling, which is sometimes referred to as resetting the PI3K signal transduction cascade[15]. PDK1 subsequently activates the serine/threonine kinase Akt/ phosphokinase B (PKB) through phosphorylation of threonine[308], an important first step in activating Akt/PKB[16]. Full activation of Akt/PKB requires a second phosphorylation event of serine[473] mediated by mammalian target of rapamycin complex 2 (mTORC2).

Activation of the critical signalling node Akt/PKB mediates several of the important downstream effects of insulin. Activated Akt/PKB phosphorylates and inhibits Akt substrate of 160 kDa (AS160/TBC1D4), which is a component in the signalling cascade leading to translocation of Glucose Transporter type 4 (Glut4)-containing vesicles from the intracellular stores to the membrane for glucose uptake[17]. In the liver, Akt/PKB phosphorylates and inhibits Forkhead box protein O1 (FOXO1) to decrease gluconeogenesis while also phosphorylating and inhibiting glycogen synthase kinase 3 (GSK3) to increase glycogen synthesis[18]. Protein synthesis is also increased by Akt/PKB phosphorylation and subsequent inhibition of tuberous sclerosis complex-1 and -2 (TSC1/2), which activates the mammalian target of rapamycin complex 1 (mTORC1) and ribosomal protein S6 kinase beta-1 (p70S6K)[19]. Insulin-induced activation of p70S6K results in phosphorylation of the S6 ribosomal

subunit and increases ribosome activity and protein translation. Overall, effective insulin signalling is a critical component of modifying many biological processes, including maintenance of euglycemia and lipid storage in adipocytes. However, impairments in key nodes of the insulin signalling cascade can contribute to metabolic dysfunction and detrimental health consequences related to insulin resistance and progression to T2D.

1.3.2 Insulin resistance

Insulin resistance is characterised by an impaired response of an organism or cell's response to insulin. Insulin resistance generally precedes T2D. Obesity is a major predictor of the development of insulin resistance, although the causes of insulin resistance are multifactorial[20]. In addition to obesity, the factors that can influence insulin sensitivity include age, genetics, diet, exercise, and medications[6]. The progression of insulin resistance towards T2D can occur over the course of several years in humans[21]. As insulin resistance increases, euglycemia can be maintained through compensatory increased production of insulin from pancreatic β-cells. In addition, higher insulin load can contribute to both obesity and insulin resistance[22–24]. Uncovering the various causes of insulin resistance during this critical window provides a therapeutic opportunity to intervene and improve insulin sensitivity with various lifestyle modification strategies and pharmacotherapies.

Insulin resistance is characterized by a reduction in signal transduction through the insulin signalling pathway. Inhibition of the insulin signalling cascade at specific nodes can relate to a decrease in the metabolic function of insulin in various tissues. Impaired insulin signalling can correlate with decreased glucose uptake in muscle and adipose tissue, decreased suppression of lipolysis from adipocytes and decreased suppression of hepatic glucose output. An important node of insulin signal regulation is through the complex balance of serine/threonine (Ser/Thr) phosphorylation of IRS-1, a protein that has more than 50 phosphorylation sites[25]. Phosphorylation of specific Ser/Thr residues on IRS-1 inhibit insulin signalling through preventing interaction with the juxtamembrane domain of the insulin receptor, blocking PI3K interaction, and through altering subcellular localisation and subsequent protein degradation[25,26]. Tyrosine phosphorylation at multiple sites of IRS proteins results in activation and facilitates binding of important proteins involved in the insulin signaling cascade such as PI3K[14].

The serine/threonine kinase Akt/PKB node is also a node and biomarker of insulin resistance. Akt/PKB phosphorylates and regulates several downstream cell processes. For example, Akt/PKB phosphorylates AS160/TBC1D4 to cause glucose uptake, PRAS40 to stimulate protein synthesis and GSK3 to cause glycogen synthesis[17,18,27,28]. A reduction in

Akt/PKB phosphorylation is observed in models of insulin resistance in liver, muscle, and adipose tissue[29–31]. Ceramide can even directly inhibit insulin stimulated phosphorylation of Akt/PKB through engagement of protein kinase C-ζ (PKCζ)[32]. However, there is evidence that the downstream pathways and effectors of Akt/PKB are not equally affected during "selective" insulin resistance. Insulin should suppress hepatic gluconeogenesis and stimulate triglyceride synthesis. Paradoxically, hepatic insulin resistance is characterised by a failure of insulin to suppress gluconeogenesis, but simultaneously insulin-stimulated hepatic lipogenesis is unaltered[33]. This difference is often termed selective insulin resistance. In muscle, phosphorylation of Akt/PKB at Ser473 (Human Ser474) and Thr308 (Human Thr309) was reduced in obese insulin-resistant and type 2 diabetic patients compared to lean and obese insulin-sensitive individuals[34]. These changes in Akt/PKB phosphorylation tracked with whole-body glucose disposal, but no other correlation was observed regarding the insulin-stimulation of GSK3, FOXO, and even the Glut4 regulator AS160/TBC1D4. In adipocytes similar selective insulin resistance is also observed[35]. Adipose tissue from lean and obese mice or models of insulin resistance in 3T3-L1 adipocytes all showed decrease insulin-stimulated glucose disposal, which correlated with the magnitude of phosphorylation of Akt/PKB. In these adipocyte experiments, insulin-

stimulated protein synthesis and insulin-suppression of lipolysis was unchanged in each model of insulin resistance. In addition, there was no strong evidence for a correlation between insulin resistance of cellular glucose uptake and insulin-stimulated phosphorylation of FOXO, GSK3, or PRAS40, whereas only changes in the activation of AS160/TBC1D4 tracked with impaired glucose uptake in adipocytes[35].

1.3.3 Inflammation and insulin resistance

Understanding the multifactorial causes in insulin resistance is important. Key nodes of insulin signaling such as IRS-1 and Akt/PKB can be perturbed and related to selective insulin resistance, where certain metabolic actions of insulin are impaired in certain cells[25,32,36]. During obesity, chronic low-grade elevation of inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6), can mediate insulin resistance[37–40]. Inflammatory cytokines can activate stress kinases[41–43]. Activation of stress kinases can cause insulin resistance through inhibition at key nodes of insulin signaling[41,44,45]. Inflammatory mediator and stress responses can disrupt insulin signaling through multiple serine/threonine phosphorylation sites on IRS proteins, blocking tyrosine phosphorylation and mitigating signal transduction[25,46]. For example, activation of c-Jun N-terminal kinase (JNK) by TNFα, causes serine 307 (Ser307) phosphorylation of IRS-1 and inhibits tyrosine

phosphorylation[47]. Different innate immune sensors such as the Nucleotide-binding oligomerization domain-containing proteins (Nods) and Nod-like receptor protein 3 (NLRP3) have been shown to mediate inflammation and promote insulin resistance in muscle and adipose tissue[31,48]. There are a plethora of triggers of innate immune proteins that can promote inflammation-induced insulin resistance. These triggers of inflammation include fatty acids, hyperglycemia, bacterial components, and potentially drug metabolites[49-52]. This thesis focuses on statins, the NLRP3 inflammasome and the inflammatory cytokine IL-1β.

1.4 NLRP3 Inflammasome

The Nod-like receptor protein 3 (NLRP3) inflammasome complex is an intracellular sensor of a wide range of both pathogen-associated molecular patterns and damage-associated molecular patterns (PAMPs and DAMPs)[53,54]. The complex is composed of (i) NLRP3, (ii) the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and (iii) procaspase-1. The NLRP3 protein structure consists of a C-terminal leucine rich repeat domain (LRR), a central nucleotide binding and oligomerization domain (NOD), and an N-terminal pyrin domain (PYD)[55]. The adaptor protein ASC contains a caspase recruitment domain (CARD) and a PYD domain, allowing for facilitating interactions between both NLRP3 and caspase-1 in this complex[56]. It was recently

demonstrated that the serine/threonine kinase NIMA-related kinase 7 (Nek7) is required for NLRP3 complex formation[57,58]. Nek7 was already known to play a role in mitosis, where it associates with microtubules and during this function of Nek7, the protein is unable to interact with NLRP3[57,59]. It is thought this Nek7 requirement for NLRP3 activation, causes inflammasome activation and mitosis to be mutually exclusive events.

Inflammasome activation requires both a (i) 'priming' step up-regulating NLRP3 protein and pro-IL-1β through NF-kB activation, and (ii) a stimulation step activating the complex and cleaving pro-caspase-1 to active caspase-1 (**Figure 1.1**)[54,60]. Assembly of these NLRP3 inflammasome components causes self-oligomerization into a multimeric 'speck' like structure containing caspase-1 and ASC. NLRP3 inflammasome assembly and activation of caspase-1 will cleave both pro-IL-1β and pro-IL-18 into their bioactive forms, which are then available to be secreted from the cytosol of the cell. Secreted IL-1β can enter the circulation or extracellular environment and can bind and signal through the IL-1R (IL-1 receptor), while IL-18 signals through the IL-18R[61]. Both of these cytokines are elevated in obese and T2D patients and each cytokine could potentially modify insulin resistance and development of T2D[62–66].

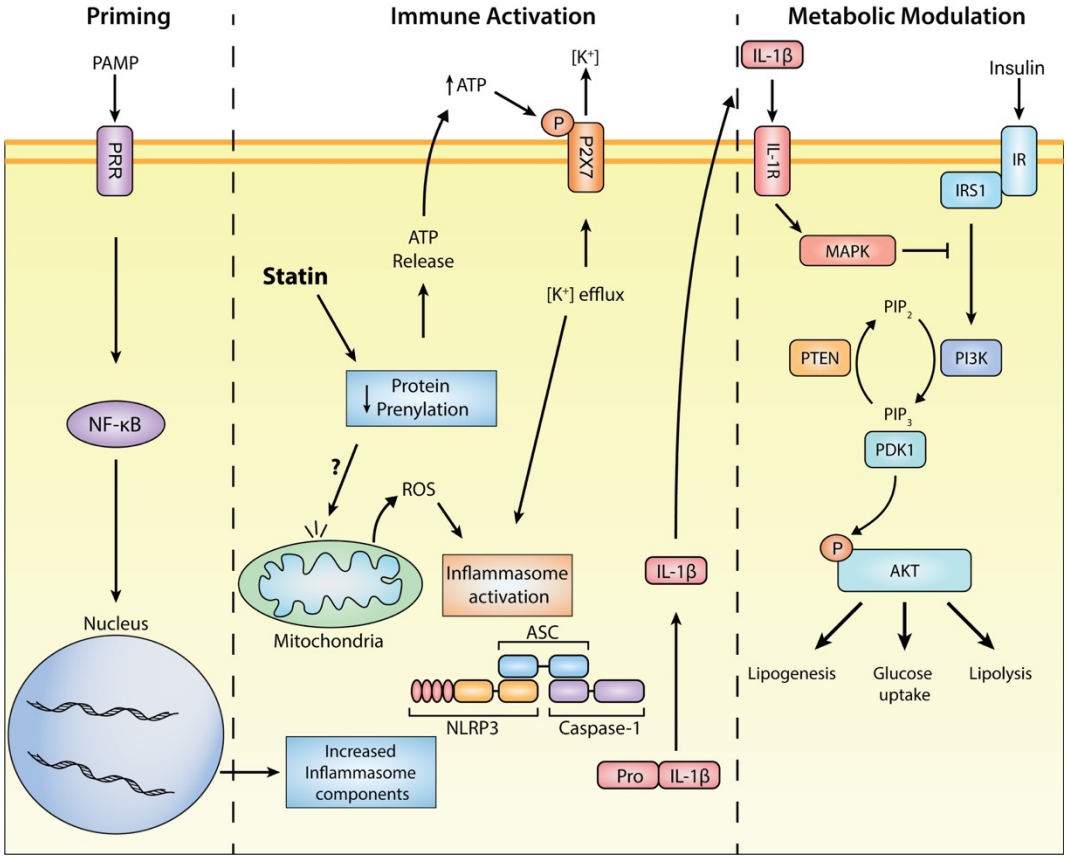

Priming
Immune Activation
Metabolic Modulation
PAMP
PRR
NF-κB
Nucleus
Increased Inflammasome components
ATP
[K+]
P
P2X7
ATP Release
[K+] efflux
Statin
Protein Prenylation
?
ROS
Mitochondria
Inflammasome activation
ASC
NLRP3
Caspase-1
Pro IL-1β
IL-1β
IL-1β
IL-1R
Insulin
IR
IRS1
MAPK
PIP2
PTEN
PI3K
PIP3
PDK1
P
AKT
Lipogenesis
Glucose uptake
Lipolysis

Fig 1.1 Mechanisms of inflammasome activation. *Priming*: Following PRR stimulation, NF-kB stimulates transcriptional events that increase levels of the inflammasome (NLRP3) and inflammasome effectors (pro-IL-1β). *Immune activation*: HMGCR inhibition with statins causes pleotropic effects through decreased protein prenylation. Decrease in protein prenylation is a suspected cause for signals to promote increase NLRP3 inflammasome activity, but how these signals conspire to activate this inflammasome is not fully understood. Statins have been shown to cause mitochondrial membrane dysfunction, increase intracellular reactive oxygen species and also promote release of cellular ATP. Extracellular ATP can bind to the P2X7 receptor and promote potassium (K^+) efflux, a key trigger for increased NLRP3 inflammasome activity. The identity of the prenylated protein(s) responsible for statin-induced inflammasome activation is not known. Following inflammasome activation, caspase-1 cleaves pro-IL-1β to biologically active IL-1β. *Metabolic modulation*: The connection between NLRP3 inflammasome activation and insulin signaling occurs through IL-1β-mediated inflammation. Activation of MAPKs (JNK, ERK, p38) inhibit insulin signalling at the level of insulin receptor substrate-1 (IRS-1); or through an alternate target (not shown) which may alter insulin signaling at the level of phosphatase and tensin homolog (PTEN). PRR, pattern recognition receptor; NLRP, NOD-like receptor family, pyrin domain containing; NF- kB, Nuclear factor-kappa B; IL-1β, interleukin-1 β; IL-1R, interleukin-1 receptor; P2X7, P2X purinoceptor 7; PI3K, Phosphoinositol-3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDK1, phosphoinositide-dependent kinase-1; MAPK, Mitogen activated protein kinase; ROS, reactive oxygen species; IR, insulin receptor; IRS1, Insulin receptor substrate-1.

1.4.1 NLRP3 inflammasome activation mechanisms

The NLRP3 inflammasome is an innate immune sensor that detects a diverse array of danger molecules. The NLRP3 inflammasome has been described as a metabolic danger sensor. Even though NLRP3 is the most studied of the inflammasome complexes, the exact mechanism of activation is not fully understood. Changes in ion concentrations, particularly K^+ efflux, Ca^{2+} signalling, Cl^- efflux, and Na^+ influx are important, but not always required changes for NLRP3 activation[67]. Activation of the purinergic P2X7 receptor by increased extracellular ATP or membrane pore formation with Nigericin, cause intracellular decrease in K^+ and trigger assembly and activation of the inflammasome complex. Excessive mobilization of Ca^{2+} from the endoplasmic reticulum (ER) causes activation of the inflammasome[68–70]. Changes in these ion concentrations have been proposed to converge on a common activation signal through generation of mitochondrial reactive oxygen species (mtROS), which is sufficient for activation of the NLRP3 inflammasome[71,72]. Damage to mitochondria and subsequent release of ROS and mitochondrial DNA (mtDNA) are potent triggers of NLRP3 activation[72]. It is not yet understood how the inflammasome senses mtROS, but some evidence suggests that a Nek7-NLRP3 is responsible for mtROS detection and inflammasome assembly and activation[58].

Following activation, oligomerization of the inflammasome occurs and induces activation of the zymogen pro-Caspase-1 into activate Caspase-1. Cleavage of both pro-IL-1β and pro-IL-18 at aspartic-acid residues is the typically studied targets of caspase-1, however there is in-vitro evidence and >20 theorized targets of caspase-1 cleavage[73,74]. The role and significance of many of these other caspase-1 substrates in insulin resistance and T2D is not known and warrants further research.

1.4.2 Regulation and function of IL-1β

The interleukin-1 family consists of ligands, receptor antagonists and receptor complexes that coordinate critical innate immune responses for host defense. The biological activity of IL-1β is regulated before and after cleavage of the cytokine and also after cytokine secretion[61]. Transcription and translation of IL-1β is controlled by NF-κB activation whereby the inactive, intracellular pro-IL-1β form is produced. Following activation of the zymogen Caspase-1, cleavage of IL-1β and release by the unconventional protein secretion (UPS) pathway occurs[75,76]. Biologically active IL-1β in the circulation is further regulated by IL-1β binding to soluble IL-1β receptors termed “decoy receptors”, which can prevent IL-1β binding to cell/membrane bound IL-1β receptors that facilitate the cellular actions of IL-1β[77]. Docking of biologically active IL-1β to membrane bound

IL-1 Receptor 1 (IL-1R1) causes a confirmation change and recruitment of IL-1R Accessory protein (IL-1RAcP) to form a trimer complex at the membrane of target cells. The intracellular domain recruits adaptor proteins, myeloid differentiation factor 88 (MyD88), IL-1 Receptor associated kinase (IRAK) 4, and TNF receptor-associated factor 6 (TRAF6)[78]. This signalling platform activates the various Mitogen-activated protein kinases (MAPK's) JNK, p38 and ERK and further activates NF-κB[79,80].

There is a wide range of biological consequences of IL-1 signalling at both the local and systemic level in the body. Although chronic IL-1β signalling can have deleterious effects including its role in septic shock, the canonical function is to illicit an immune response to resolve infection or sterile insult[81]. IL-1β also causes immune cell recruitment to the target site by stimulating expression of adhesion proteins on monocytes such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin 1 (ELAM-1)[82,83]. Following resolution of the infection or sterile insult, inflammasome complex is degraded resulting in reduction in IL-1β secretion.

1.4.3 NLRP3 Inflammasome in metabolic disease

The inflammasome and IL-1β are important mediators of homeostasis responding to a wide array of danger signals. Unchecked and chronic

activation of the NLRP3 inflammasome can result in chronic inflammation and can contribute to many disease processes. Predominately, auto-inflammatory diseases like cryopyrin-associated periodic syndrome (CAPS), are associated with gain of function mutations in NLRP3 and characterised by increased IL-1-realted inflammation[84]. Overt inflammasome activation has also been associated with cancer, atherosclerosis, Alzheimer, arthritis and development of T2D in mice[85]. Increased inflammasome activation has been found in both humans and rodents with T2D and inhibition or deletion of NLRP3 protects against development of insulin resistance, dysglycemia and pancreatic beta-cell death; each of which are mechanisms underpinning the progression towards T2D[31,86–89]. Vandanmagsar *et al* showed rodents fed a fat-laden diet for 6 months had increased expression and activation of the inflammasome and IL-1β in adipose tissue, liver and muscle[31]. Genetic deletion of NLRP3 protected mice against high fat diet-induced whole body insulin resistance and restored insulin signalling, in particular insulin-stimulated phosphorylation of Akt/PKB in adipose, liver and muscle. NLRP3 deletion also reduced hepatic steatosis in the high fat-fed rodents[31]. Similar findings were also shown by Stienstra *et al* where the NLRP3 inflammasome regulated aspects of obesity during high fat diet feeding in mice[89]. Genetic deletion of caspase-1 showed a small reduction

in weight-gain and adipocyte hypertrophy in rodents fed a 45% high fat diet, despite the same food intake. Activation of the NLRP3 inflammasome in muscle can cause insulin resistance and is also linked to age-related muscle sarcopenia[90]. Reduction in muscle glycolytic capacity and decreased GAPDH was associated with increased caspase-1 activity which may explain the link between the inflammasome and muscle loss during ageing. Activation of muscle catabolic processes by IL-1β may also be involved in age-related muscle loss[91]. Obesity-associated increases in caspase-1 activity have been shown to regulate both insulin sensitivity and differentiation of adipocytes[92]. High fat diet feeding is known to increase caspase-1 activity in adipocytes, however treatment with a caspase-1 inhibitor protected mice from high fat diet-induced insulin resistance[92]. The NLRP3/casaspe-1 inflammasome appears to have cell autonomous effects on adipocyte differentiation and metabolism. Differentiation of pre-adipocytes derived from caspase-1 or NLRP3-knockout mice displayed higher insulin sensitivity and had higher fat oxidation rates. Incubation of caspase-1 deficient pre-adipocytes with IL-1β during differentiation reduced adipogenesis demonstrating these cell autonomous effects of caspase were conveyed through IL-1β[92]. All of these findings demonstrate the importance of the NLRP3/caspase-1 inflammasome in adipose tissue and insulin resistance.

1.5 Adipose tissue function and inflammation

Adipose tissue is a critical energy storage site, where lipids are primarily stored as triglycerides in adipocytes. Adipocyte-derived lipids are released during periods of nutrient scarcity or by triggers of lipolysis. Throughout the body there are several types of adipose tissue, where white adipose tissue (WAT) is a main storage site for lipids. WAT expansion in obesity has a well documented relationship with the co-morbidities of obesity, including the progression toward T2D[93–97]. Adipose tissue is composed of multiple different cell types including preadipocytes, vasculature cells, fibroblasts, and resident immune cells commonly referred to as adipose tissue macrophages (ATM). Adipose tissue is now recognized as an endocrine organ, capable of secreting a variety of important hormones such as Leptin, Adiponectin and other adipokines[98,99]. The seminal discovery that adipose tissue can secrete pro-inflammatory cytokines such as TNFα, added to the concept that adipose tissue is an endocrine organ capable of integrating immune responses and signals, which can alter adipose tissue metabolic function[100]. Recent evidence suggests this tissue can secrete approximately 600 different proteins[98].

Adipokines can signal in an auto/paracrine manner causing local alterations to the adipose tissue environment, which influence adipocyte metabolism. These adipokines can also act in an endocrine manner by

entering circulation and allow for cross-talk with distal organs, such as the liver, muscle, brain and pancreas[101,102]. Adipokines can influence food satiety, weight gain, inflammation and immune cell recruitment, and insulin sensitivity[101,102]. The important satiety hormone leptin was the first adipokine discovered and plays an important role in energy balance[103]. Activation of the leptin receptor in the hypothalamus, results in activation of STAT3, suppression of neuropeptide-Y (NPY) and suppression of hunger[104]. Leptin sensitivity is often lower during obesity, which is one connection between an adipokine and obesity because leptin resistance may equate to increased food intake even though adipose tissue energy stores are full[105–107].

Adipose tissue expansion occurs during periods of excess energy balance. In an attempt to buffer and store of the increased amount of lipids during nutrient excess, adipose tissue expansion occurs by: a) increased lipid loading of mature adipocytes, known as cell hypertrophy or b) increased adipogenesis, which is the process of differentiation of pre-adipocytes into new mature adipocytes, termed hyperplasia. Adipocytes undergoing hypertrophy can increase their volume several fold, however this increase in adipocyte size is associated with an increase in adipose tissue-derived inflammatory cytokines[108,109]. Both forms of adipose tissue expansion also require extra cellular matrix and vasculature remodelling to

support the nutrient requirements of the expanded adipose tissue. Failure to maintain suitable remodeling can lead to adipocyte hypoxia, leading to further inflammatory cytokine production, immune cell infiltration and apoptosis[110,111]. Though inflammatory cytokines are often regarded for their ability to lower insulin sensitivity, recent evidence demonstrates a requirement for specific inflammatory mediators to induce sufficient adipose tissue remodelling and support the nutrient holding capacity of the expanding tissue[112]. Asterholm *et al*, showed RIDtg mice (an adenoviral protein complex that suppresses TLR4, TNFα, and IL-1β mediated signalling in adipocytes) on an HFD had less adipose tissue expansion, but greater ectopic fat deposition in muscle and liver and worse glucose tolerance, insulin sensitivity and hepatic steatosis than WT mice[112]. This demonstrates a need for an acute inflammatory response to maintain lipid homeostasis through adipose tissue expansion. However, this result does not negate the well documented effects of increased inflammation that increase insulin resistance. Important considerations appear to include the timing, duration, resolution and compartmentalization of immune responses when comparing inflammation induced adipose tissue expansion versus inflammation-induced insulin resistance[113]. In fact, the short term versus long term impact of TLR4 deletion from adipocytes has recently been investigated in high fat diet fed mice[114]. TLR4 deletion

protected mice from inflammation-induced insulin resistance during an acute lipid challenge. However, during chronic high fat feeding where adipocyte expansion is required for maintenance of insulin sensitivity, TLR4 deletion worsened insulin resistance[114]. This result appears inconsistent with the concept that inflammation is necessary for long-term, but not short-term high fat diet-induced insulin resistance in mice, where Rag1 mice lacking adaptive immune responses were used[115]. However, these results highlight the complexity of innate versus adaptive immune responses that are engaged during diet-induced insulin resistance.

Proper lipid storage and release from adipocytes is linked to insulin sensitivity. A hallmark of obesity and prediabetes is ectopic fat deposition in muscle and liver, which can promote further inflammation in these tissues[116]. The importance of this is best characterized with the lipoatrophic A-ZIP/F-1 mice, which lack functional adipocytes. Lipid accumulation in the muscle and liver is increased and lipid overload in these tissues leads to insulin-resistance and liver steatosis[117,118]. Interestingly, transplantation of adipose tissue into A-ZIP/F-1 mice lowers ectopic lipid deposition and lowers insulin resistance[117]. Further evidence supporting that ectopic lipid deposition in liver and muscle contributes to tissue specific insulin resistance, is demonstrated with transgenic mice that overexpress lipoprotein lipase in the liver or muscle. Each of these

mouse models have pronounce lipid accumulation in the respective tissue with concomitant insulin resistance[119,120]. Both pieces of evidence point to the importance of lipid storage within adipocyte as a protective factor, where inability to store lipids in the adipocytes leads to insulin resistance.

Lipolysis is also an important factor in lipid storage in adipocytes. Release of fatty acids (FA) from adipose tissue occurs in response to negative energy balance (i.e. fasting) and is regulated by several hormones and inflammatory factors. Glucagon release during a fasted state or stress-induced epinephrine secretion are two examples of hormones that stimulate lipolysis in adipocytes through activation of beta-adrenergic receptors[121,122]. These hormonal signals can engage beta-adrenergic receptors and promote activation of adenylyl-cyclase, increasing intracellular cAMP thereby activating protein kinase A (PKA)[122]. Activation of PKA activates hormone sensitive lipases (HSL) and regulates release of fatty acids from adipocyte lipid droplets[123–125]. Conversely, insulin inhibits lipolysis creating a dichotomy between the fasted state (where glucagon is more active) and fed state (where insulin is more active). Insulin stimulation of Akt/PKB causes activation of phosphodiesterase (PDE) and reduces intracellular concentration of cAMP leading to lower activation of PKA and decreased lipolysis[126,127]. Further, insulin also decreases expression and activation of the lipases, HSL and

adipose triglyceride lipase (ATGL), which inhibits lipolysis in adipocytes[128,129].

During obesity, increased lipolysis results in higher circulating free fatty acids (FFAs), which coincides with increased circulating inflammatory cytokines[130,131]. The insulin resistance associated with higher levels of circulating pro-inflammatory cytokines manifest as higher lipolysis in adipose tissue due to a reduced ability of insulin to suppress of lipolysis. However, the extent that insulin resistance reduces the suppression of lipolysis by insulin is dependent on the model system used to measure lipolysis. In particular, 3T3-L1 adipocytes rarely demonstrate a measurable difference in the insulin suppression of lipolysis in the presence of TNFα[35]. TNFα can still stimulate lipolysis in adipocytes, it is often just measured independently of insulin suppression of lipolysis. In particular, treatment of adipocytes with TNFα can increase lipolysis through MAPK activation, which involves activation of endoplasmic reticulum (ER) stress[132–134]. Dysregulation of adipocyte lipolysis is important since it can contribute FFA the fuel ectopic fat deposition in muscle and liver[116]. These FAs and excess nutrient load in liver and muscle can promote inflammation and insulin resistance in these tissues[120,135].

Importantly, saturated fatty acids have been associated with an inflammatory response from immune cells and insulin resistance in muscle

and liver tissue. Early research suggested direct interaction between the saturated fatty acid palmitate and Toll Like Receptor 4 (TLR4) to induce inflammation from immune cells[136]. Recent evidence has argued against a direct activation of TLR4 by saturated fatty acids, including palmitate. However, it is now thought that reprogramming of cellular metabolism does play a role in inflammation, where palmitate can induce activation of JNK and IL-6 release[137]. Under conditions of adequate priming, palmitate can activate the NLRP3 inflammasome in macrophages and dendritic cells (DC) causing IL-1β release and subsequent insulin resistance in mice[138,139]. The mechanism of palmitate-inflammasome activation was shown to involve mtROS generation and was exacerbated by adenosine monophosphate kinase (AMPK) inhibition, which reduced autophagy and mitochondria turnover[138]. Further, palmitate loading of macrophages causes lysosome destabilization and inflammasome activation through a cathepsin-B dependent mechanism, suggesting multiple routes of lipotoxicity to activate the NLRP3 inflammasome[140]. The secondary lipid messenger ceramide can also cause activation of the inflammasome[31,138,141]. Obesity and consumption of obesogenic diet in both mice and humans show elevated levels of ceramide in circulation and adipose tissue, in particular for the ceramide 18:0[142–145]. Differences in the ability of specific ceramide species to promote insulin resistance, suggest

further understanding of how specific ceramides could engage immune responses such as the NLRP3 inflammasome in the development of T2D is required[141,146].

Activation of the NLRP3 inflammasome and subsequent IL-1β release in adipose tissue has multiple metabolic consequences. Deletion of NLRP3 and the inflammasome components shows marked improvement in insulin sensitivity in adipose tissue of mice on a HFD[138]. Addition of IL-1β to 3T3-L1 adipocytes and human adipocytes promotes insulin resistance, which is evident by lower insulin stimulated phosphorylation of Akt/PKB and decreased insulin stimulated lipogenesis[43,147]. Deletion of NLRP3 and inhibition of the NLRP3 inflammasome component caspase-1, also improved adipogenesis and insulin sensitivity in adipose tissue of high-fat fed mice[148].

In summary, adipose tissue stores and buffers lipid requirements of the body, but adipose tissue is also an important organ in the production of many adipokines that control whole-body energy balance. Fatty acid storage is highly regulated and ectopic lipid deposition in non-adipose tissue can have lipotoxicity effects linked to insulin resistance. Increased levels of circulating fatty acids derived from adipocyte lipolysis or lower lipogenesis is associated with inflammation and insulin resistance. Inflammation plays an important role in tissue remodelling during adipose

tissue expansion that occurs during obesity. Conversely, chronic inflammation can promote insulin resistance.

1.6 Statin drug class and cholesterol lowering mechanism

Statins are the most widely prescribed cholesterol lowering drug and effectively reduce risk of mortality from cardiovascular disease[149,150]. Statins inhibit 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) thereby lowering cholesterol biosynthesis and promoting hepatic cholesterol uptake[151,152]. Circulating cholesterol in the form of low-density lipoprotein (LDL) is mainly cleared by the liver through hepatocyte LDL-receptor (LDLR)-mediated uptake and statins increase the levels of LDLR[153,154]. Normal or elevated cholesterol production in the liver, causes insulin induced gene 1 (INSIG1) protein to bind to (sterol regulatory binding protein) SREBP cleavage association protein (SCAP) and prolong the residency of SREBP1 and 2 in the endoplasmic reticulum (ER)[155]. A decrease in sterol concentration causes dissociation of INSIG1 from SCAP and subsequent translocation of SCAP-SREBP to the Golgi, where cleavage of SREBP occurs to produce a transcription factor nSREBP. Translocation to the nucleus results in upregulation in genes responsible for cholesterol and lipid synthesis as well as the LDL receptor and its counter regulatory protein Proprotein convertase subtilisin/kexin type 9 (PCSK9). Increased expression of LDLR mediates greater clearance of

LDL cholesterol from circulation[156]. Following endocytosis and dissociation of the LDL particle and receptor, the receptor may return to the cell surface for further LDL particle capture, or upon association with PCSK9, become targeted for degradation. In addition to statins, current atherosclerosis research aims to decrease levels of PCSK9 to increase the recycling of LDLR to the surface and improve the clearance of LDL particles by the liver[157]. Following uptake, cellular cholesterol deemed in excess is secreted through the conversion to bile salts and excretion through the intestines. Conversion of cholesterol into bile salts involves at least 16 enzymes, followed by secretion from the hepatocyte through the bile salt export protein (BSEP)[158,159]. However, much of the bile salts are reabsorbed in the ileum and are returned to the liver for possible utilization or for excretion. A single bile salt can traverse this cycle anywhere from 4 to 20 times in a day before final excretion in the stool. Bile acid sequestrants limit the amount of reabsorption and contribute to the lowering of circulating cholesterol through increased excretion.

1.6.1 Statin mechanism

The first committed step in cholesterol synthesis is the reduction of HMG-CoA to mevalonate catalyzed by HMG-CoA reductase. The structure of all statins in their active form have an HMG-like moiety, allowing for competitive inhibition of HMGCR through binding to the enzyme active site

and excluding HMG-CoA interaction (**Figure 1.2**)[160]. Statins inhibit the rate limiting step of the mevalonate pathway that is catalyze HMG-CoA, which lowers cholesterol biosynthesis and increases levels of LDLR. Covalently linked to the HMG-like moiety, statins have similar hydrophobic ring structures that result in different pharmacokinetics between them as well as different binding affinities for HMGCR[161]. These differences in hydrophobicity likely cause the differences in potency and degree of pleotropic effects of statins.

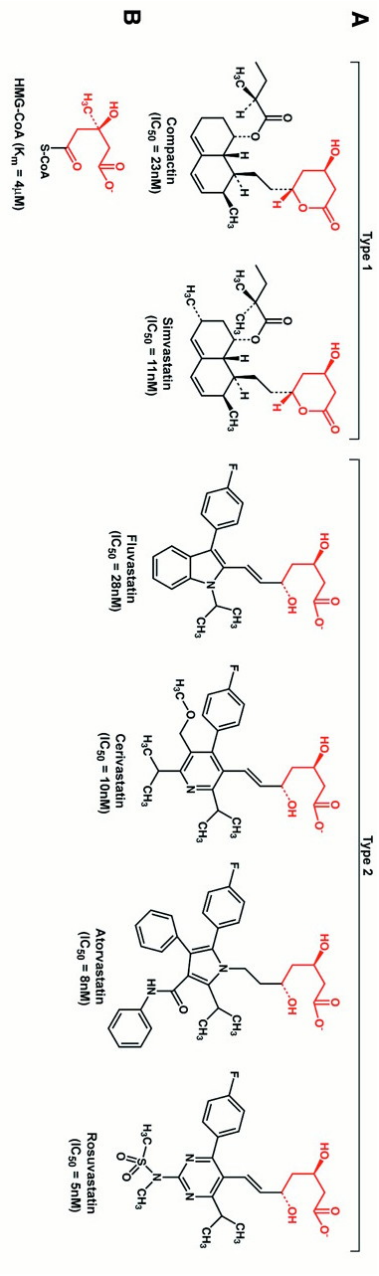


Figure 1.2 Statin and HMG-CoA structure.
Adapted from Istvan S. and Deisenhofer J. 2001, *Science. A:* Structure of several statins with the HMG moiety highlighted in red with the median inhibitory concentration (IC_{50}). Type 1 and type 2 classifications denote the statin is a derivative of the first isolated compactin (Type 1) or fully synthetic (Type 2). *B:* Structure of HMG-CoA and its K_M value.

1.6.2 Pleotropic effects of statins

Statin treatment is an efficacious and cost-effective way to reduce LDL cholesterol and lower cardiovascular disease risk and adverse events. However, some of the benefits and potential side effects are due to cholesterol-independent pleotropic effects of statins. Statin inhibition of the mevalonate pathway lowers not only cholesterol production, but inhibition of HMG-CoA reductase can also lower any of the downstream metabolites that are produced in the mevalonate pathway. These mevalonate pathway metabolites include, but are not limited to isoprenoids for protein prenylation, dolichols for protein folding, ubiquinone for electron transport in mitochondria, and various cholesterol derivatives such as 25-hydroxycholesterol. Much of the current evidence points to modulation of protein prenylation as a mediator of statin-induced pleotropic effects. There is a long list of immune modulatory effects observed with statin treatment, predominately due to alteration of small GTPase protein activity[162]. In clinical trials, statins are regarded as having widespread anti-inflammatory effects since statin treatment is associated with lower circulating IL-6, TNFα and C-reactive protein (CRP) in humans[163–165]. Statins also reduce T-cell and lymphocyte proliferation, reduce expression of MHCII antigen presentation complex, and reduce immune cell motility

and migration[162,166–169]. Alterations in protein prenylation have been associated with immunomodulation and some of the anti-inflammatory effects of statin may be linked to changes in isoprenoids rather than cholesterol[162].

However, not all statin-mediated pleotropic effects equate to lowering of immune responses. Statin treatment of immune cells from both mice and humans show an increase in secretion of IL-1β and increased caspase-1 activation[170–173]. Interestingly, in each of these series of experiments, statins only stimulated IL-1β secretion from THP-1 or human monocytes with an adequate priming signal, usually in the form of LPS, but also with *M. tuberculosis* and lipid loading. This suggests an inflammasome-caspase-1 mechanism regulating statin-induced IL-1β release[85]. Statins can also increase production of neutrophil extra cellular traps (NET), which are an important method of bacterial killing[174]. These increased immune responses may explain the reduced mortality of infection from sepsis, pneumonia, and bacteremia seen in patients undergoing statin therapy[175–178].

1.6.3 Statins, and diabetes

Recent clinical and epidemiological data demonstrated an association between statin use and increased risk of developing new onset T2D[179–181]. Statins have been shown to increase the risk of new onset T2D in up to

9% of patients on intensive statin therapy[179]. Studies have also shown a correlation of statin treatment with increased blood glucose levels[182,183]. This is very important because epidemiological evidence suggests that increased fasting blood glucose is sufficient to increase the risk of all-cause mortality interpedently of T2D status[184]. It is not yet clear if higher potency statins (often defined in terms of greater cholesterol lowering) equate to increased diabetes risk[185,186]. There is evidence suggesting that an increase in the dose of statin does increase risk of developing insulin resistance[187]. Also, there is not yet a clear consensus if the use of specific statins confers greater risk of new onset T2D[188,189]. To date, epidemiological evidence shows an association between increased risk of new onset T2D and use of fluvastatin, atorvastatin, rosuvastatin or simvastatin[185]. These statins are also associated with an increase in insulin resistance[187]. However, the association of pravastatin with increased risk of new onset T2D insulin resistance is not yet clear[189,190]. We hypothesized that it was important to understand *why* a drug class that is generally given to individuals that are already at risk for various metabolic disease characteristics, including T2D and has widespread anti-inflammatory effects, and improves circulating lipid profile, does not improve blood glucose control and may actually worsen glucose control in some patients.

Statins decrease many markers of inflammation, but recent evidence shows that the exception to the anti-inflammatory role of statins is IL-1β[172,173,191]. Since IL-1β and its processing via the NLRP3 inflammasome have been linked to insulin resistance and subsequent dysglycemia, we hypothesized that statins engage this inflammasome and cytokine to precipitate insulin resistance.

1.7 Mevalonate pathway metabolism

The mevalonate pathway is responsible for producing a diverse set of metabolites. The initial steps in the pathway involve conversion of acetyl-CoA to mevalonate (**Figure 1.3**)[192,193]. Two acetyl-CoA molecules are utilized to form acetoacetyl-CoA where HMG-CoA synthase catalyses the formation of HMG-CoA with another acetyl-CoA unit. The rate limiting step is carried out by HMG-CoA reductase, which converts HMG-CoA to mevalonate. Mevalonate is then phosphorylated, followed by a decarboxylation step to form the important five-carbon isopentenyl pyrophosphate (IPP), which is the building block used to produce growing isoprenoid chains. Condensation of two IPP units (following isomerisation of one of the units) yields geranyl pyrophosphate (GPP). Synthesis of farnesyl pyrophosphate (FPP) is achieved through addition of another IPP unit to GPP. FPP is an important branch point in the mevalonate pathway. Squalene synthase can condense two FPP molecules together, directing it

towards cholesterol and sterol production. FPP is utilized for protein prenylation to farnesylate proteins with farnesyl transferase (FTase)[193]. Protein prenylation is an important post-translational protein modification that modulate a protein's activity, localization, complex formation with membranes and other proteins[194–197]. FPP molecules are also diverted towards dolichol production. These substrates are added to nascent proteins currently undergoing folding in the ER, where sugar molecules decorate the dolichol molecule to track maturation progress. Addition of a final IPP molecule to FPP yields geranylgeranyl pyrophosphate (GGPP). Substrates of GGPP are also utilized for protein prenylation termed geranylation. Further, through additional steps, GGPP is also a precursor for ubiquinone (CoQ10). Ubiquinone is important for mitochondrial respiration acting as an electron transporter between complex I and III for ATP production[198,199]. There are also several further hormones and vitamins that are synthesized from the mevalonate pathway. Inhibition at different points of the mevalonate pathway reduces the production of the respective downstream molecules. Many metabolites are generated from mevalonate, which can alter cellular function and metabolism, especially considering the requirement for isoprenoids in the post-translational modification of hundreds of cellular proteins via prenylation with FPP and GGPP.

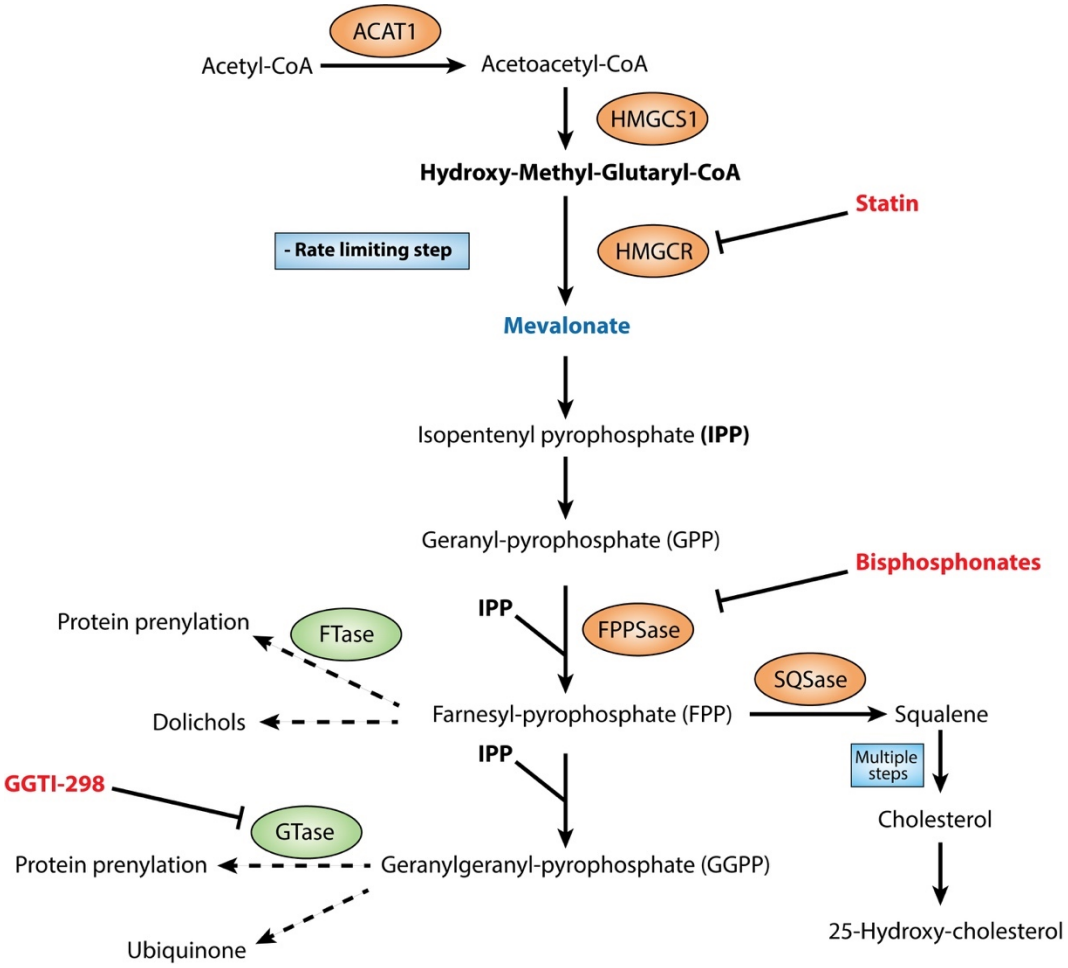


Figure 1.3 Mevalonate pathway metabolism.
Synthesis of cholesterol from the mevalonate pathway is initiated with Acetyl-Coa Acetyltransferase 1 (ACAT1) conversion of Acetyl-CoA to Acetoacetyl-CoA. Next 3-Hydroxy-3-Methylglutaryl-CoA Synthase 1 (HMGCS1) converts Acetoacetyl-CoA to Hydroxy-Methyl-Glutaryl-CoA where 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) catalyzes the rate limiting step to produce Mevalonate. Statins inhibit at HMGCR. Farnesyl-pyrophosphate (FPP) is committed to cholesterol synthesis following conversion with Squalene synthase (SQSase). Prenylation of proteins (farnesylation and geranylation) is catalyzed by FTase and GTase. GGTase inhibitor-298 (GGTI-298) inhibits geranylgeranylation of proteins. IPP- Isopentenyl pyrophosphate. Bisphosphonates inhibit FPP synthase (FPPSase).

1.8 Isoprenoid protein modification

There are a vast array of post-translational modifications that modulate a protein's activity, localization, complex formation, and degradation. One important post-translational modification is protein prenylation[200]. This process involves the irreversible attachment of a lipophilic isoprenoid chain of 15 or 20-carbon length to a protein. There are over 200 proteins that are predicted to be prenylated, many of which are in the large Ras, Rho, and Rab family of proteins[201,202]. The biological function of prenylation is predominately for cellular localization, but prenylation may also facilitate protein-protein interactions. Addition of a lipophilic isoprenoid chain on the c-terminal portion of a protein facilitates membrane anchoring and subsequent membrane trafficking.

There are three enzymes responsible for catalysing prenylation: Farnesyltransferase (FTase), and Geranylgeranyltransferase I and II (GGTase I and II), which carry out farnesylation and geranylgeranylation respectively. The most well characterized prenylation recognition sequence is a CAAX motif (Cysteine-Aliphatic-Aliphatic-X amino acid), facilitating the interaction with FTase or GGTase I. The amino acid in the X position dictates farnesylation or geranylgeranylation[203]. The isoprenoid group is covalently linked to the sulfur of the cysteine group, and the AAX

motif is cleaved off by RAS-converting CAAX endopeptidase 1 (RCE1). Prenylation of Rab family proteins is catalysed by GGTase II, and requires a Rab escort binding protein (Reb)[204,205]. Rab proteins will bind to Reb in the cytosol and are then prenylated by GGTase II. Often the prenylation sequence will contain two cysteine residues, and both will be prenylated.

Many Rho GTPase proteins require geranylgeranylation in order to translocate to the membrane where critical association factors reside for activation. RhoA associates with Rho guanine dissociation inhibitor (RhoGDI) to mediate movement from the ER membrane where prenylation occurred to the plasma membrane for insertion, whereby present guanine nucleotide exchange factors (GEFs) prime RhoA for activation[206,207].

Conversely, inhibition of prenylation can disrupt the function of the proteins dependent on the post-translation modification. Due to the complexity of the mevalonate pathway, multiple points of inhibition exist that culminate in a reduction prenylation. Inhibition of HMGCR by statins or inhibition of farnesyl diphosphate synthase (FPPS) by bisphosphonates, subsequently decrease downstream metabolites, including farnesyl and geranylgeranyl[208]. Chemical inhibitors of the prenyl-transferase proteins, such as GGTI-298, will prevent addition of the isoprenoid onto the receiving protein[209]. Much of the current research on prenylation inhibition is related cancer. Many of the Ras, Rho, and Rab family proteins have

oncogenic properties and upon dysregulation contribute to unchecked cell proliferation. Statins and prenyl-transferase inhibitors have been shown to inhibit NRas and KRas, two important oncogene regulators, and suppress tumor growth[210,211]. However, very little is known about how statins, prenylation and immunity are linked with respect to insulin resistance, lipid metabolism or T2D.

1.9 Objectives and Hypothesis

Preliminary findings from our lab and previous published work, demonstrated that several statins can cause IL-1β secretion from macrophages. Recent epidemiological evidence suggests there is a significant population of patients prescribed statins that are at risk for developing increased circulating blood glucose and T2D. This is concerning and perplexing for a drug class that improves blood lipid profiles and is generally regarded as anti-inflammatory. Our work attempts to uncover a possible mechanistic explanation to explain the relationship between statins, immunity and insulin resistance during metabolic syndrome. Understanding these relationships may allow for improvements to this drug class by mitigating pleiotropic side effects and maintaining lipid lowering properties of statins.

We hypothesized that:

1) the NLRP3 inflammasome mediates statin-induced insulin resistance in adipose tissue.
2) IL-1β mediates statin-induced insulin resistance in adipose tissue.
3) Lower protein prenylation mediates statin-induced insulin resistance in adipose tissue.

The objectives of this thesis were to determine if the NLRP3 inflammasome contributes to statin-induced insulin resistance, and evaluate the mevalonate pathway intermediate that propagate statin-mediated activation of this inflammasome. Last, we sought to determine what cytokine effectors downstream of the inflammasome were required for statin-induced insulin-resistance in adipose tissue and characterize functional changes in adipose metabolism. The contributions of this thesis were the discoveries that NLRP3 and IL-1β are both involved in statin-induced insulin resistance in adipose tissue as a result of lower protein prenylation, but not cholesterol. (**Figure 1.4**)

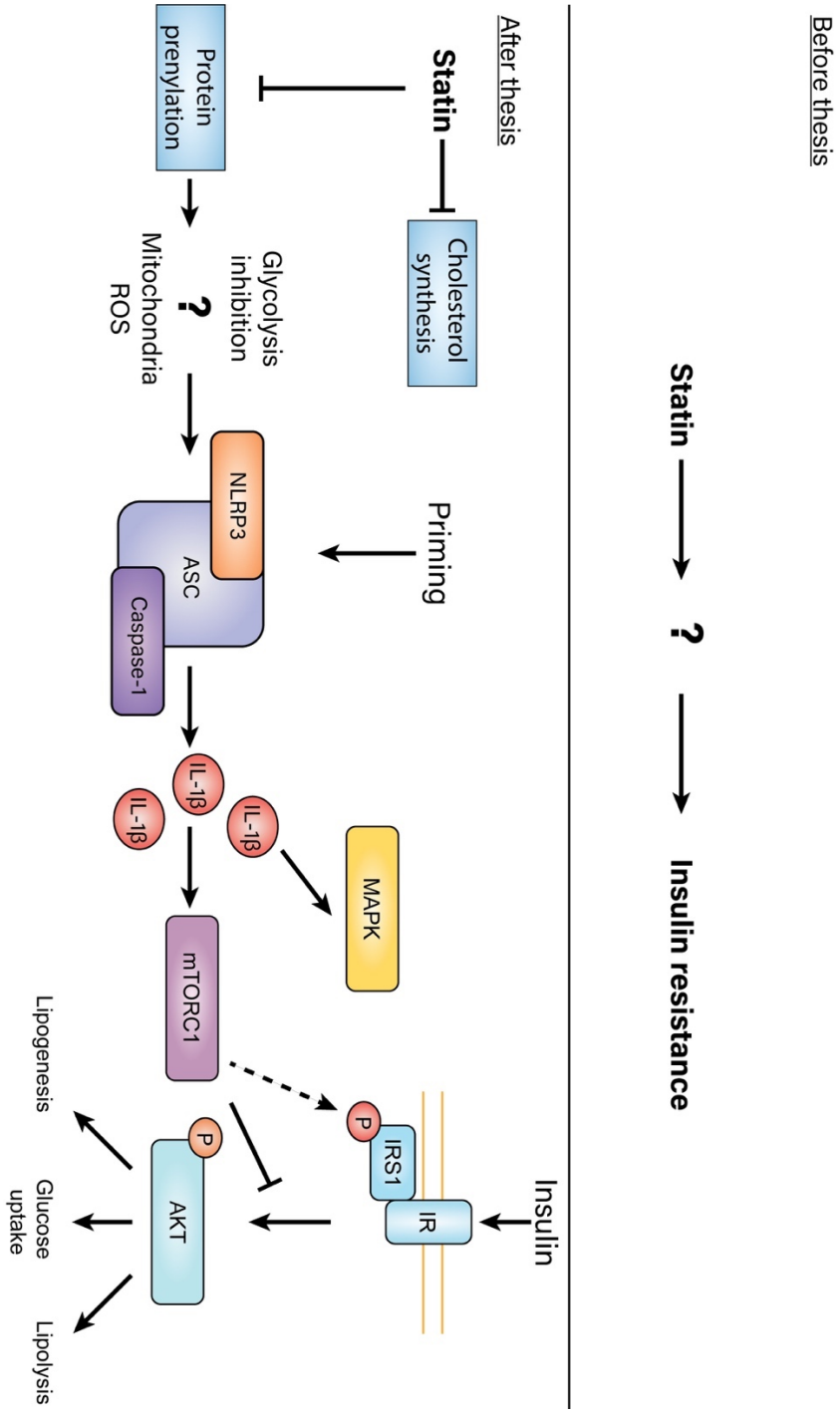


Fig 1.4. Understanding of statin-induced insulin resistance before and after thesis.

Chapter Two: Methods

2.1 Mice and Materials

The McMaster University Animal Ethics Review Board approved all procedures. Male wild-type (WT) C57BL/6J (#000664) mice were born from in-house colonies at McMaster University, but originally purchased from The Jackson Laboratory. Ob/Ob (#000632) mice where from Jackson Laboratory. NLRP3$^{-/-}$ mice (10 generations backcrossed to C57BL/6J) were from Professor Nicolas Fasel (Université de Lausanne, Lausanne, Switzerland) and were provided by Dr. Dana Philpott (University of Toronto, Toronto, ON, Canada). IL-1β$^{-/-}$ mice were kindly provided by Dr. Yoichiro Iwakura (University of Tokyo, Minato-ku, Tokyo, Japan) and bred in-house at McMaster University. IL-18$^{-/-}$ mice were kindly provided by Ali Ashkar (McMaster University). Statins, GGPP and GGOH were from Cayman Chemical (Ann Arbor, MI). InvivoGen (San Diego, CA) supplied ultra-pure LPS (Escherichia coli 0111:B4). z-YVAD-FMK was from Abcam (Cambridge, MA). (^{3}H)-2-Deoxy-glucose (#NET328001MC) and (^{14}C)-U-Glucose (#NEC042V250UC) were from Perkin Elmer (Waltham, MA). Farnesol, Low Density Lipoprotein (LDL, L8292), Cholesterol, 25-Hydroxycholesterol and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Insulin was Novorapid (#02245397) from Novo Nordisk (Mississauga, ON). Antibodies were from Cell Signalling Technologies

(Danvers, MA)(Table 1). Amplex Red Cholesterol assay kit (#A12216) was from Thermo Fisher (Waltham, MA).

2.2 Adipose Explants, Adipocytes and Macrophages

Mice were killed by cervical dislocation, and PBS-rinsed gonadal adipose tissue was minced into 5-mg pieces in DMEM containing 10% FBS. After 2 hours of equilibration, ~30 mg explants were placed in serum-free DMEM and exposed to 1 μM atorvastatin or the indicated dose of statin or zoledronate for 18 hours. During the final 4 hours adipose tissue explants were exposed to 2 μg/mL LPS (where indicated) and were left unstimulated or stimulated with 0.3 nM insulin for the final 10 min. Adipose tissue explants were supplemented with cholesterol, 25-HC, GGPP, LDL or z-YVAD at the indicated doses during the entire statin exposure. LDL was resuspended as per the manufacture's recommendations, by rotating overnight in DMEM carefully to avoid vortex/vigorous agitation. Adipose tissue lysates were prepared and immunoblotted, as described previously[48]. Caspase-1 and caspase-3 activity was quantified using the R&D Caspase activity assay. Change in the fluorescence of the probe was tracked over the course of 4 hours, measured every 30 minutes. For each experiment at least 2 mice were used to generate the explant adipose tissue for treatment. Each N replicate represents an individually treated well of adipose tissue for quantification.

3T3-L1 pre-adipocytes (ATCC, Rockville, MD) were differentiated for 10-12 days, as described[212]. In brief, cells were seeded in 12 well plates and allowed to grow to confluency. Following 48 hours of confluency, differentiation media 1 (DM I: DMEM, FBS 10%, Penicillin streptomycin 1%, GlutaMAX x1, Rosiglitazone, Insulin, Dexamethasone, IBMX) was added for 48 hours, followed by a media change to DM II (DMEM, FBS 10%, Penicillin streptomycin 1%, GlutaMAX x1, Insulin) for two 48 hour periods. Media was changed two more times in growth media (DMEM, FBS 10%, Penicillin streptomycin 1%, GlutaMAX x1) before cells were assessed for differentiation and used for experiments. 3T3-L1 adipocytes were exposed to atorvastatin (10 μM) for 18 hours and were left unstimulated or stimulated with 1 nM insulin for the final 10 min before lysates were prepared and immunoblotted, as described (See Immunoblotting protocol for full details below)[212]. 3T3-L1 adipocyte experiments treated with FOH was confirmed in 2 separate passages of cells. All other 3T3-L1 adipocyte experiments were confirmed in 3 separate passages of cells. Each experimental replicate (i.e. “N” value) represents an individually treated and quantified culture well containing 3T3-L1 adipocytes or adipose tissue explant-derived material.

Bone marrow–derived macrophages (BMDMs) were cultured as previously described[213]. For transcript analysis, BMDMs, differentiated

3T3-L1 adipocytes and adipose tissue explants were treated in serum-free media and exposed to statin (10 μM atorvastatin or fluvastatin) for 18 hours or LPS (200 ng/mL or 2 ug/mL) for 4 hours. Transcript levels were analyzed by quantitative PCR, as described previously[212].

2.3 Macrophages

Bone marrow–derived macrophages (BMDMs) were cultured for 7–10 days in DMEM containing 10% FBS and 15% L929 conditioned media. BMDMs were washed in serum-free media and exposed to statin (1 μM fluvastatin, unless otherwise stated) for 18 hours in serum- free DMEM and LPS (200 ng/mL) was added during the final 4 hours. GGPP (10 μM), z-WEHD-FMK (10 μM), and glyburide (200 μM) were used during the statin treatment period. IL-1β and IL-6 were quantified by ELISA.

2.4 Total Cholesterol quantification

3T3-L1 adipocytes were treated with Atorvastatin (10 μM) for 1, 3, and 22 hours followed by removal of media, PBS washed and replaced with isopropanol overnight at 4 °C with gentle rocking. Isopropanol was transferred to clean tubes and evaporated in a Speedvac at room temperature. Samples were resuspended in Amplex Red kit reaction buffer and cholesterol concentration was determined using the Amplex Red Cholesterol Assay kit (Thermo Fisher). Following isopropanol removal

from cells, cell lysates were made as previously described[212], and protein was measured using the BCA assay to correct cholesterol concentration between wells based on protein levels.

2.5 Adipose tissue segmentation and ELISA

Adipose tissue explants were cultured and treated as previously described with atorvastatin and LPS. Explants were transferred to 5 mL DMEM with 1 mg/mL collagenase and 1% BSA and shaken at 200 RPM at 37 °C until adipose was dissolved (~45 minutes). Samples were then filtered through a 100-micron mesh and centrifuged at 1000g for 10 minutes at RT. Floating adipocytes were removed into a new tube for ELISA or transcript analysis. Lysates for ELISA assay of adipocytes were made with 1% Triton x-100 in PBS with protease inhibitor and homogenized using a MP Bio FastPrep-24 shaker. Stromal vascular fraction (SVF) pellet was resuspended in lysis buffer and homogenized using the same technique as for adipocytes. Total IL-1β was quantified by ELISA (R&D systems, DY401) and corrected to lysate protein concentration. For transcript analysis adipocytes and SVF fraction were lysed with Trizol and analyzed by quantitative PCR, as described previously[212]. Please refer to the section containing the Gene expression assay protocol for full details.

2.6 Immunoblotting

Protein was extracted from approximately ~35 mg of adipose tissue by mechanical homogenization in SBJ lysis buffer (0.05M HEPES, 0.15M NaCl, 0.1M NaF, 0.01M Na pyrophosphate, 0.0005M EDTA*2H20, 0.25M Sucrose, 1M DTT, 20% Triton-X, 200 mM Na-orthovanadate, and 100% Protease inhibitor solution) at 4.5 meters/second for 30 seconds using a FastPrep-24 tissue homogenizer (MP Biomedicals) and one plastic bead. Samples were at centrifuged at 4 ^{o}C at 13000 x g for 15 minutes and supernatant transferred to clean sample tubes. Protein concentration was quantified with the Pierce BCA protein assay (#23225, Thermo Fisher Scientific). Samples were prepared in Laemmli Sample buffer and resolved on 7.5% poly-acrylamide gels using the Biorad Mini-Protean Tetra Cell Western blotting system. Samples were transferred to PVDF membrane (0.45 μm, Millipore Immobilon-P, #IPVH00010) using the Bio-rad Trans-Blot turbo transfer system. Following transfer, membrane was blocked for 1 hour while rocking using a %5 BSA solution, followed by overnight incubation with the designated antibody. Please refer to Table 1 for antibody list and dilutions. Antibody solutions were recovered and reused while membrane was washed 4 x 15 minutes with TBS-Tween 0.05%, followed by a 1 hour incubation with Anti-Rabbit secondary antibody and repeat washing step. Membranes were then incubated in 7 ml of Bio-rad Clarity max ECL regent while rocking for 5 minutes followed

by imaging with the Bio-rad ChemiDoc XRS+. Experiments comparing phosphorylated protein to total protein, were striped of antibodies and re-probed with new antibodies (as indicated) by incubating membranes with pre-warmed Gentle ReView Stripping Buffer (#N552, VWR) at 30 ^{o}C for 27 minutes. Blocking, antibody incubation, wash and imaging was conducted the same during re-probing step. Quantification of bands was performed using the Bio-Rad ImageLab software (6.0.1).

2.7 Lipolysis

Differentiated 3T3-L1 adipocytes were first exposed with or without Atorvastatin 18 hours and then left unstimulated or stimulated with isoproterenol (10 nM) for 5 hours of exposure with LPS. Free glycerol was determined in the media at 0, 1, 3, and 5 hours following isoproterenol exposure and free glycerol was quantified to calculate glycerol release rate, as described[214].

2.8 Insulin-stimulated Lipogenesis

White adipose tissue explants were exposed to 1 μM atorvastatin (18 hours) and 2 μg/mL LPS (final 4 hours) and 3T3-L1 adipocytes were exposed to 10 μM atorvastatin (18 hours) prior to performing insulin-stimulated lipogenesis. Subsequently, (^{14}C)-U-Glucose (2 μCi/mL) with and without insulin (0.3 nM) was added to culture for the final 2 hours. For 3T3-L1 adipocytes, following treatment, (^{14}C)-U-Glucose (1 μCi/mL) with

and without insulin (0.3 nM) was added to culture for the final 1 hour. Tissue and cells were washed with PBS and lipids were extracted by the Folch method using a chloroform/methanol (2:1) solution and the entire chloroform layer was counted for radioactivity. For adipose tissue explant experiments, radioactivity was corrected to tissue weight. For 3T3-L1 adipocyte experiments, protein content was quantified by BCA assay and radioactivity was corrected to protein concentration.

2.9 Glucose uptake

Glucose uptake was measured as done previously[48]. 3T3-L1 adipocytes were treated with atorvastatin (10 μM,18 hours) and then cells were stimulated with and without insulin (0.3 nM) for 20 minutes. Cells were washed with Hepes-buffered saline (HBS; 140 mM NaCl, 20 mM Hepes-Na pH 7.4, 5 mM KCl, $MgSO_4$, 1.0 mM $CaCl_2$), and replaced with glucose transport solution (TS; In HBS, 10 μM 2-deoxy-glucose, 0.5 μCi/mL (^{3}H)-2-deoxy-glucose) for 5 minutes. TS solution was removed followed by 3 washed with ice-cold stop solution (0.9% NaCl, 25 mM glucose). Cells were lysed with 0.05 N NaOH, and radioactivity was quantified and corrected to protein concentration using the Bradford method.

2.10 Fluvastatin serum quantification

Fluvastatin was quantified in 50 μL of serum. Mice were gavaged a known dose of fluvastatin (as indicated) and venous blood was collected 2 hours later and 24 hours later (data not shown at 24 hours since it was not detectable at 24 hours). Atorvastatin at 40 ng/ml was spiked in as an internal standard before sample preparation. Acetonitrile (3:1) was added and centrifuged to remove particulates. Sample were evaporated and resuspended in running conditions (70:30, 10mM ammonium acetate and 90% Acetonitrile, 10% 10mM ammonium acetate). Protocol followed the details outlined by Agilent Techonologies Application Notes by Srividya Kailasam. Samples were separated by HPLC using an Eclipse XDB-C18 column (3.5μm, 2.1 × 100mm) and detected with the Bruker micrOTOF II (Bruker; Billerica, MA).

2.11 In-vivo 2DG uptake

To determine the effect of long-term statin treatment on insulin-stimulated tissue glucose uptake, ob/ob mice were orally administered 40–50 mg/kg fluvastatin or vehicle 5 days a week for 6 weeks. Twenty-four hours after the last dose, mice were injected with 2 μCi of 3H-2-deoxy-D-glucose (2DG) via tail vein, immediately followed by the administration of insulin (4 units/kg i.p.). Blood samples were taken at baseline, 5, 10, 15, and 20 min, and were analyzed for 2DG radioactivity. Mice were killed by cervical dislocation, and tissues were snap frozen in liquid nitrogen. Brown

adipose tissue (BAT) and gonadal white adipose tissue (WAT) were analyzed for 2DG radioactivity with and without deproteinization (0.3 mM BaOH and 0.3 mM ZnSO4) to calculate the rates of tissue-specific glucose uptake.

2.12 Gene expression analysis

Total ribonucleic acid (RNA) was extracted from ~50 mg of adipose tissue using mechanical homogenization in TRIzol reagent (#15596018, Thermo Fisher Scientific) at 4.5 meters/second for 30 seconds using a FastPrep-24 tissue homogenizer (MP Biomedicals) and glass beads followed by phenol-chloroform extraction. Homogenates were centrifuged at 12,000xg for 10 minutes at 4 °C and the aqueous phase was taken and added to equal volume isopropanol. Following mixing, samples were centrifuged and the precipitated RNA pellet was washed twice with 75% ethanol/water solution. RNA pellet was left to dry and resuspended in ultrapure water.

cDNA was prepared using 1000 ng of extracted total RNA. Sample was treated with DNAse I (#18068015, Thermo Fisher Scientific) and incubated at room temperature for 15 minutes. Random hexamer primers and dNTPs were added and solution was incubated at 95 °C for 10 minutes to inactivate DNAse, followed by incubation at 55 °C for 10 minutes. cDNA synthesis was completed using SuperScript III Reverse

Transcriptase (#18080044, Thermo Fisher Scientific). This solution was incubated at 55 °C for 50 minutes, and 70 °C for 15 minutes and samples were diluted 1/25 in ultrapure water.

Transcript expression was measured using TaqMan Gene Expression Assays (Thermo Fisher Scientific) with AmpliTaq Gold DNA polymerase (#4311818, Thermo Fisher Scientific). Please refer to Table 2 for a list of primers and targets. Synthesized cDNA was incubated with polymerase and TaqMan probe, and was placed in a Rotor-Gene Q real-time PCR cycler (QIGEN). Samples complete 45 cycles at 95 °C for 5 seconds and incubation at 58 °C for 10 seconds. Each gene was compared to the house keeper *Rplp0* using the ΔΔCT method.

2.13 Statistical Analysis

Significance was determined by unpaired, two-tailed t-tests where only 2 conditions are compared and by one-way ANOVA when more than two conditions are compared. Tukey's post hoc test was used when appropriate (Prism 7, GraphPad Software).

Table 2.1: Antibody reagent list
CS- Cell Signalling Technologies (Danvers, MA)

Antibody	Species	Dilution	Company
pAkt (Ser473)	Rabbit	1:1000	CS-#4058
pAkt (Thr308)	Rabbit	1:1000	CS-#4056
Total Akt	Rabbit	1:1000	CS-#9272
pJNK (Thr183)	Rabbit	1:1000	CS-#9251
JNK	Rabbit	1:1000	CS-#9252
pERK (Thr202)	Rabbit	1:1000	CS-#4370
ERK	Rabbit	1:1000	CS-#4695
p38	Rabbit	1:1000	CS-#9212
p-p38 (Thr180)	Rabbit	1:1000	CS-#4511
p-p70s6k (Thr389)	Rabbit	1:1000	CS-#9250
p70s6k	Rabbit	1:1000	CS-#9202
PTEN	Rabbit	1:1000	CS-#9552
β-Actin	Rabbit	1:5000	CS-#4970
Secondary Anti-rabbit	Goat	1:5000	CS-#7074

Table 2.2: TaqMan qPCR probe list
From Thermofisher TaqMan Gene Expression Assays.

Gene	Probe Catalog #
Nlrp3	Mm00840904_m1
Rplp0	Mm01974474_gH
Pyrin	Mm01342372_m1

Chapter Three: Results

3.1 Mevalonate pathway inhibition causes inflammasome-dependent insulin resistance

3.1.1 Statins promote NLRP3-dependent release of IL-1β in macrophages

Activation of the inflammasome in macrophages derived from mice generally requires a two-step process that includes (i) priming of the inflammasome components with a stimulus that increases NF-κB activity such as LPS and (ii) a second stimulus to activate the assembly of the NLRP3/ASC/Casapse-1 inflammasome complex. Priming in BMDM experiments was achieved with 4-hour treatment with 200 ng/mL LPS. Exposure of primary macrophages derived from WT mice to 10 μM of one of four different statins (lovastatin, simvastatin, atorvastatin, fluvastatin) increased IL-1β secretion, but only when combined with the LPS/immunogenic priming in BMDMs (Fig. 3.1 A). Fluvastatin increased IL-1β secretion in a dose dependent manner, but only when combined with LPS priming in BMDMs (Fig. 3.1 B). LPS plus 1 μM fluvastatin increased IL-1β to greater than 800 pg/mL in the media of BMDMs, whereas controls conditions or fluvastatin without LPS priming was less than ~200 pg/mL. LPS alone increased IL-6 secretion in BMDMs (Fig. 3.1 C). These divergent cytokine responses are indicative of statins acting on the NLRP3 inflammasome, which regulates cytokines such as IL-1β (and IL-18),

whereas the NLRP3 inflammasome does not regulate IL-6. Fluvastatin did not increase IL-1β secretion above levels seen during LPS treatment alone in BMDMs derived from NLRP3$^{-/-}$ mice (Fig. 3.1 D). However, LPS still increased IL-6 secretion in BMDMs derived from NLRP3$^{-/-}$ mice (Fig. 3.1 E). Treatment of LPS-primed wild type (WT) BMDMs with the NLRP3 inhibitor, glyburide at 200 μM, or the Caspase inhibitor, Z-WEHD at 10 μM prevented statin-induced IL-1β secretion (Fig 3.1 F). Fluvastatin and LPS at the doses used in these experiments did not cause cell death in BMDMs (Fig. 3.1 G). This data shows that statin-induced IL-1β secretion in LPS-primed macrophages is NLRP3 inflammasome-dependent.

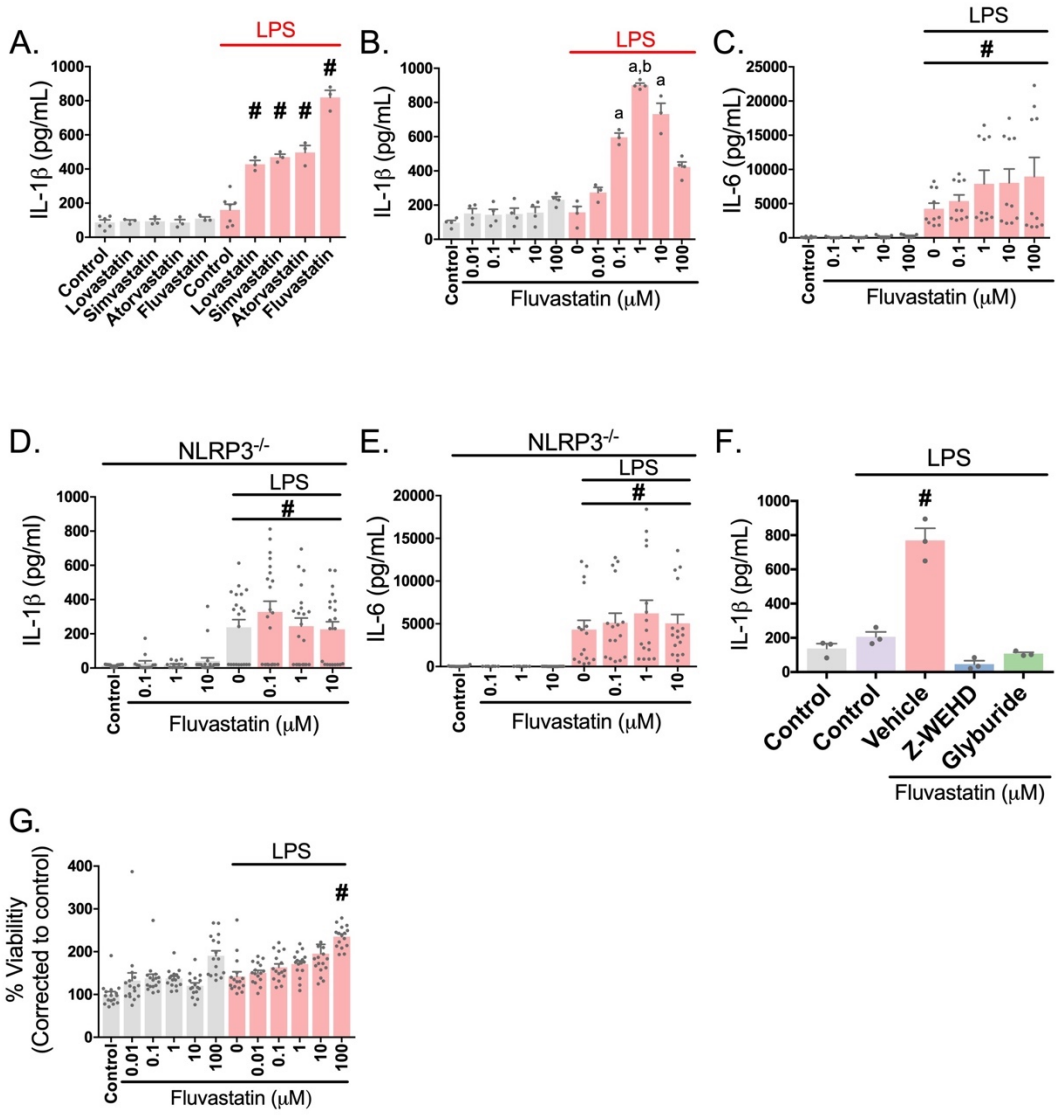

A.
LPS
IL-1β (pg/mL)
Control
Lovastatin
Simvastatin
Atorvastatin
Fluvastatin
B.
LPS
IL-1β (pg/mL)
Fluvastatin (μM)
C.
LPS
IL-6 (pg/mL)
Fluvastatin (μM)
D.
NLRP3-/-
LPS
IL-1β (pg/ml)
Fluvastatin (μM)
E.
NLRP3-/-
LPS
IL-6 (pg/mL)
Fluvastatin (μM)
F.
LPS
IL-1β (pg/mL)
Control
Control
Vehicle
Z-WEHD
Glyburide
Fluvastatin (μM)
G.
LPS
% Viability
(Corrected to control)
Fluvastatin (μM)

Fig 3.1. Statins promote NLRP3-dependent release of IL-1β in macrophages

BMDMs from wild type or NLRP3$^{-/-}$ mice and primed with LPS (200 ng/mL, 4 hours) where indicated. *A:* Macrophages were treated with various statins (10 μM, 18 hours) or *(B)* with indicated fluvastatin dose (18 hours) and IL-1β *(A, B)* or IL-6 *(C)* in the media was quantified. *D, E:* BMDMs from NLRP3$^{-/-}$ mice were treated with indicated fluvastatin dose (18 hours) and IL-1β or IL-6 in the media was quantified. *F:* BMDM from WT mice treated with fluvastatin and LPS similar to the conditions described in (*A)* and treated with Z-WEHD (10 μM) or glyburide (200 μM, 18 hours) and IL-1β in media was quantified. *G:* Cell viability was quantified with an MTT assay following treatment with fluvastatin (indicated dose, 18 hours) and LPS (200 ng/mL, 4 hours). Each value from a given well and mean ± SEM is shown. # Significantly different from control; a- significantly different from control, b- significantly different from 0.1 μM fluvastatin with LPS.

3.1.2 Statins impair adipose tissue insulin signalling via the NLRP3 inflammasome.

We next tested whether statin-induced activation of the inflammasome could impair insulin action in adipose tissue. We first determined the relative levels of IL-1β in adipocytes versus other adipose-resident cells, such as the immune cell-enriched stromal vascular fraction (SVF) in gonadal adipose tissue depot from male WT mice. These WT mice were between 20 and 40 weeks of age. When LPS-primed adipose tissue explants were treated with atorvastatin, we found that both adipocytes and SVF had higher levels of IL-1β (Fig. 3.2 A). IL-1β was ~8 fold higher in the SVF compared to adipocytes (Fig. 3.2 A). Transcripts of NLRP3, but not PYRIN were increased with LPS treatment in both the SVF and adipocyte fraction (Fig. 3.2 B). These data indicate that LPS can increase levels of (i.e. prime) the NLRP3 inflammasome, but LPS does not prime the PYRIN inflammasome in adipocytes and adipose resident cells. Further, both adipocytes and adipose resident cells can increase IL-1β protein levels in response to statins given adequate LPS priming in adipose tissue.

Fluvastatin increased caspase-1 activity by 1.5 fold in LPS-primed adipose tissue derived from WT, but not adipose tissue from NLRP3$^{-/-}$ mice (Fig. 3.2 C, E). Glyburide at 10 μM administered with fluvastatin, prevented statin-induced increase of caspase-1 activity in adipose tissue explants (Fig.

3.2 C). Fluvastatin also increased caspase-3 activity in LPS-primed adipose tissue from both WT and NLRP3$^{-/-}$ mice and also fluvastatin also increased caspase-3 activity independently of glyburide treatment (Fig. 3.2 F, G). These results show that fluvastatin activates a NLRP3-dependent, glyburide-sensitive caspase-1 inflammasome in adipose tissue, whereas the effects of a statin on other caspases (such as caspase-3) are not regulated by this inflammasome.

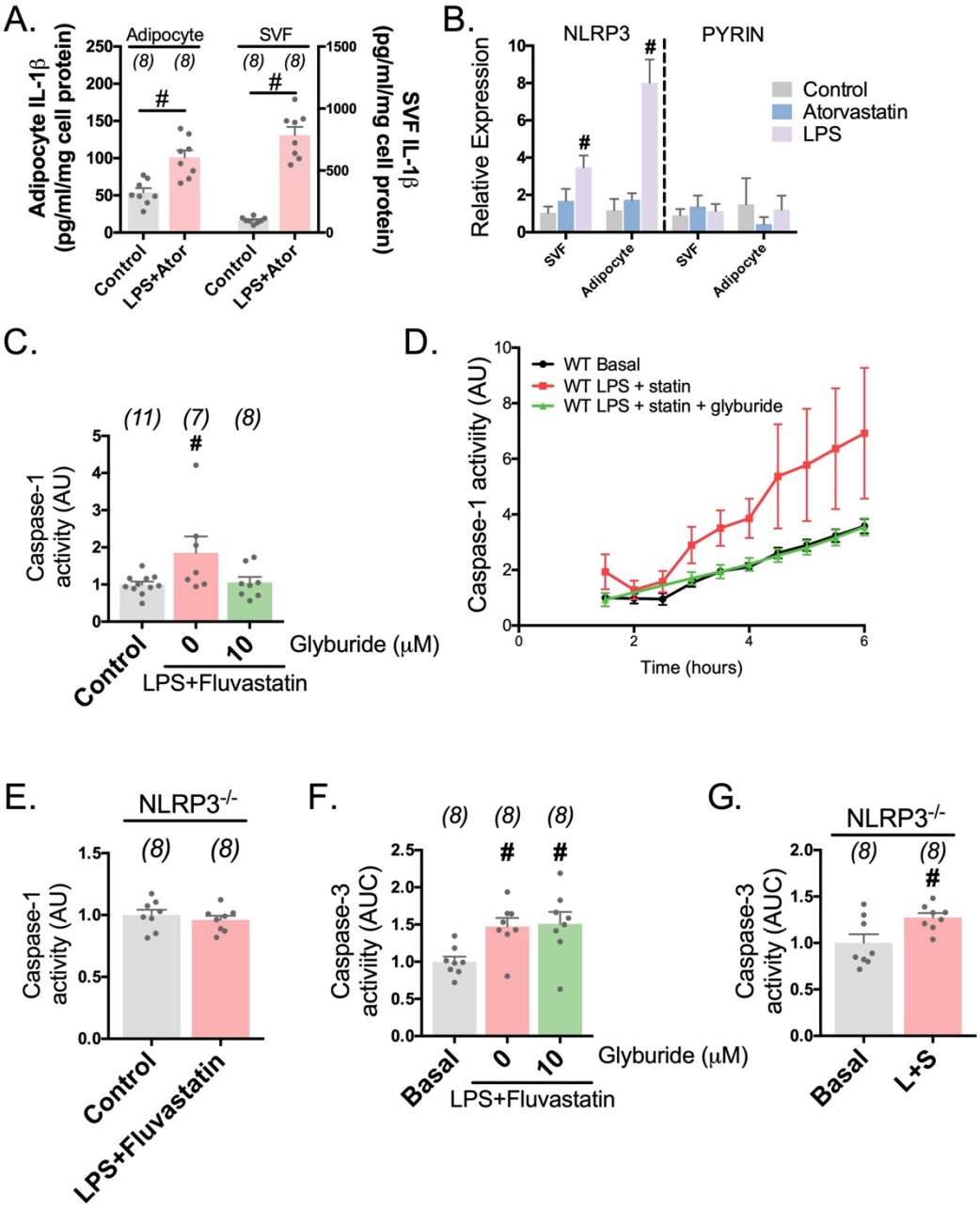

A.
Adipocyte
SVF
(8) (8) (8) (8)
#
#
Adipocyte IL-1β (pg/ml/mg cell protein)
SVF IL-1β (pg/ml/mg cell protein)
250
200
150
100
50
0
1500
1000
500
0
Control
LPS+Ator
Control
LPS+Ator
B.
NLRP3
PYRIN
Relative Expression
10
8
6
4
2
0
#
#
Control
Atorvastatin
LPS
SVF
Adipocyte
SVF
Adipocyte
C.
(11) (7) (8)
#
Caspase-1 activity (AU)
5
4
3
2
1
0
Control
0
10
Glyburide (μM)
LPS+Fluvastatin
D.
Caspase-1 activiity (AU)
10
8
6
4
2
0
WT Basal
WT LPS + statin
WT LPS + statin + glyburide
0
2
4
6
Time (hours)
E.
NLRP3-/-
(8) (8)
Caspase-1 activity (AU)
1.5
1.0
0.5
0.0
Control
LPS+Fluvastatin
F.
(8) (8) (8)
# #
Caspase-3 activiity (AUC)
2.5
2.0
1.5
1.0
0.5
0.0
Basal
0
10
Glyburide (μM)
LPS+Fluvastatin
G.
NLRP3-/-
(8) (8)
#
Caspase-3 activity (AUC)
2.0
1.5
1.0
0.5
0.0
Basal
L+S

Fig 3.2. Statins activate the inflammasome and caspase-1 in adipose tissue

A and B: Quantification of IL-1β by ELISA and transcript levels of NLRP3 and pyrin in adipocytes and the SVF from adipose tissue explants treated with atorvastatin (1 μM, 18 hours) and LPS (2 μg/mL for another 4 hours). *C-E:* Caspase-1 activity at the 4 hour time-point of the assay *(C, E)* and over the course of the assay *(D)* in adipose tissue explants derived from WT or *(E)* NLRP3$^{-/-}$ mice. Adipose tissue explants were treated with fluvastatin (18 μM, 18 hours), LPS (2 μg/ml) and Glyburide (10 μM, 18 hours). *F and G*: Caspase-3 activity at the 4 hour time-point of the assay in adipose tissue explants derived from WT *(F)* or NLRP3$^{-/-}$ mice *(G)*. Adipose tissue explants were treated with fluvastatin (18 μM, 18 hours), LPS (2 μg/ml) and Glyburide (10 μM, 18 hours). Data are mean ± SEM. The number above each experimental condition indicates the number of adipose tissue explants used in the quantification. n ≥ 5 replicates if not otherwise indicated. #Significantly different from control. Ator, atorvastatin; SVF, Stromal vascular fraction; L+S, LPS + Statin (Fluvastatin); AU, arbitrary unit.

We next tested if exposure of adipose tissue explants to fluvastatin impaired insulin-mediated signals that occur during insulin resistance (Fig. 3.3 A). LPS alone did not change the ability of insulin to phosphorylate Akt (pAkt) at serine473 in adipose tissue explants. Fluvastatin at 10 μM alone impaired insulin-mediated pAkt in adipose tissue from WT, but not NLRP3$^{-/-}$ mice. Further, the combination of LPS and fluvastatin at 10 μM decreased insulin stimulated pAkt compared to fluvastatin alone and completely prevented insulin's ability to significantly increase pAkt in adipose tissue explants from WT, but not NLRP3$^{-/-}$ mice (Fig. 3.3 A).

Next, we showed that multiple statins impair insulin signalling in adipose tissue, indicating a drug class effect on adipose tissue insulin sensitivity. We found that 1 μM atorvastatin or 1 μM pravastatin (Fig. 3.3 B, C) decreased insulin-stimulated Ser473 phosphorylation of Akt/PKB in LPS-primed explanted adipose tissue derived from WT mice. It was found that 1 μM or as little as 0.1 μM cerivastatin decreased insulin-stimulated Ser473 phosphorylation of Akt/PKB in LPS-primed adipose explants (Fig. 3.3 D). The bisphosphonate zoledronate (1 and 5 μM), which inhibits the mevalonate pathway distal to HMG-CoA reductase, also lowered insulin stimulated Ser473 phosphorylation of Akt/PKB in LPS-primed explanted adipose tissue (Fig. 3.3 E), suggesting this effect is specific to mevalonate pathway inhibition.

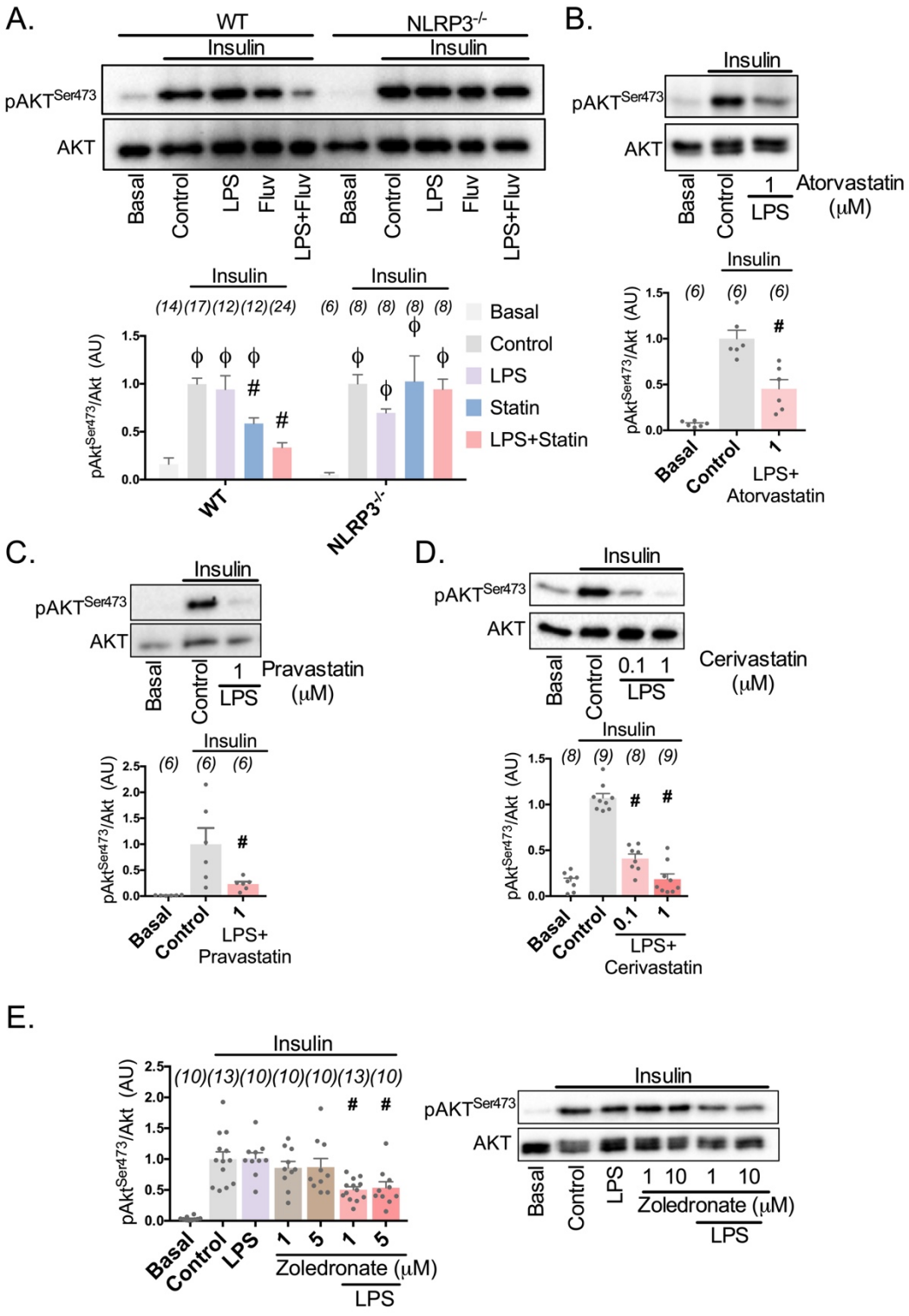

A.
WT
NLRP3-/-
Insulin
pAKTSer473
AKT
Basal
Control
LPS
Fluv
LPS+Fluv
pAktSer473/Akt (AU)
(14)(17)(12)(12)(24)
(6) (8) (8) (8) (8)
Basal
Control
LPS
Statin
LPS+Statin
B.
Atorvastatin (μM)
(6) (6) (6)
LPS+ Atorvastatin
C.
Pravastatin (μM)
(6) (6) (6)
LPS+ Pravastatin
D.
Cerivastatin (μM)
(8) (9) (8) (9)
LPS+ Cerivastatin
E.
(10)(13)(10)(10)(10)(13)(10)
Zoledronate (μM)
LPS

Fig 3.3. Mevalonate pathway inhibition impairs insulin action in adipose tissue.
Adipose tissue explants were from WT or NLRP3$^{-/-}$ C57BL/6J mice and treated with LPS (2 ug/mL for the final 4 hours) where indicated. *A*–*E*: Representative immunoblots (top) and quantification (bottom) of phosphorylated Akt (pAkt)/PKB (Ser473) from basal (i.e., no insulin) and insulin-stimulated (0.3 nM) conditions after treatment of adipose tissue explants with vehicle (control) or fluvastatin (10 μM), atorvastatin (1 μM), pravastatin (1 μM), cerivastatin (0.1 or 1 μM) or zoledronate (1 or 5 μM) for 18 hours. Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control; ϕsignificantly different from basal control. Ator, atorvastatin; AU, arbitrary unit.

Glyburide treatment at 10 μM (but not 1 μM) reversed fluvastatin-induced suppression of insulin-mediated pAkt in LPS-primed adipose explants. This glyburide dose (10 μM) did not modulate adipose tissue insulin-stimulated pAkt on its own (Fig. 3.4 A). Finally, a functional outcome of insulin in adipocytes was tested in the presence of a statin by measuring insulin-stimulated lipogenesis. Atorvastatin impaired insulin-stimulated lipogenesis in LPS-primed white adipose tissue explants (Fig 3.5 A). These results show that inhibiting the mevalonate pathway at multiple steps or with multiple statins impair insulin signalling through activation of the NLRP3 inflammasome. We discovered that one functional outcome is lower insulin-stimulated adipose tissue lipogenesis due to statin exposure.

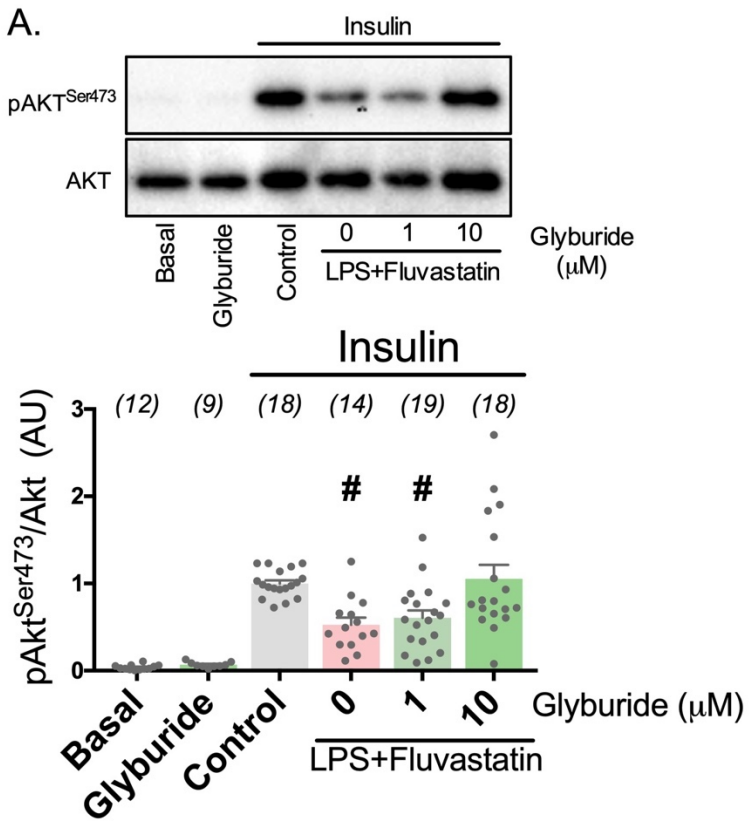


Fig 3.4. The NLRP3 inhibitor glyburide rescues fluvastatin-mediated insulin resistance in adipose tissue.
Adipose tissue explants from WT C57BL/6J mice were treated with fluvastatin (10 μM, 18 hours) and LPS (2 μg/ml) and/or glyburide (indicated, 18 hours). Representative immunoblots (top) and quantification (bottom) of phosphorylated Akt (pAkt)/PKB (Ser473) from basal (i.e., no insulin) and insulin-stimulated (0.3 nM) conditions. Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control; AU, arbitrary unit.

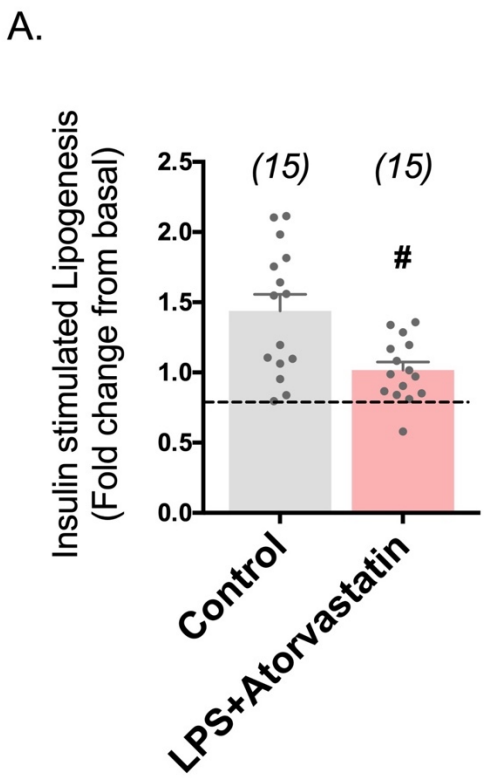


Fig 3.5. Atorvastatin inhibits insulin-stimulated lipogenesis in adipose tissue.
Adipose tissue explants were from WT mice. *A:* Fold-change insulin-stimulated lipogenesis in adipose tissue explants treated with vehicle (control) or atorvastatin (1 μM, 18 hours) and LPS (2 ug/mL, final 4 hours). Fold change was calculated as the increase relative to adipose tissue explants not exposed to insulin. Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control.

3.1.3 Statins activate the inflammasome and impair insulin signalling in 3T3-L1 adipocytes.

There are many non-adipocyte cell types and potential sources of IL-1β processing in adipose tissue. Even though our data show that the SVF contains a higher level of IL-1β compared to adipocytes in adipose tissue, it is important to consider adipocyte-autonomous effects of statins. Adipocytes comprise most of the cellular volume in adipose tissue and are the cells responsible for the majority of lipid metabolism in adipose tissue. We had already found that the adipocytes from LPS-primed adipose tissue had increased production of IL-1β with statin treatment (Fig. 3.2 A). We next tested the adipocyte cell-autonomous response to statins using the 3T3-L1 adipocyte model. Treatment with fluvastatin plus LPS increased caspase-1 activity by 1.4 fold in 3T3-L1 adipocytes (Fig. 3.6 A). Fluvastatin plus LPS decreased insulin-mediated Ser473 phosphorylation of Akt/PKB 3T3-L1 adipocytes (Fig. 3.6 B). Since fatty acids can contribute to activation of the NLRP3 inflammasome and perturb insulin signalling, we determine if these adipocyte autonomous statin-mediated responses were driven by increases in lipolysis. We found that atorvastatin did not alter lipolysis (with or without isoproterenol) in 3T3-L1 adipocytes (Fig 3.7). These results show that

statins promote activation of NLRP3-caspase-1 inflammasome and inhibit insulin signalling in an adipocyte cell-autonomous manner.

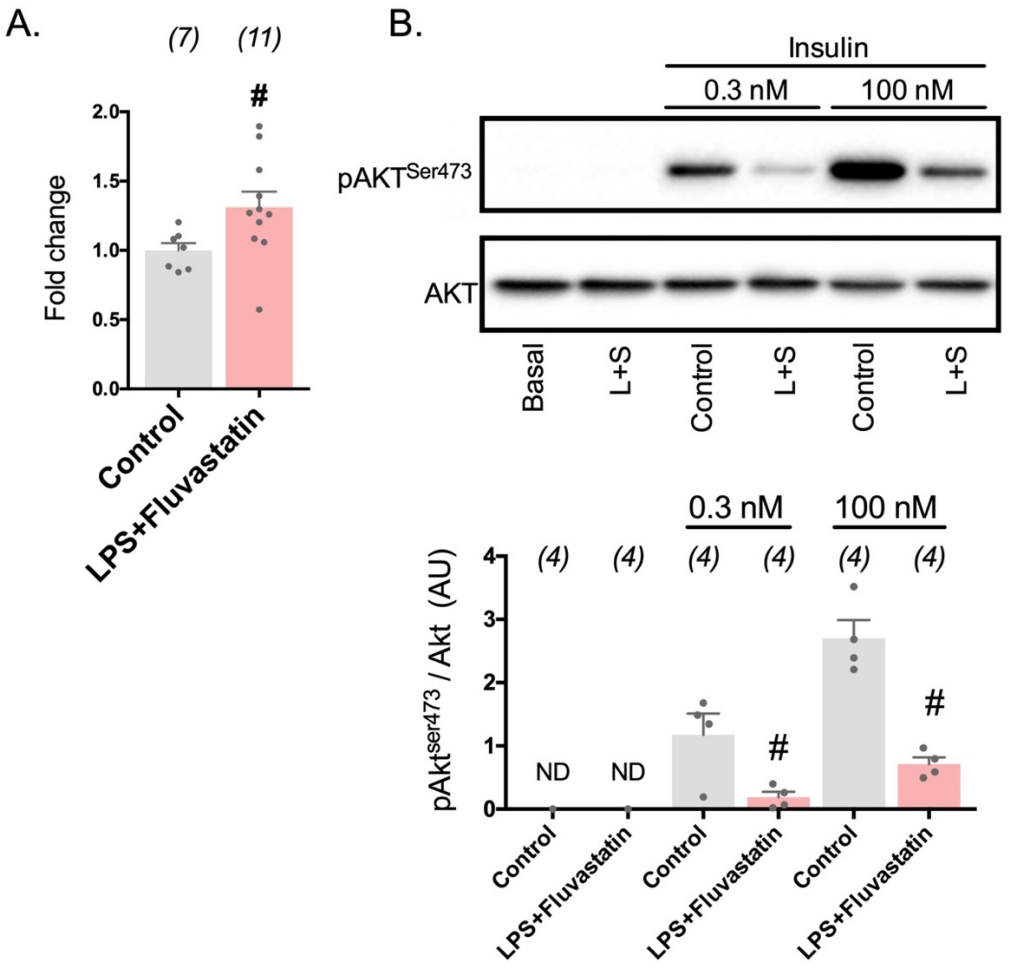


Fig 3.6. Fluvastatin activates caspase-1 and causes insulin resistance in 3T3-L1 adipocytes

A: Relative caspase-1 activity in 3T3-L1 adipocytes after treatment with vehicle (Control) or LPS plus fluvastatin. *B:* Representative immunoblots (top) and quantification (bottom) of 0.3 and 100 nM insulin-stimulated phosphorylation of Akt (serine 473) in 3T3-L1 adipocytes after treatment with control or LPS with fluvastatin 10 μM. Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control; L+S, LPS + Statin (Fluvastatin); AU, arbitrary unit. ND, not detected phosphorylation.

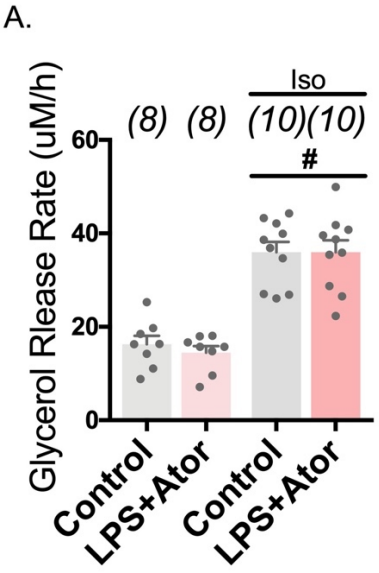


Fig 3.7. Atorvastatin does not alter basal or stimulated lipolysis in 3T3-L1 adipocytes.

A: 3T3-L1 adipocytes were treated with atorvastatin (10 μM, 18 hours) and LPS (2 ug/mL, 4 hours) with or without isoproterenol (Iso) (10 nM, 5 hours), and lipolysis was determined by glycerol release rate. Each value from a given treatment well and mean ± SEM is shown. The number above each experimental condition indicates the number of treated wells used in quantification. #Significantly different from basal control. Iso, isoproterenol; Ator, Atorvastatin.

3.1.4 Fluvastatin serum concentrations in mice.

In order to test if statins promote insulin resistance *in-vivo,* it was important to select a drug dose that is physiologically and/or clinically relevant. Patients given the highest prescribed dose of 40 mg of fluvastatin showed a range of 200 nM to 1 μM in circulation[215]. The time to maximum concentration (T_{max}) for fluvastatin is ~2 hours. We measured fluvastatin concentration in serum 2 hours following oral gavage of 10, 20 and 50 mg/kg fluvastatin by HPLC/MS. We also measured fluvastatin serum concentration of mice fed ad-libitum with a fluvastatin-diet mixture (600mg/kg food). Average fluvastatin serum concentration by oral gavage was 0.4, 1.8 and 6 μM respectively and through diet delivery was ~4 μM (Fig 3.8 B, C).

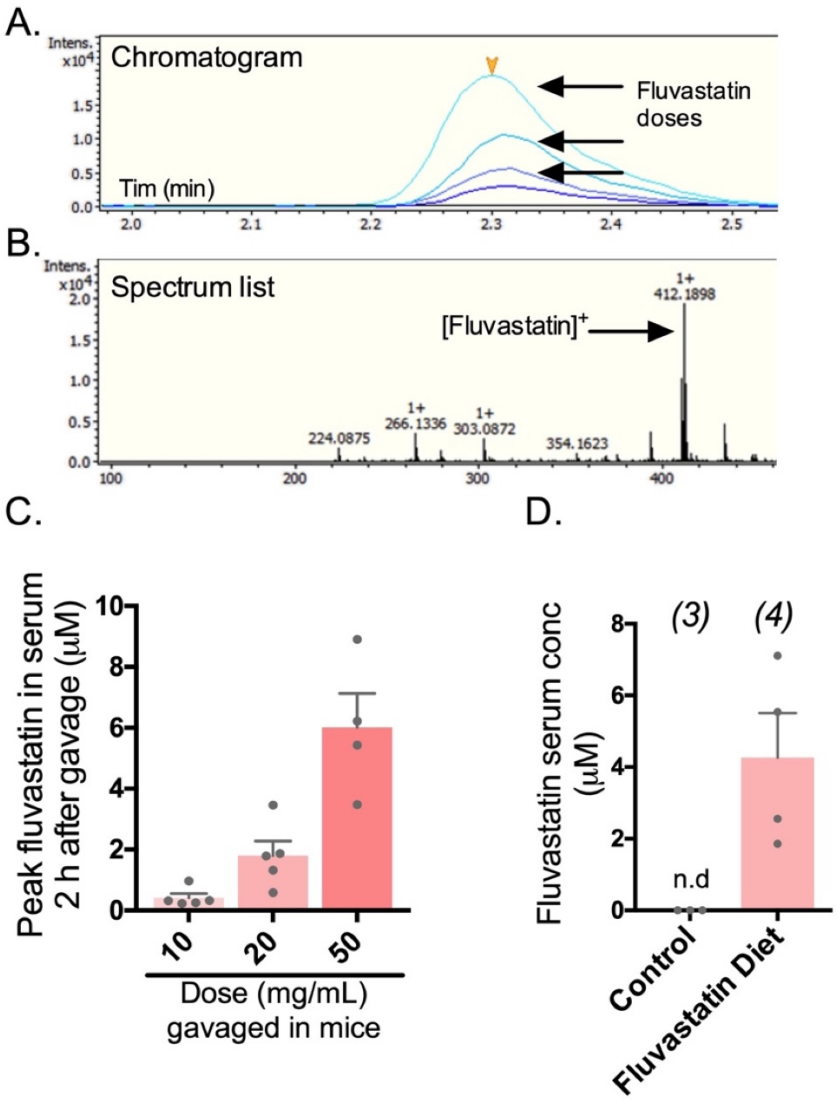


Fig 3.8. Fluvastatin serum concentration in wild-type C57BL/6J mice. *A and B:* Typical chromatogram and m/z spectrum of fluvastatin standards detected with HPLC/MS. *C:* Mice were gavaged the indicated dose (mg/kg) and serum was collected 2 hours later. *D:* Mice were fed a 600 mg/kg fluvastatin diet for # weeks and blood was collected from control (no fluvastatin in the diet) and fluvastatin fed mice. Fluvastatin serum concentration was quantified with HPLC/MS. The number above each experimental condition indicates the number of serum samples collected from different mice used in quantification. n.d, Not detected.

3.1.5 Fluvastatin supplementation does not alter blood glucose homeostasis in lean, chow-fed WT mice

Wild type male mice (n=5) were fed either a control diet or fluvastatin supplemented diet (600 mg/ kg food) for 4 weeks. Blood glucose was measured post 6-hour fast and a glucose tolerance test was performed (Fig 3.9). No difference in body mass (Fig. 3.9 A), fasting blood glucose (Fig. 3.9 B) or glucose tolerance was observed between the fluvastatin diet-fed or control diet-fed groups of mice (Fig. 3.9 C, D).

3.1.6 Fluvastatin supplementation induces glucose intolerance in obese (ob/ob) mice

Male ob/ob mice (n=4) were fed either a control diet or fluvastatin supplemented diet (600 mg/ kg food) for 28 days (Fig. 3.10). Fluvastatin supplemented diet was removed for 1 week and glucose tolerance test was performed. Body mass of the fluvastatin-fed ob/ob mice was significantly lower compared to ob/ob mice fed the control diet (Fig. 3.10 A) ($P < 0.05$). However, despite this moderate weight loss, fasting blood glucose was higher in the fluvastatin-fed ob/ob mice (8 mmol/L) compared to control diet-fed ob/ob mice (7 mmol/L) ($p < 0.05$) (Fig 3.10 B). Further, glucose intolerance was worse (25% increase in AUC) in fluvastatin-fed ob/ob mice compared to control-diet fed ob/ob mice (Fig. 2.10 C, D) ($p < 0.05$). These

data show that oral fluvastatin (feeding) can increase fasting blood glucose and worsen blood glucose control in obese mice despite moderate weight loss. These effects were not seen in lean, chow fed WT mice.

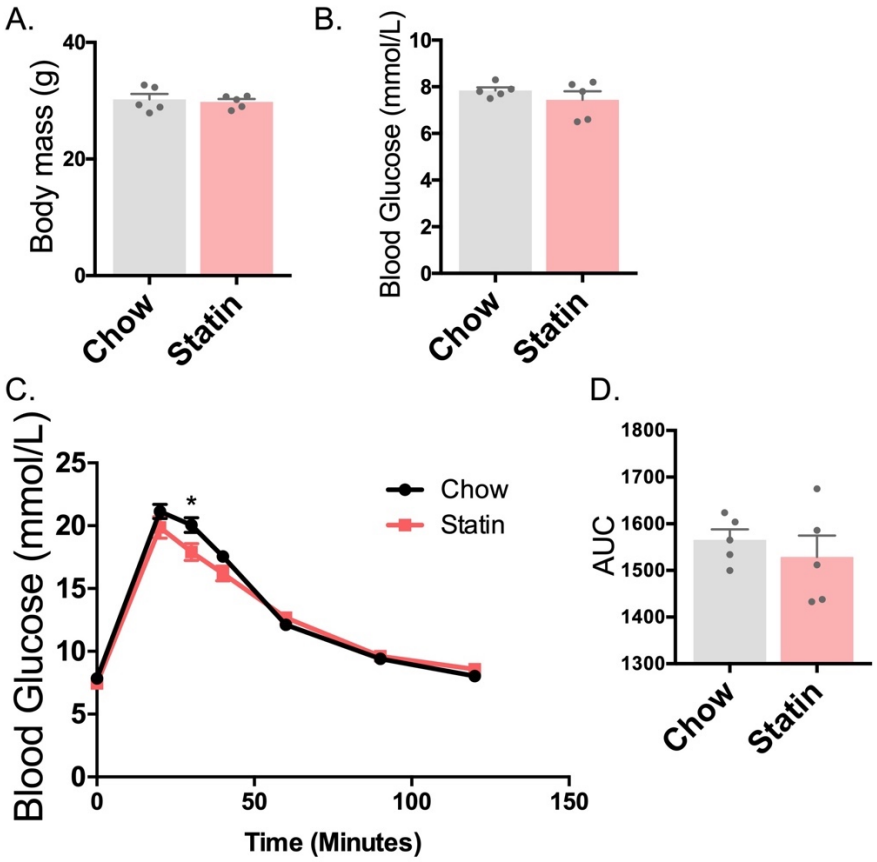


Fig 3.9. Fluvastatin supplemented, low-fat diet fed C57BL/6J mice do not develop glucose intolerance.
A-D: WT C57BL/6J male mice (n-5) were fed either a control low-fat diet or fluvastatin supplemented low-fat diet (600 mg/ kg food) for 4 weeks. *A, B:* Body weight and 6 hour fasting blood glucose on day 30. *C, D:* Glucose tolerance test (glucose, 2 g/kg) and AUC quantified from blood glucose measured at 0, 20, 30, 40, 60, 90, and 120 minutes following injection. *Significantly different between groups at that point, quantified by T-test. Statin, fluvastatin. AUC; area under the curve.

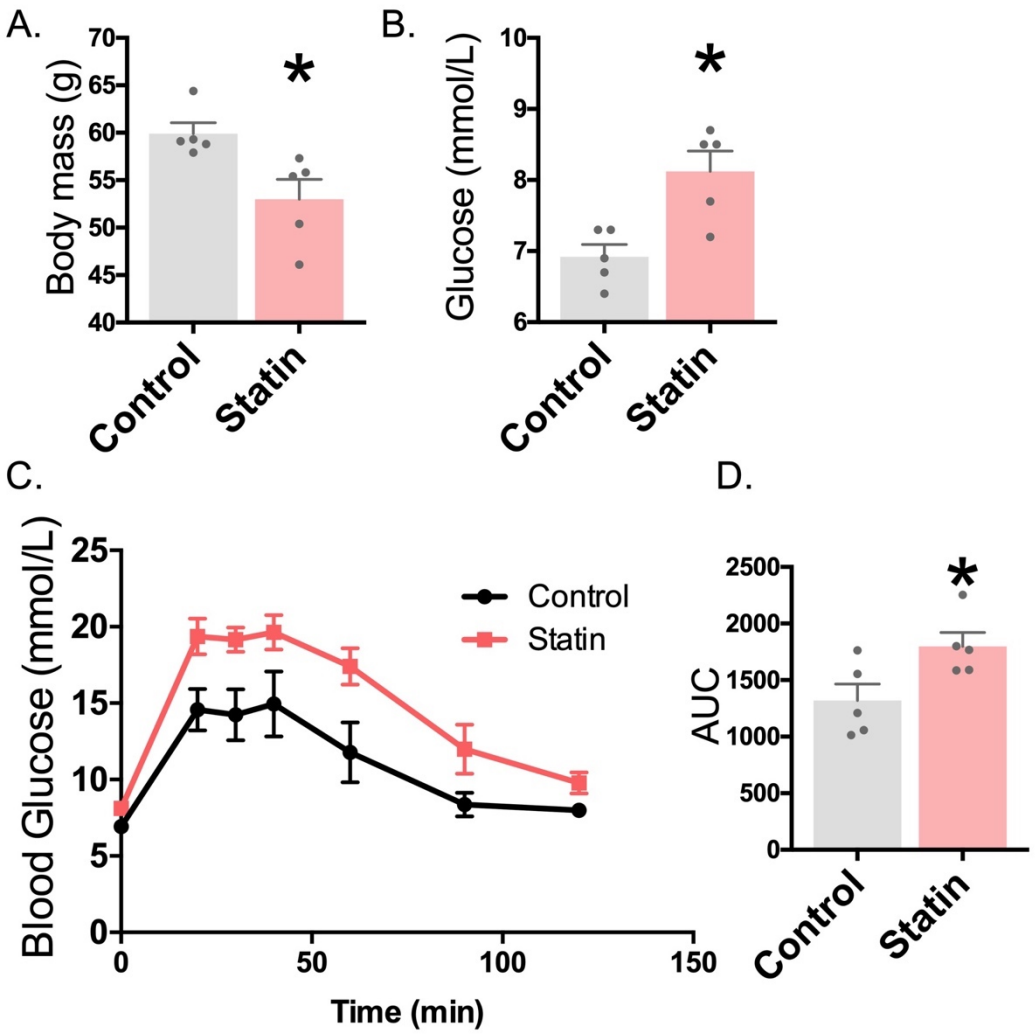


Fig 3.10. Fluvastatin supplementation induces glucose intolerance in obese (ob/ob) mice

A-D: Ob/Ob mice at 14 weeks of age were fed either a control diet or fluvastatin supplemented diet (600 mg/ kg food) for 28 days . *A, B:* Body weight and 6 hour fasting blood glucose on day 45. *C, D:* Glucose tolerance test (glucose, 0.5 g/kg) and AUC quantified from blood glucose measured at 0, 20, 30, 40, 60, 90, and 120 minutes following injection. *Significantly different between groups, determined by t-test. Statin, fluvastatin. AUC; area under the curve.

3.1.7 Fluvastatin supplementation impairs glucose uptake in adipose tissue in ob/ob mice

We next measured if delivering a statin once a day via gavage, similar to a dose taken by a patient, could alter tissue-specific insulin action or glucose control. To measure uptake into specific tissues in ob/ob mice, insulin and ^{3}H-2-deoxyglucose (2DG) was infused via tail vein injection. Insulin stimulated uptake of 2DG into certain tissues was quantified by measuring radioactivity of different tissues. We found that oral gavage of fluvastatin (50mg/kg) once a day, 5 days a week for 6 weeks impaired insulin-stimulated glucose disposal into specific adipose tissue depots (i.e. WAT) of ob/ob mice ($n \geq 3$) (Fig. 3.11 A). Glucose uptake was 50% lower in WAT, but not brown adipose tissue (BAT) or muscle tissue of fluvastatin-treated ob/ob mice (Fig. 3.11 B). Glucose uptake was also reduced in the liver of fluvastatin-treated ob/ob mice (Fig. 3.11 A) ($P < 0.05$). Overall, these results demonstrate that fluvastatin administration impairs both whole body glucose tolerance and insulin-stimulated glucose uptake in the WAT of ob/ob mice.

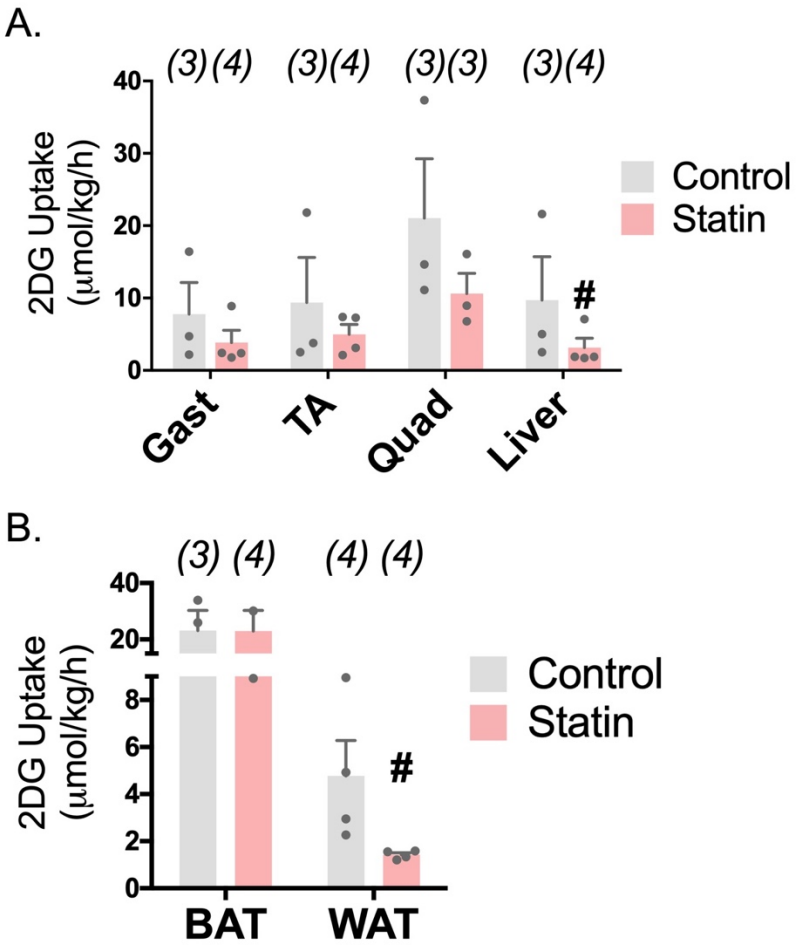


Fig 3.11. Fluvastatin supplementation impairs glucose uptake in adipose tissue in ob/ob mice.
A: In vivo insulin-stimulated 2DG uptake in muscle (Gast, gastrocnemius; TA, tibialus anterior; Quad, quadricep), liver, and adipose tissue (BAT, brown adipose tissue; WAT, white adipose tissue) from ob/ob mice orally gavaged with vehicle (Control) or fluvastatin (Statin) for 6 weeks ($n \geq 3$ mice per group). *B:* Data of adipose tissue alone from figure (*A)* demonstrating difference with alternate scale. #Significantly different from control treatment.

3.2 Identification of the mediators of statin-induced insulin-resistance following inflammasome activation

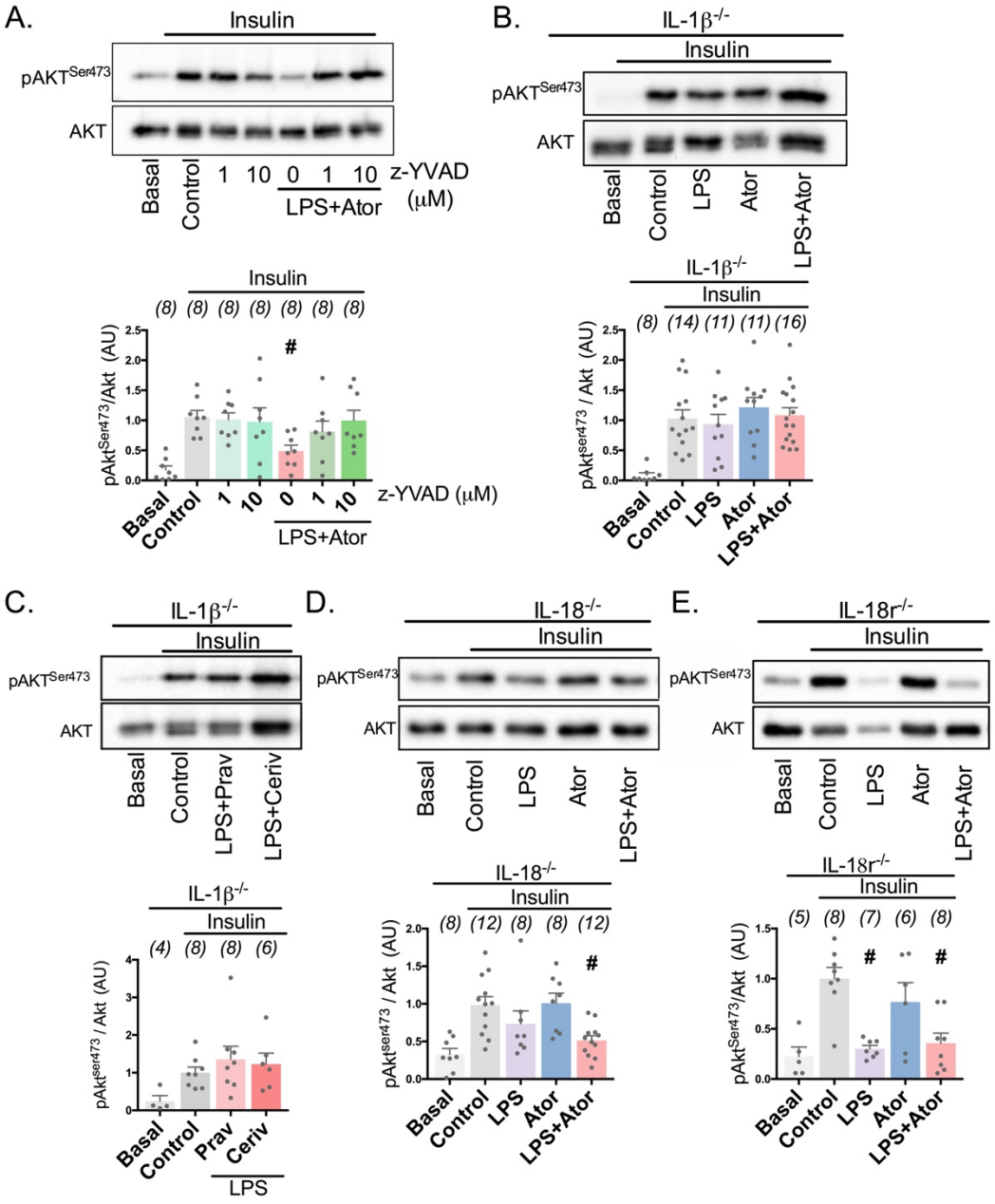
3.2.1 Statin-activation of a NLRP3-Caspase-1 mechanism is required for IL-1β secretion in macrophages

To determine what factor(s) mediate statin-induced insulin resistance following inflammasome activation, we first tested if active Caspase-1 is required in immune cells. Treatment of LPS-primed BMDMs derived from WT mice with the Caspase inhibitor z-WEHD, prevented statin-induced IL-1β secretion (Fig. 3.1 F). This was one of the first results in the course of the thesis and the result shows that statin-induced IL-1β secretion in LPS-primed macrophages utilizes a caspase-1 mechanism for activation. We next tested the relevance of caspase-1 and IL-1β in adipose tissue.

3.2.2 IL-1β and caspase-1 is required for statin-mediated insulin resistance in adipose tissue

We next tested if caspase-1 and IL-1β or IL-18 is involved in statin-induced insulin resistance in adipose tissue (Fig. 3.12). Inhibition of caspase-1 with 1 or 10 μM z-YVAD restored atorvastatin-mediated lowering of Ser473 phosphorylation of Akt/PKB in LPS-primed adipose tissue explants (Fig. 3.12 A). Atorvastatin did not lower insulin-stimulated Ser473 phosphorylation of Akt/PKB in LPS-primed adipose tissue explants derived from IL-1β$^{-/-}$ mice (Fig. 3.12 B). Furthermore, both pravastatin and

cerivastatin also did not lower insulin-stimulated Ser473 phosphorylation of Akt/PKB in adipose tissue explants derived from IL-1β$^{-/-}$ mice (Fig. 3.12 C). Similar to adipose tissue derived from WT mice, atorvastatin lowered Ser473 phosphorylation of Akt/PKB in LPS-primed adipose tissue explants derived from IL-18$^{-/-}$ mice (Fig. 3.12 D). Interestingly, testing adipose tissue explant tissue derived from IL-18 receptor$^{-/-}$ (IL-18r$^{-/-}$) mice revealed that LPS alone lowered of insulin-stimulated Ser473 phosphorylation of Akt/PKB (Fig. 3.12 E #) ($P < 0.05$). This increased LPS sensitivity in adipose tissue derived from IL-18r$^{-/-}$ mice should be followed up in future testing. Overall, these findings demonstrate that statins require caspase-1 and IL-1β to impair insulin signalling in adipose tissue.


A.
Insulin
pAKTSer473
AKT
Basal
Control
1
10
0
1
10
z-YVAD (μM)
LPS+Ator
Insulin
(8) (8) (8) (8) (8) (8) (8)
pAktSer473/Akt (AU)
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Basal
Control
1
10
0
1
10
z-YVAD (μM)
LPS+Ator
B.
IL-1β-/-
Insulin
pAKTSer473
AKT
Basal
Control
LPS
Ator
LPS+Ator
IL-1β-/-
Insulin
(8) (14) (11) (11) (16)
pAktSer473 / Akt (AU)
Basal
Control
LPS
Ator
LPS+Ator
C.
IL-1β-/-
Insulin
pAKTSer473
AKT
Basal
Control
LPS+Prav
LPS+Ceriv
IL-1β-/-
Insulin
(4) (8) (8) (6)
pAktSer473 / Akt (AU)
Basal
Control
Prav
Ceriv
LPS
D.
IL-18-/-
Insulin
pAKTSer473
AKT
Basal
Control
LPS
Ator
LPS+Ator
IL-18-/-
Insulin
(8) (12) (8) (8) (12)
pAktSer473 / Akt (AU)
#
Basal
Control
LPS
Ator
LPS+Ator
E.
IL-18r-/-
Insulin
pAKTSer473
AKT
Basal
Control
LPS
Ator
LPS+Ator
IL-18r-/-
Insulin
(5) (8) (7) (6) (8)
pAktSer473/Akt (AU)
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#
Basal
Control
LPS
Ator
LPS+Ator

Fig. 3.12. Statins impair adipose insulin action through IL-1β. Adipose tissue explants were treated with LPS (2 ug/mL for the final 4 hours) where indicated. Explants derived from WT C57BL/6J or IL-1$\beta^{-/-}$ or IL-18$^{-/-}$ mice as indicated. *A:* Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated phosphorylated Akt (pAkt) (Ser473) after treatment of adipose tissue explants with vehicle (control) or atorvastatin (1 μM) supplemented with or without the caspase-1 inhibitor z-YVAD at the dose indicated. *B and C*: Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated pAkt (Ser473) after treatment with either atorvastatin (1 μM) or pravastatin (1 μM) or cerivastatin (0.1 μM) in adipose tissue explants derived from IL-1$\beta^{-/-}$ mice. *D:* Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated pAkt (Ser473) after treatment with atorvastatin (1 μM) in adipose tissue explants derived from IL-18$^{-/-}$ mice. Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control tested by one-way ANOVA. Ator, atorvastatin; AU, arbitrary unit; Ceriv, cerivastatin; Prav, pravastatin.

3.2.3 Statin-mediated IL-1β does not engage MAPKs to decrease insulin signalling

Since IL-1β is required for statin-induced impairments in insulin-signalling, we then sought to uncover the intermediary immune or stress kinase cellular mediators that could lower insulin-simulated Akt/PKB phosphorylation. Increased IL-1β can lead to downstream activation of MAPK proteins, which have all been associated with insulin resistance in adipose tissue[43,147]. The MAPKs JNK, ERK, and p38 are all potential downstream mediators of IL-1β-mediated insulin resistance. We measured activation/phosphorylation of these proteins in both WT and IL-1β$^{-/-}$ adipose tissue explants treated with LPS and/or atorvastatin (Fig. 3.12). Increased activation of p38 was detected in WT adipose tissue treated with atorvastatin and LPS (Fig. 3.12 A). However, deletion of IL-1β, did not reduce this statin-mediated elevation in p38 activation suggesting that this is not the key intermediate involved in IL-1β-dependent statin-mediated insulin resistance (Fig 3.12 A). A small but signification increase in JNK and ERK activation was seen with LPS alone, but neither of these stress kinases had higher phosphorylation that was exacerbated by statin exposure (Fig. 3.12 B, C). Further, JNK nor ERK phosphorylation by LPS was not decreased in adipose tissue explants from IL-1β$^{-/-}$ mice (Fig. 3.12 B, C).

These results suggest that these MAPKs are not involved in statin-mediated insulin resistance driven by IL-1β in adipose tissue explants.

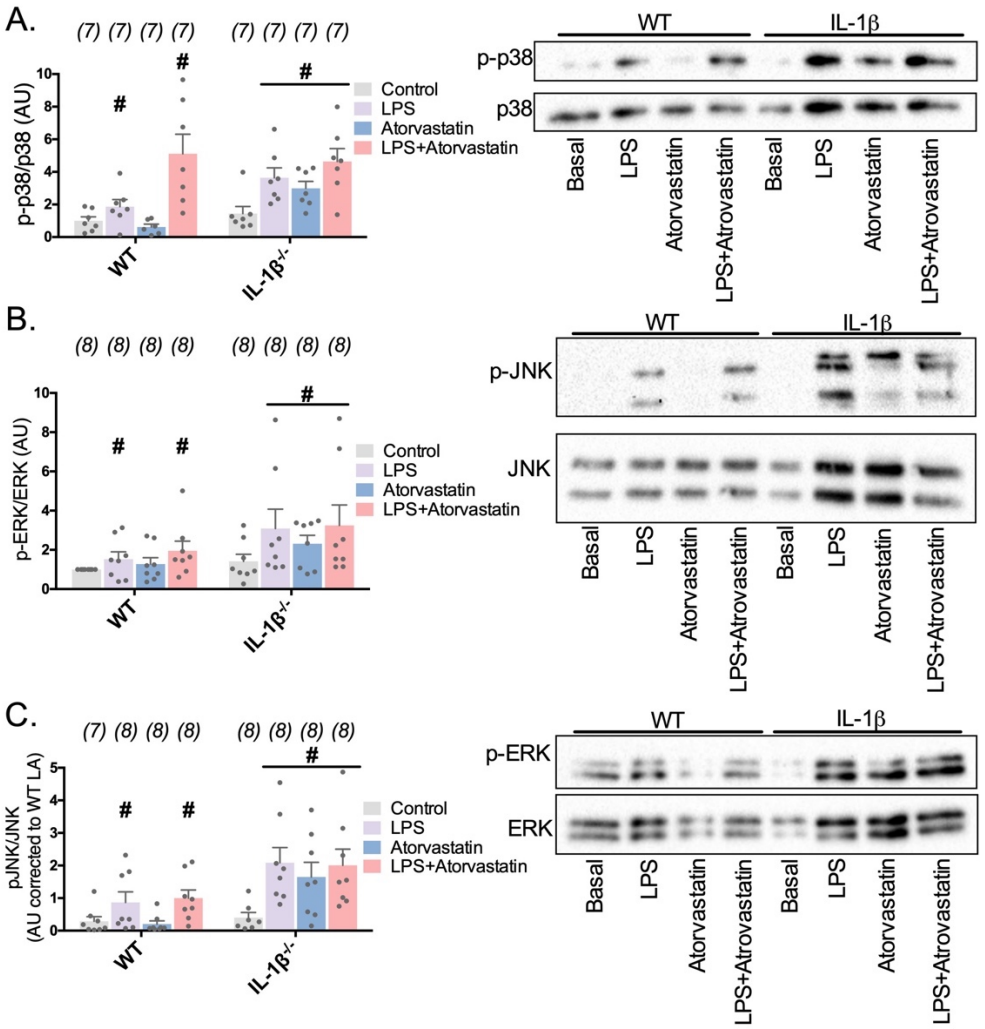

Fig 3.13. Statin-mediated IL-1β does not engage MAPKs to decrease insulin signalling

Adipose tissue explants were treated with LPS (2 ug/mL for the final 4 hours) where indicated. Explants derived from WT C57BL/6J or IL-1$\beta^{-/-}$ mice as indicated. *A-C:* Representative immunoblots (right) and quantification (left) of *(A)* phosphorylated p38 (p-p38), *(B)* phosphorylated ERK (pERK), or *(C)* phosphorylated JNK (pJNK) after treatment of adipose tissue explants with vehicle (control) or atorvastatin (1 μM). Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control tested by one-way ANOVA. AU, arbitrary unit;

3.2.4 iNOS is not required in statin-induced insulin signalling suppression

Previous work has shown inflammasome-derived IL-1β can activate inducible nitric oxide synthase (iNOS)[216,217]. Metabolic stress can increase iNOS activity, leading to tyrosine nitrotrosylation of IRβ, IRS1, and Akt, preventing propagation of the insulin signalling cascade[217]. To test if statin-mediated IL-1β causes decreased insulin action through iNOS activation, adipose tissue explants derived from WT mice were treated with the iNOS inhibitor 1400w. We found that treatment with 500 μM 1400w did not restore statin-mediated lowering of Ser473 phosphorylation of Akt/PKB in LPS-primed adipose tissue explants (Fig 3.14 A).

3.2.5 Rapamycin restores statin-suppression of insulin-mediate pAkt

Since our findings suggest statin-derived IL-1β does not cause decreased insulin signalling through iNOS, stress kinases and MAPKs known to impair insulin signalling, we next examined mTOR. Activation of mTORC1 complex causes activation of S6K and both mTORC1 and S6K can inhibit IRS-1 tyrosine phosphorylation through serine phosphorylation at Ser-632/5 and S265, S302 and S522 respectively[25,218,219]. Inflammatory cytokines such as TNFα and IL-1β can increase

mTORC1 activity[220]. To test if mTORC1 mediates statin-suppression of insulin-signalling, adipose tissue explants from WT mice were primed with LPS and treated with atorvastatin combined with a low dose of rapamycin (20 nM). Low dose rapamycin preferentially inhibits mTORC1 complex, but not mTORC2 signalling, which is required for insulin-mediated Ser473 phosphorylation of Akt/PKB[221]. Treatment of LPS-primed adipose tissue explants with 20 nM rapamycin restored atorvastatin-induced suppression of insulin-mediated Ser473 phosphorylation of Akt/PKB (Fig. 3.15 A). Rapamycin, at this dose, alone did not alter insulin-mediated phosphorylation of Akt/PKB.

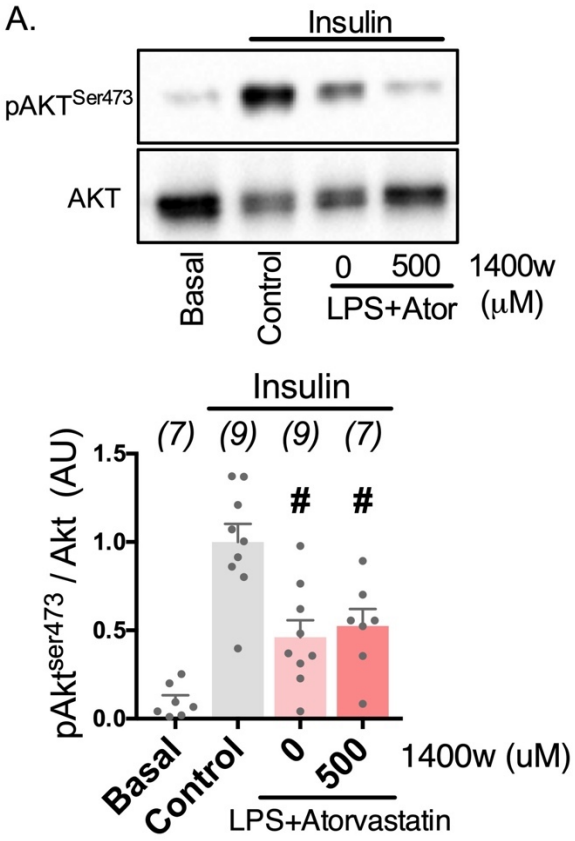

Fig 3.14. iNOS is not required in statin-induced insulin signalling suppression

Adipose tissue explants were from WT mice. *A:* Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated phosphorylated Akt (pAkt) (Ser473) after treatment of adipose tissue explants with vehicle (control) or atorvastatin (1 μM) supplemented with or without the iNOS inhibitor 1400w (500 μM, 18 hours). Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants or wells used in quantification. #Significantly different from control tested by one-way ANOVA. AU, arbitrary unit; Ator, Atorvastatin.

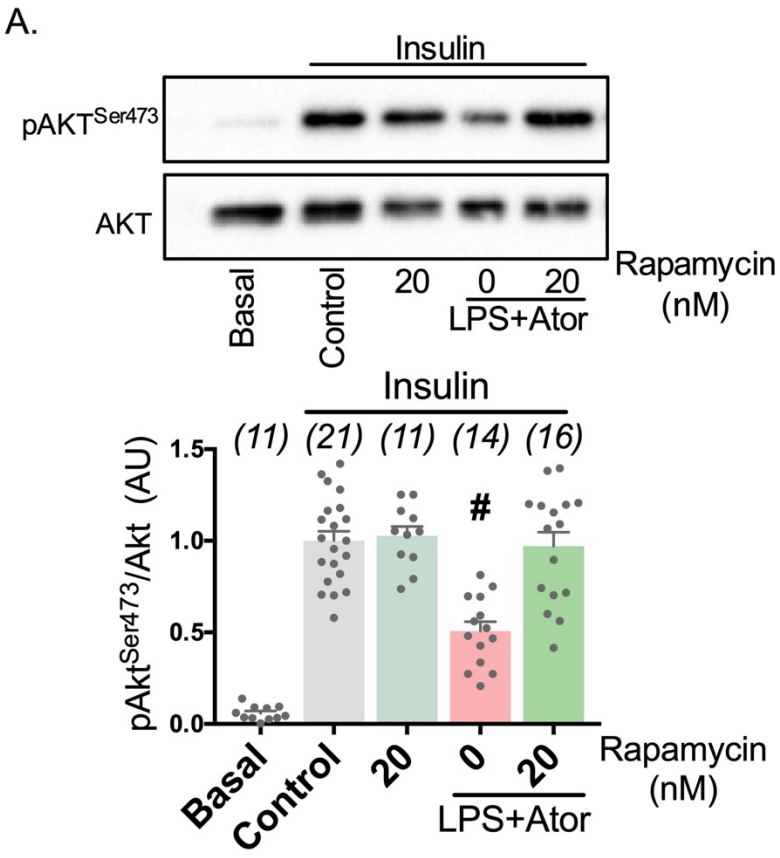

Fig 3.15. Rapamycin restores statin-mediated insulin resistance in adipose tissue

Adipose tissue explants were from WT mice. *A:* Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated phosphorylated Akt (pAkt) (Ser473) after treatment of adipose tissue explants with vehicle (control) or atorvastatin (1 μM) supplemented with or without Rapamycin (20 nM, 18 hours). Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants or wells used in quantification. #Significantly different from control tested by one-way ANOVA. Ator, atorvastatin; AU, arbitrary unit.

3.2.6 p70S6 kinase is not correlated with statin-induced lowering of insulin-stimulated pAkt in adipose tissue

Restoration of statin-suppression of insulin-signalling by rapamycin suggests mTORC1 may be an intermediate between statin-mediate IL-1β and inhibition of insulin signalling. Activation of mTORC1 leads to activation of S6K, which can serine phosphorylate IRS-1 and inhibit insulin-signal transduction. Treatment of LPS primed adipose explants with atorvastatin showed a small increase in phosphorylation of p70S6K (Fig 3.16 A). However, this small increase in phosphorylation of p70S6K was also seen with LPS alone and not exacerbated by exposure to a statin (Fig 3.16 A), suggesting p70S6K is not a key signal involved in statin-mediated insulin resistance.

3.2.7 PTEN is not correlated with statin-induced lowering of insulin-stimulated pAkt in adipose tissue or 3T3-L1 adipocytes

Previous findings have shown mice fed an HFD treated with atorvastatin develop glucose intolerance in adipose tissue and have elevated PTEN protein[222]. PTEN inhibits insulin signalling through conversion of PIP_3 to PIP_2 (opposing role of PI3K, which converts PIP_2 to PIP_3). mTORC1 activation can stimulate activation of PTEN[223]. We measured PTEN expression in both adipose tissue explants and 3T3-L1

adipocytes to understand if PTEN is involved in the mechanism induced by rapamycin/mTORC1 to restore statin-induced insulin resistance. Treatment of LPS-primed adipose tissue with atorvastatin (1 μM, 18h) did not increase PTEN protein abundance compared to controls (Fig. 3.16 B). Similarly, 3T3-L1 adipocytes treated with LPS and atorvastatin showed no change in PTEN protein levels (Fig. 3.16 C), suggesting that changes in the levels of PTEN do not contribute to statin-mediated suppression of insulin signalling in adipocytes.

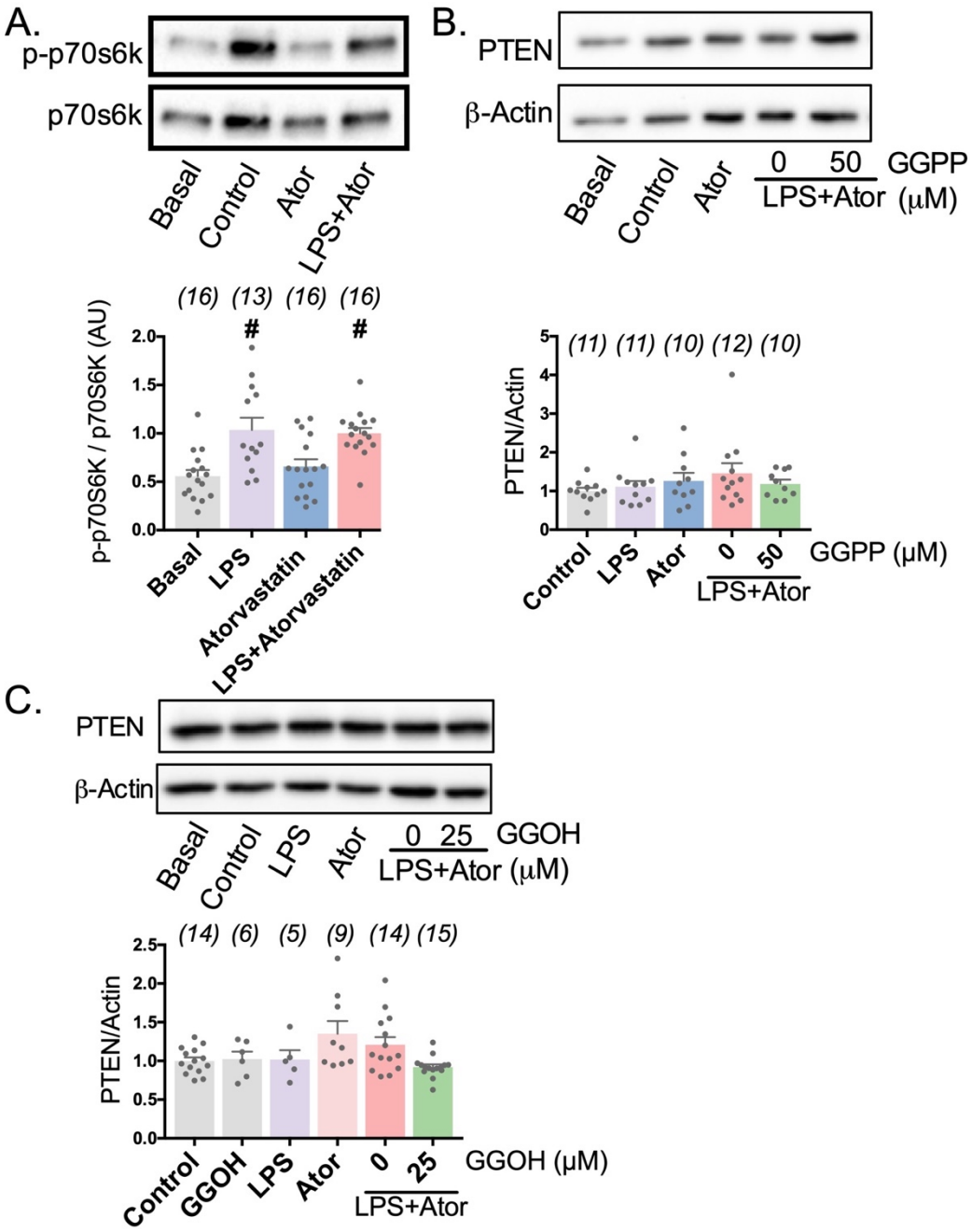
A.
p-p70s6k
p70s6k
Basal
Control
Ator
LPS+Ator
(16) (13) (16) (16)
#
#
p-p70S6K / p70S6K (AU)
2.0
1.5
1.0
0.5
0.0
Basal
LPS
Atorvastatin
LPS+Atorvastatin
B.
PTEN
β-Actin
Basal
Control
Ator
0
50
GGPP
LPS+Ator (μM)
(11) (11) (10) (12) (10)
PTEN/Actin
5
4
3
2
1
0
Control
LPS
Ator
0
50
GGPP (μM)
LPS+Ator
C.
PTEN
β-Actin
Basal
Control
LPS
Ator
0 25
GGOH
LPS+Ator (μM)
(14) (6) (5) (9) (14) (15)
PTEN/Actin
2.5
2.0
1.5
1.0
0.5
0.0
Control
GGOH
LPS
Ator
0
25
GGOH (μM)
LPS+Ator

Fig 3.16. p70S6 kinase and PTEN are not correlated with statin-induced lowering of insulin-stimulated pAkt in adipose tissue
Adipose tissue explants were treated with LPS (2 ug/mL for the final 4 hours) where indicated. *A:* Representative immunoblots (top) and quantification (bottom) of phosphorylated p70S6k/ p70S6K after treatment of adipose tissue explants with vehicle (control) or atorvastatin (1 μM, 18 hours). *B:* Representative immunoblots (top) and quantification (bottom) of PTEN corrected to actin after treatment with vehicle (control) or atorvastatin (1 μM, 18 hours) with GGPP (50 μM, 18 hours). *C:* Representative immunoblots (top) and quantification (bottom) of PTEN in 3T3-L1 adipocytes treated with vehicle (control) or atorvastatin (10 μM, 18 hours) with GGOH (25 μM, 18 hours). Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants or wells used in quantification. #Significantly different from control tested by one-way ANOVA. Ator, atorvastatin; GGPP, Geranylgeranylpyrophosphate; GGOH, Geranylgeraniol; AU, arbitrary unit.

3.3 Statin lowering of protein prenylation activates the NLRP3 inflammasome

3.3.1 Statin-mediated decreased protein prenylation promotes IL-1β secretion in macrophages

Statins decrease important mevalonate pathway metabolites in parallel with reducing cholesterol biosynthesis. Some of these metabolites, such as isoprenoids are critical substrates for protein prenylation, and have proven immunomodulatory properties[162,167,168,170,171]. To assess how statins promoted IL-1β secretion and subsequent inhibition of insulin signalling, we first tested if isoprenoids were involved with inflammasome activation in immune cells. Priming in BMDM experiments was achieved with 4-hour treatment with 200 ng/mL LPS. Fluvastatin (1 μM) and LPS in combination increased IL-1β secretion. Addition of the isoprenoid intermediate GGPP (10 μM) to BMDMs treated with fluvastatin significantly decreased IL-1β section (Fig. 3.17 A) ($P < 0.05$). GGPP and LPS treatments alone did not change the level of IL-1β secreted from BMDMs. These data show that providing exogenous isoprenoids prevents statin-mediated activation the NLRP3 inflammasome to produce IL-1β.

3.3.2 Statin-mediated mitochondrial ROS promotes IL-1β secretion in macrophages

Although the mechanisms of activation of the NLRP3 inflammasome is not fully understood, evidence suggests one possible mechanism is through production of mtROS. Fluvastatin treated BMDMs further treated with the mitochondrial reactive oxygen species scavenger MitoTempo showed fluvastatin-induced IL-1β secretion was decreased by 1 μM MitoTempo in BMDMs (Fig. 3.17 B). These data suggest statins activate the inflammasome through a mitochondrial ROS-related mechanism, which may be linked to a reduction of the isoprenoid GGPP. The connection between mitochondrial ROS and isoprenoids warrants formal testing.

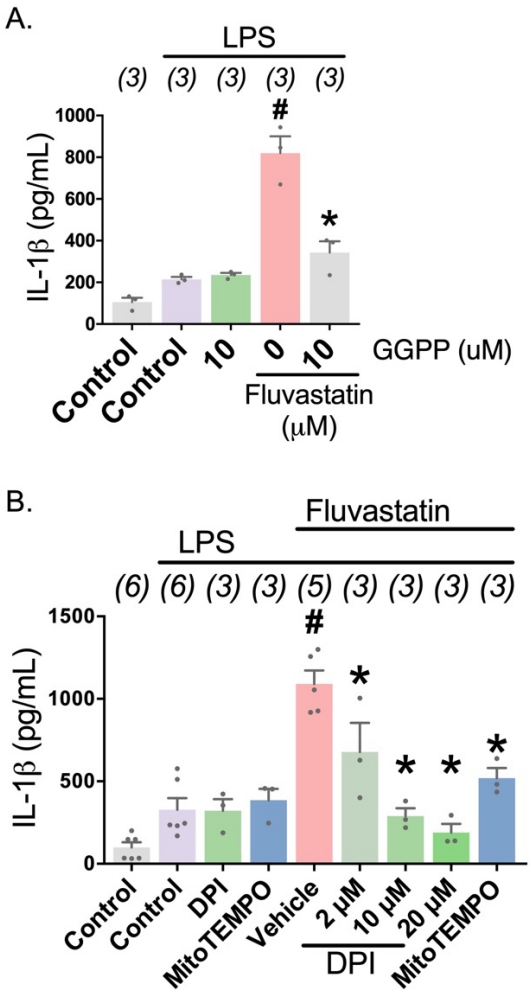

Fig 3.17. Statin-mediated decreased protein prenylation and mitochondrial ROS promotes IL-1β secretion in macrophages. BMDM derived from WT C57BL/6J mice were treated with LPS (200 ng/mL for a final 4 hours) where indicated. *A:* BMDM were treated with vehicle (control) or fluvastatin (1 μM, 18 hours) with or without GGPP (5 μM, 18 hours) and IL-1β in media was quantified. *B:* BMDM were treated with vehicle (control) or fluvastatin (1 μM, 18 hours) with or without either MitoTEMPO (18 hours) or DPI (indicated dose, 18 hours) and IL-1β in media was quantified. Each value from a given well and mean ± SEM is shown. The number above each experimental condition indicates the number of individual wells treated and quantified. #Significantly different from Control, *Different from LPS + fluvastatin. DPI, diphenyleneiodonium.

3.3.3 Providing isoprenoids required for protein prenylation, but not cholesterol or 25-HC restores statin-mediated insulin-resistance in adipose tissue

Statins lower cholesterol and isoprenoids in multiple cell types. We next tested if lowering cholesterol metabolites or isoprenoids underpinned statin-mediated insulin resistance in adipose tissue. We found that supplementation with LDL-cholesterol (0.01 and 1 mg/ml) or free cholesterol (1 and 20 μM) did not promote a further reduction (and did not restore) statin-mediated lowering of Ser473 phosphorylation of Akt/PKB in LPS-primed adipose explants derived from WT mice (Fig. 3.18 A). LDL cholesterol treatment alone (1 mg/mL) lowered insulin signalling, independently of LPS priming or statin treatment (Fig 3.18 A) ($P < 0.05$). This is an important finding since it shows that higher LDL cholesterol can promote insulin resistance through a pathway separate from statins and protein prenylation-linked to the NLRP3 inflammasome. It was known that lower 25-HC can lead to activation of caspse-1 and increased IL-1β in macrophages[224]. However, we found that supplementation of LPS-primed adipose tissue explants with 25-HC (1 and 20 μM) did not restore impaired insulin signalling due to atorvastatin exposure in adipose tissue explants (Fig 3.18 B). Conversely, supplementation of LPS-primed adipose tissue explants with the isoprenoid geranylgeranyl pyrophosphate (GGPP) at 50

μM (but not 5 μM) restored atorvastatin-induced suppression of insulin-stimulated phosphorylation of Akt/PKB at two activation sites (Ser473 and Thr308) (Fig. 3.19 A, B). These data show that a statin-mediated reduction in a geranylgeranyl isoprenoid is required for impaired insulin signalling, which occurs independently of cholesterol lowering in adipose tissue.

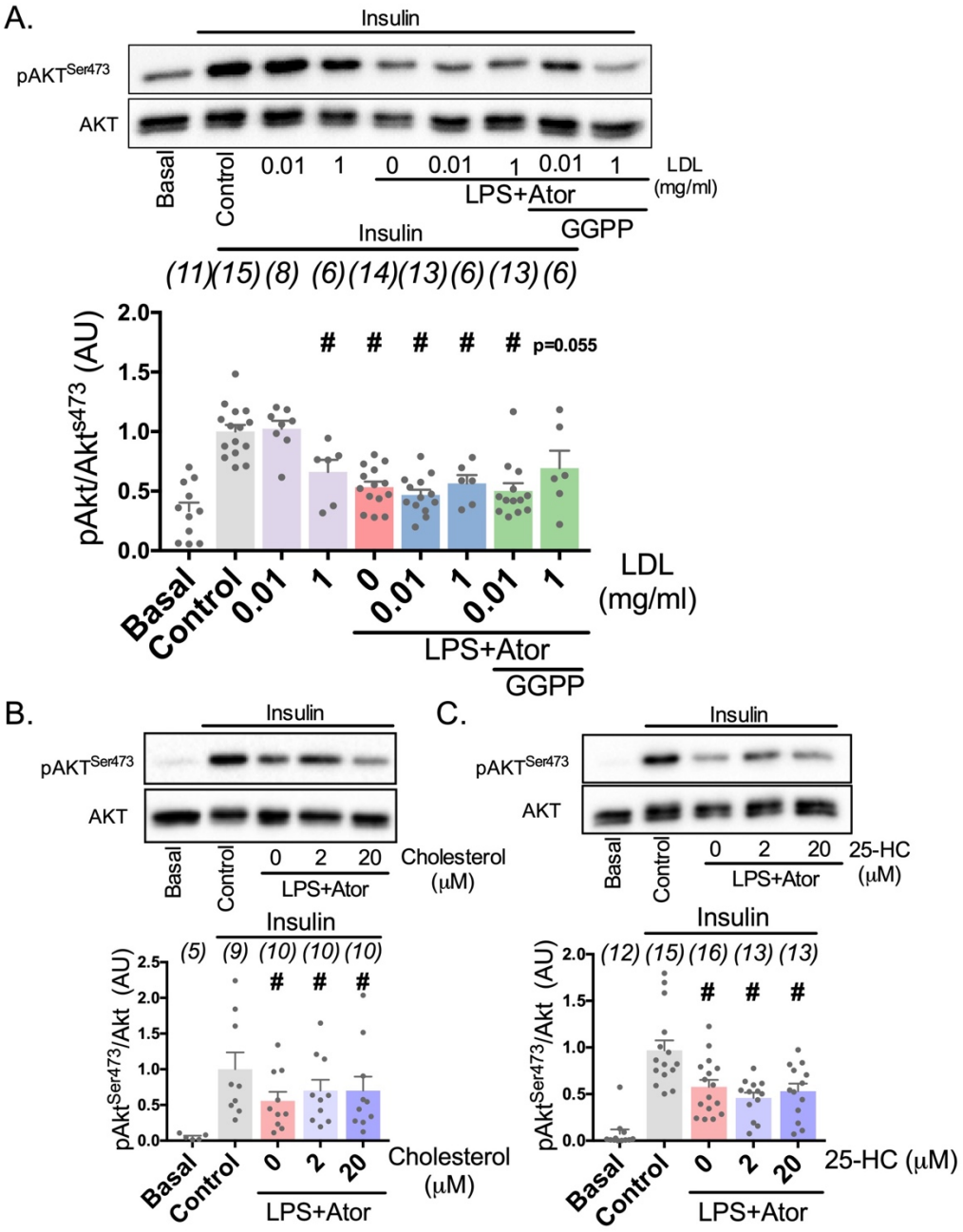
A.
Insulin
pAKTSer473
AKT
Basal
Control
0.01
1
0
0.01
1
0.01
1
LDL (mg/ml)
LPS+Ator
GGPP
Insulin
(11)(15)(8) (6)(14)(13)(6)(13)(6)
pAkt/AktS473 (AU)
2.0
1.5
1.0
0.5
0.0
p=0.055
Basal
Control
0.01
1
0
0.01
1
0.01
1
LDL (mg/ml)
LPS+Ator
GGPP
B.
Insulin
pAKTSer473
AKT
Basal
Control
0
2
20
Cholesterol (μM)
LPS+Ator
Insulin
(5) (9) (10) (10) (10)
pAktSer473/Akt (AU)
2.5
2.0
1.5
1.0
0.5
0.0
# # #
Basal
Control
0
2
20
Cholesterol (μM)
LPS+Ator
C.
Insulin
pAKTSer473
AKT
Basal
Control
0
2
20
25-HC (μM)
LPS+Ator
Insulin
(12) (15) (16) (13) (13)
pAktSer473/Akt (AU)
2.0
1.5
1.0
0.5
0.0
# # #
Basal
Control
0
2
20
25-HC (μM)
LPS+Ator

Fig 3.18. Providing cholesterol does not mitigate statin-induced insulin resistance in adipose tissue.
Adipose tissue explants were from WT C57BL/6J mice and treated with LPS (2 μg/mL for a final 4 hours) where indicated. *A*–*C*: Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated phosphorylated Akt (pAkt) (Ser473) after treatment of adipose tissue explants with vehicle (control) or atorvastatin (1 μM, 18 hours) with and without LDL cholesterol (A), free cholesterol (B), or 25-Hydroxycholesterol (25-HC, C) at the dose indicated. *A:* LDL cholesterol–treated explants were also treated in combination with GGPP (50 μM, 22 hours). Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control insulin stimulated, tested by one-way ANOVA. Ator, atorvastatin; AU, arbitrary unit.

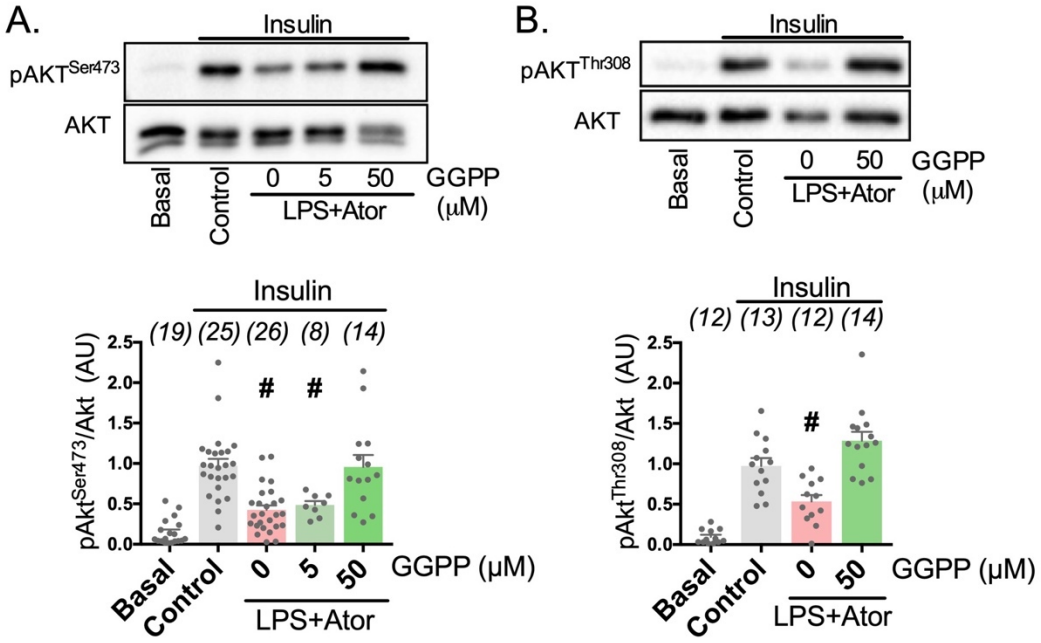

Fig 3.19. Providing isoprenoids, mitigates statin-induced insulin resistance in adipose tissue.
Adipose tissue explants were from WT C57BL/6J mice and treated with LPS (2 μg/mL for the final 4 hours) where indicated. *B and C:* Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated pAkt at Ser473 and Thr308 after treatment of adipose tissue explants with atorvastatin (1 μM, 18 hours) plus supplementation with and without GGPP at the dose indicated. Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control insulin stimulated, tested by one-way ANOVA. Ator, atorvastatin; AU, arbitrary unit.

3.3.4 Inhibition of GGTase-I is sufficient to impair insulin signalling in adipose tissue

Given that statin-mediated insulin-resistance in adipose tissue explants is prevented with the isoprenoid, GGPP, we next tested if inhibiting the transferase responsible for attaching GGPP to proteins was sufficient to impair insulin signalling. The transferase GGTase-I prenylates proteins with a CAAX motif on the cysteine residue with GGPP. Treatment of primed LPS-primed adipose tissue explants with GGTI-298 (GGTase inhibitor, 10 μM) lowered insulin-stimulated Ser473 phosphorylation of Akt/PKB (Fig. 3.20 A).

3.3.5 Activation of MAPKs do not correlate with statin-mediated decreases in protein prenylation or insulin signalling

The MAPKs JNK, ERK, and p38 are all potential downstream mediators of IL-1β-mediated insulin resistance. Given that statin-mediated insulin-resistance requires IL-1β is dependent on reduction of the isoprenoid GGPP, we next tested activation (i.e. phosphorylation) of the stress kinases JNK, ERK, and p38 in adipose tissue explants primed with LPS and treated with atorvastatin (10 μM) and GGPP (50 μM) (Fig. 3.21). LPS alone promoted phosphorylation of JNK and ERK (Fig. 3.21 B, C). LPS also increase phosphorylation of p38, which was further elevated with LPS + atorvastatin in adipose explants (Fig. 3.21 A). Although p38 activation was

increased with the combinations LPS and atorvastatin, no reduction in the phosphorylation of p38 was evident with addition of GGPP (Fig. 3.21 A). Overall, this data shows that the phosphorylation of MAPKs (JNK, ERK, p38) do not correlate with the ability of statins to impair insulin signalling via lower prenylation in adipose tissue.

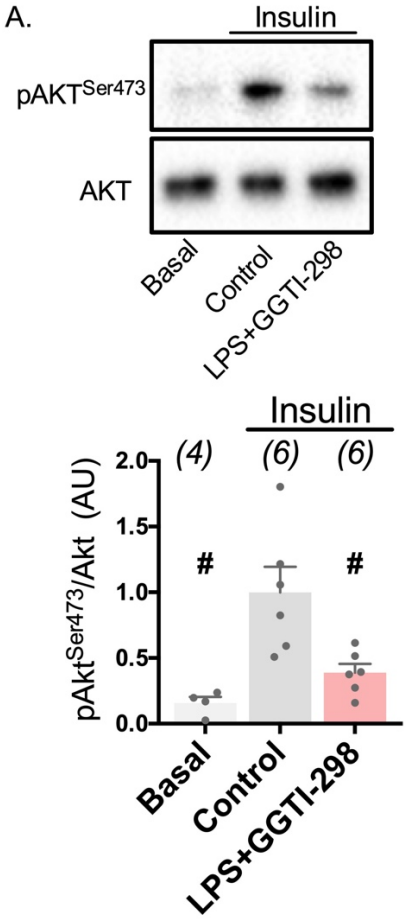

Fig 3.20. GGTase I inhibitor promotes insulin resistance in adipose tissue

Adipose tissue explants were from WT C57BL/6J mice and treated with LPS (2 μg/mL for a final 4 hours) where indicated. *A:* Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated pAkt at Ser473 and Thr308 after treatment of adipose tissue explants with GGTI-298 inhibitor (10 μM, 18 hours). Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control insulin stimulated, tested by one-way ANOVA. Ator, atorvastatin; AU, arbitrary unit.

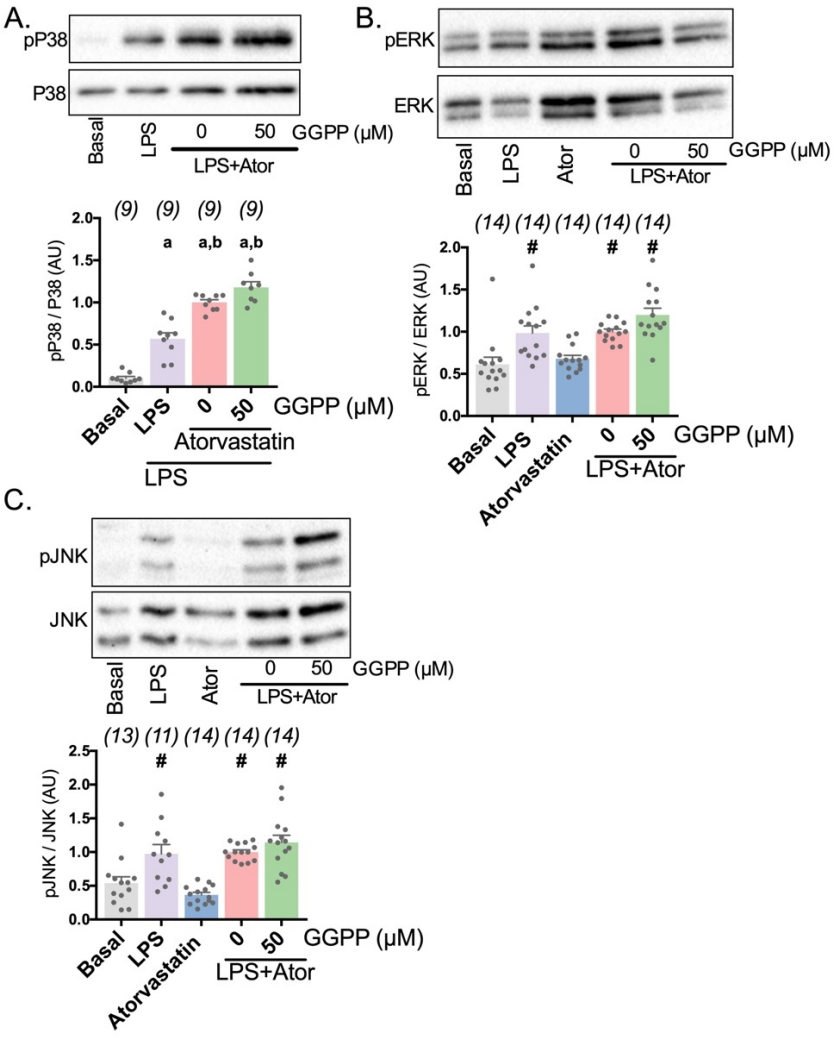

Fig 3.21. Activation of MAPKs do not correlate with isoprenoid-mediated restoration of insulin resistance caused by statins.
Adipose tissue explants were treated with LPS (2 ug/mL for the final 4 hours) where indicated. Explants derived from WT C57BL/6J mice. *A-C:* Representative immunoblots (top) and quantification (bottom) of *(A)* phosphorylated p38 (p-p38), *(B)* phosphorylated ERK1 (pERK), or *(C)* phosphorylated JNK (pJNK) after treatment of adipose tissue explants with vehicle (control) or atorvastatin (1 μM) with or without GGPP (50 μM, 18 hours). Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control tested by one-way ANOVA. a- difference from control. b- different from LPS alone. AU, arbitrary unit.

3.3.6 Geranylgeranylation but not farnesylation rescues statin-mediated insulin-resistance in 3T3-L1 adipocytes

Our previous results indicate that treatment of adipose tissue explants with the isoprenoid GGPP, but not cholesterol rescued statin-mediated insulin resistance in adipose tissue. Furthermore, cholesterol content of 3T3-L1 adipocytes did not change during treatment with atorvastatin (Fig. 3.22 A). We next tested if statin could induce cell-autonomous insulin resistance in adipocytes via lower prenylation. Atorvastatin lowered insulin-stimulated Ser473 phosphorylation of Akt/PKB in 3T3-L1 adipocytes and supplementation with the isoprenoid geranylgeraniol (GGOH) (25 μM) restored this aspect of insulin signalling (Fig. 3.22 B). Supplementation with farnesol (FOH) did not restore atorvastatin-mediated lowering of insulin-stimulated Ser473 phosphorylation of Akt/PKB in 3T3-L1 adipocytes (Fig. 3.22 C).

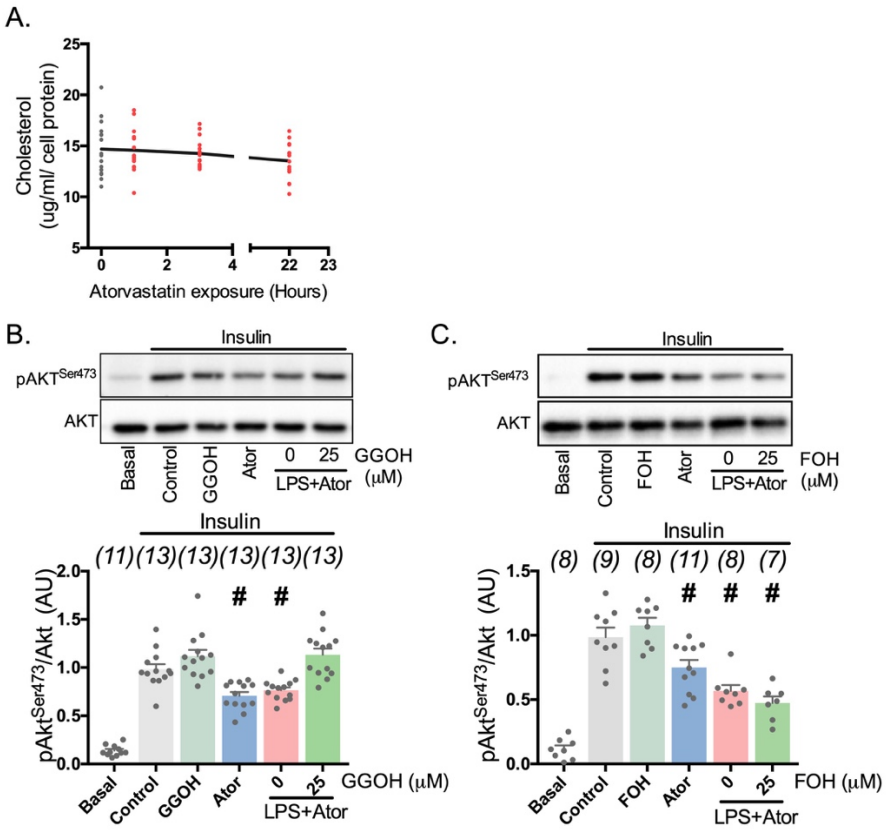

Fig 3.22. Providing isoprenoids required for geranylgeranylation but not farnesylation rescues statin-mediated insulin-resistance in 3T3-L1 adipocytes.

A: Cholesterol concentration of 3T3-L1 adipocytes after treatment with atorvastatin (10 μM) for 1, 3, and 22 hours. *B and C:* Representative immunoblots (top) and quantification (bottom) of basal (i.e., no insulin) and insulin-mediated phosphorylated Akt (pAkt)/PKB (Ser473) after treatment of 3T3-L1 adipocytes with atorvastatin (10 μM, 18 hours), LPS (2 μg/mL), and/or GGOH (25 μM, 18 hours) or farnesol (FOH) (25 μM, 18 hours). Each value from a given explant and mean ± SEM is shown. The number above each experimental condition indicates the number of adipose tissue explants used in quantification. #Significantly different from control insulin stimulated, tested by one-way ANOVA. Ator, atorvastatin; AU, arbitrary unit.

3.3.7 Statins do not alter insulin-stimulated glucose uptake in 3T3-L1 adipocytes

We next tested if statin-mediated changes in insulin signalling corresponded to changes in the cellular function of insulin in adipocytes. We tested if basal or insulin-stimulated glucose uptake into 3T3-L1 adipocytes is altered in the presence of atorvastatin with and without GGOH. We found no difference in basal or insulin-stimulated glucose uptake at a low or high dose of insulin (0.3 nM and 10 nM insulin) (Fig 3.23 A, B).

3.3.8 Statins lower insulin-stimulated lipogenesis via lower isoprenoids in adipocytes

Insulin is known to alter adipocyte lipid metabolism by suppressing lipolysis and stimulating lipogenesis[126,127,225]. We next tested if either of these functions of insulin are correlated with atorvastatin-suppression of insulin signalling and lowering of protein prenylation. Atorvastatin did not alter lipolysis (with or without isoproterenol) in 3T3-L1 adipocytes (Fig 3.7 A). However, insulin-stimulated lipogenesis was decreased by atorvastatin, an effect that was not observed when 3T3-L1 adipocytes were supplemented with 25 μM GGOH (Fig. 3.24 A).

Taken together, the findings of this thesis show that statin-induced adipocyte insulin-resistance occurs via activation of the NLRP3 inflammasome and one of this inflammasomes effectors IL-1β. Statins

activate the inflammasome through decreased protein prenylation, and activation of the inflammasome results in decreased insulin-stimulated lipogenesis in adipose tissue.

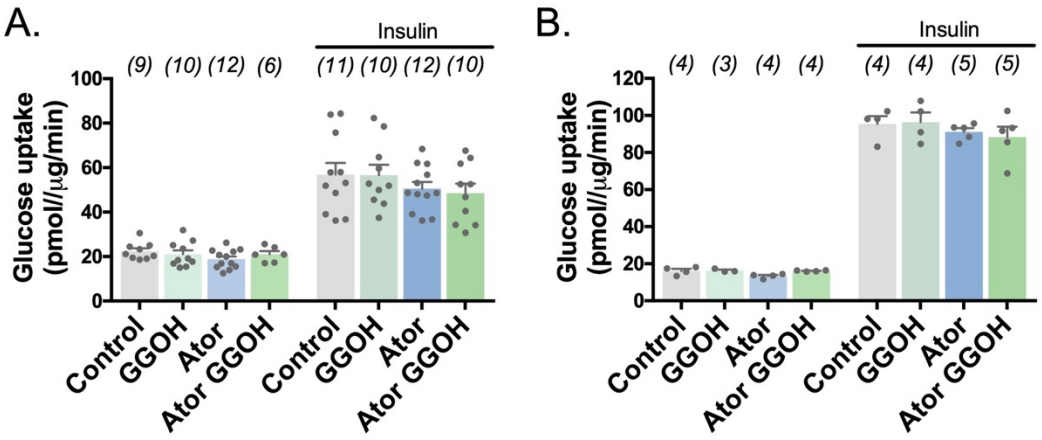

Fig 3.23. Statins do not alter glucose uptake in 3T3-L1 adipocytes. *A, B:* Glucose uptake was determined in 3T3-L1 adipocytes following treatment with atorvastatin (10 μM) and/or GGOH (25 μM) for 18 hours before the addition of insulin at *(A)* (0.3 nM, 20 min) or *(B)* (10 nM, 20 minutes) and [^{3}H]-2-deoxyglucose (0.5 μCi/mL) uptake for 5 minutes. Data are mean ± SEM. The number above each experimental condition indicates the number of wells used to quantify. Significance was test by one-way ANOVA. Ator, atorvastatin; GGOH, Geranylgeraniol,

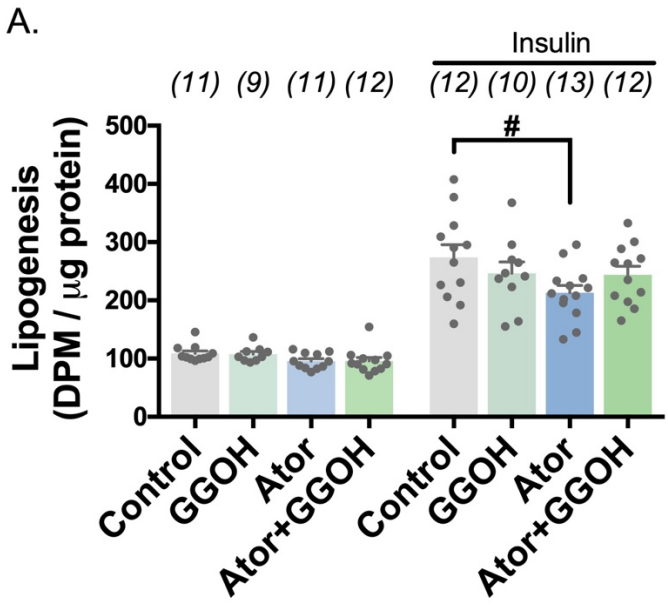

Fig 3.24. Statins decrease insulin-stimulated lipogenesis in 3T3-L1 adipocytes.

A: 3T3-L1 adipocytes were exposed to atorvastatin (10 μM, 18 hours) supplemented with or without GGOH (25 μM) for 18 hours followed by the addition of [14C]-U-glucose (1 μCi/mL) ± insulin (0.3 nM) for 1 h, and lipogenesis was quantified using the radiolabeled lipid pool. Data are mean ± SEM. The number above each experimental condition indicates the number of wells used to quantify. #Significant from control insulin stimulated tested by one-way ANOVA. Ator, atorvastatin; DPM, disintegrations per minute; GGOH, Geranylgeraniol.

Chapter Four: Discussion

4.1 Introduction

Statins are one of the most widely prescribed drugs, used for the treatment of cardiovascular disease. Statins lower circulating LDL cholesterol, by lowering cholesterol production in the liver, and increasing uptake of LDL particles from circulation. However, statins have been shown to have a multitude of pleotropic effects in multiple tissues, which can occur independent of cholesterol lowering. These changes can have beneficial effects, although they are potentially the cause for the side effects observed in patients taking statins. The findings of this thesis show that statins decrease protein prenylation and activate the NLRP3 inflammasome resulting in IL-1β-mediated insulin resistance, which decreases the ability of insulin to promote lipogenesis in adipose tissue.

4.2 Statins activate the inflammasome

4.2.1 Statins activate the inflammasome in immune cells

Statins have recently been shown to increase the risk of developing type 2 diabetes and insulin resistance in a small portion of patients[226]. The causal link is poorly understood and somewhat paradoxical considering statins generally improve circulating lipid profiles in patients, which is an important factor in development and consequences of type 2 diabetes[227]. Previous findings had demonstrated that statins can stimulate IL-1β release from THP-1 and human monocytes and that a priming factor (such

as LPS or bacteria) was required[171,173,228]. We confirm that statins increased IL-1β secretion in adequately primed macrophages, and we demonstrated the requirement of the NLRP3 inflammasome[213]. All statins tested activated NLRP3-mediated increases in IL-1β, which is similar to the class effect of these HMG-CoA reductase inhibitors increasing the risk of diabetes, independently of potency or lipophilic properties[229]. Deletion of the NLRP3 inflammasome and inhibition of caspase-1 both reduced fluvastatin-mediated IL-1β secretion. Similar findings show this to be a mevalonate pathway effect as LPS primed RAW .264 macrophages treated with bisphosphonates also increase IL-1β in a dose dependent manner[230–232]. Bisphosphonates are prescribed to treat osteoporosis and prevent bone loss. They inhibit farnesyl-pyrophosphate synthase, which in the mevalonate pathway is distal to HMG-CoA reductase, but bisphosphonates still inhibit isoprenoids required for protein prenylation (Figure 1.3).

The diabetic drug glyburide, is also an NLRP3 inflammasome inhibitor[233]. Interestingly, glyburide prevented fluvastatin-induced IL-1β secretion from LPS primed macrophages. Assessing if patients taking statins that are currently on glyburide have a lower risk of developing type 2 diabetes should be of interest.

In patients taking statins, depending on the pharmacokinetics, the circulating concentrations are typically in the nanomolar range, although they can be as high as 1 μM[161,234,235]. Fluvastatin has been measured in the micromolar range in human serum after a standard dose and our data suggests this concentration is sufficient to stimulate IL-1β secretion from LPS primed macrophages[234]. Importantly, we did not observe overt cell death from the tested concentrations of fluvastatin in our experiments using BMDMs. Signals from dying cells can be activators of the NLRP3 inflammasome and although we cannot absolutely rule out any cell death mediators in our experiments, we found no change in BMDM viability[236–238].

4.2.2 Statins activate the inflammasome in adipose and cause insulin resistance

Our next point of interest to test was if statins can activate the inflammasome in white adipose tissue. We hypothesized that cell type was a key determinant of how statins engage the NLRP3 inflammasome. Others have shown in immune cells, statins can alternatively activate the PYRIN inflammasome to cause IL-1β secretion[239]. In adipose tissue, transcript analysis of the SVF and adipocytes showed only LPS increased levels of NLRP3 and not levels of pyrin. These data suggested that the NLRP3 inflammasome regulates statin-induced capase-1 activation in

adipose tissue. Statin treatment of white adipose tissue predominately increased IL-1β secretion in the stromal vascular fraction (SVF), however statin treatment also increased IL-1β production by 2-fold in the adipocytes. This aligns with other findings that the predominate source of IL-1β in adipose tissue is generated from resident immune cells[240]. This also set the stage for testing adipocyte-autonomous actions of statins. First, we probed the activation of the key NLRP3 inflammasome component, caspase-1. Statin treatment increased caspase-1 activity in whole adipose tissue lysates and was inhibited by glyburide and NLRP3 deletion. Therefore, statins activated caspase-1 in an NLRP3-depdent manner in adipose tissue.

We next tested how statins and other inhibitors of the mevalonate pathway influenced insulin resistance in adipose tissue. Activation of the inflammasome has been implicated in insulin resistance[31,86,138]. Since statins can activate the inflammasome in both immune cells and adipose tissue, we tested if this NLRP3 inflammasome activation promoted insulin resistance. All statins that we tested showed decrease insulin signalling in adipose tissue explants. Cerivastatin was removed from the market because of side effects. Our results show that cerivastatin was the most potent statin in promoting adipose insulin resistance and the most potent activator of IL-1β release from BMDMs. The bisphosphonate

Zoledronate also caused decreased insulin signalling in primed adipose tissue explants in a dose dependent manner. Taken together these suggest a drug class effect and mevalonate pathway effect on insulin action. Deletion of NLRP3 and inflammasome inhibition with glyburide prevented statin-induced insulin resistance in adipose tissue, suggesting this effect is NLRP3 inflammasome dependent.

Interestingly, there does not seem to be an association of increased incidence of type 2 diabetes with patients taking bisphosphonates. This may occur because bisphosphonates have a high affinity for bone tissue[241]. Oral administration of bisphosphonates result in a poor absorption (<2%), and are often delivered through intravenous (IV) injection, monthly or even yearly[242–244]. Though other tissues such as adipose, may have brief acute exposures to bisphosphonates, this is unlikely enough exposure to alter mevalonate metabolism in any tissue other than bone.

Our *ex-vivo* adipose tissue explant model tested inflammasome activation using lean gonadal adipose tissue. During obesity, adipose tissue undergoes hypertrophy, with increased lipid loading occurring to mature adipocytes and pre-adipocytes differentiate to increase the number of adipocytes able to store lipids[108,109]. These adipocytes are more pro-inflammatory. This expansion also stimulates macrophage recruitment and

infiltration, increasing the number of immune cells present[245]. Further testing of inflammasome activation should be done by treating adipose tissue explants derived from HF fed obese or ob/ob mice to test if obesity further exacerbates statin-induced inflammasome activation and IL-1β secretion. Interestingly, patients with familial hypercholesteremia on lifelong statins can have a low prevalence of type 2 diabetes as opposed to patients who are obese or with metabolic disease in whom stains can increase the risk of type 2 diabetes[246]. Only a small portion (~9%) of patients taking statins develop new onset type 2 diabetes[179]. The inflammatory state and cell composition of the adipose tissue of each patient taking a statin, may be a factor influencing the risk of developing type 2 diabetes.

Adipocytes residing in the adipose tissue explants treated with atorvastatin had increased levels of IL-1β. However, this test does not discriminate the source of increased levels of IL-1β and if there is an adipocyte-autonomous response to statins. To test if adipocytes alone are sufficient to observe statin-induced insulin resistance, we used 3T3-L1 adipocytes. Fluvastatin increased caspase-1 activation in 3T3-L1 adipocytes, however, interestingly we did not detect increased IL-1β secretion. Fluvastatin and atorvastatin did however cause decreased insulin signalling in 3T3-L1 adipocytes. It is possible the amount of IL-1β

secreted or present at that time point was below detection and warrants further testing. Increased caspase-1 activity though is still important as it can dictate insulin-sensitivity in adipocytes[148]. Inflammasome-caspase-1 activation during pre-adipocyte differentiation was shown to result in a more insulin resistant mature adipocyte[148]. Testing if the presence of statins during 3T3-L1 adipocyte differentiation causes insulin resistance should be of interest and could suggest one mechanism underlying the connection between long-term statin consumption and increased risk of new onset T2D.

4.3 IL-1β is required for statin-induced insulin resistance

Activation of the inflammasome results in cleavage and secretion of the IL-1β and IL-18. Caspase-1 also has other targets such as IL-33, and GAPDH[74]. This cleavage may activate or inhibit the activity of the target protein. Since statin-induced insulin resistance is dependent on inflammasome activation, we sought to determine what downstream effectors from the inflammasome were required. Then we attempted to understand the cellular mediators of this effector that link to statin-induced insulin resistance. Finally, we sought to determine the functional outcome of this decreased insulin signalling in adipocytes.

4.3.1 Caspase-1 and IL-1β are required for statin-induced insulin resistance

Activation of the NLRP3 inflammasome results in caspase-1 activation and activation of IL-1β, IL-18, IL-33 and potentially other proteins that could be activated or inacatived[247]. Each of these proteins have complex downstream signalling pathways. We first tested the next step in the inflammasome pathway following NLRP3 activation, caspase-1 activation. Our results showed inhibition of caspase-1 activity with z-YVAD restored insulin signalling in LPS primed adipose tissue treated with atorvastatin. This result solidified that caspase-1 was involved in statin-mediated insulin resistance, but the effector was still unknown. Next we found that genetic deletion of IL-1β restored insulin signalling when adipose tissue was exposed to one of several different statins, but IL-18 was dispensable. IL-1$\beta^{-/-}$ also showed full restoration of insulin signalling to levels comparable to controls, which is similar to our findings with adipose tissue derived from NLRP3$^{-/-}$ mice. This suggests that IL-1β is the key effector of NLRP3/Caspase-1 inflammasome during statin-induced insulin resistance in adipose tissue. Interrogation of all of the results in adipose tissue explants shows that statin treatment alone can moderately lower insulin signalling. This suggests this adipose tissue explant model may have some self-priming of the inflammasome, possibly with lipids, that enables statin alone to cause some decreased insulin signalling.

Importantly, when either NLRP3 or IL-1β are not present, fluvastatin and atorvastatin alone did not cause this mild insulin resistance.

Recent interest in IL-1β and its potential link to atherosclerosis and diabetes has resulted in development of antibodies to inhibit circulating IL-1β. In rodents, inhibition of IL-1β with antibody therapy improved insulin sensitivity in diet induced obese mice[248]. Treatment of diabetic KK-A^y mice with IL-1β antibody, improved insulin sensitivity, beta-cell mass and insulin secretion, and reduced circulating free FA[249]. Early human trial evidence with the IL-1β antibody therapy anakinra, showed a small but significant reduction in glycated hemoglobin (HbA1C) after 13 weeks of treatment[250]. The completion of the Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) trial demonstrated a causal link between IL-1β and atherosclerosis, as well as lung cancer[251,252]. However, no improvement was observed in HbA1C or insulin sensitivity following Canakinumab long term treatment[253]. Statins are pervasive in these clinical trials and should be considered as a confounding variable when assessing the potential glycemic control improvement with IL-1β antibody therapy.

We confirmed that IL-18 was not a mediator of statin-induced insulin resistance in adipose tissue using mice that had Il-18 deleted. This

further supports the key role played by IL-1β and argues against a widespread immune effect of statins on cytokines that could cause insulin resistance. To further test if IL-18 was required for statin-mediated insulin resistance we also tested adipose tissue explants derived from mice that have the IL-18 receptor (IL-18r) deleted. Interestingly, insulin signalling in adipose tissue from IL-18r$^{-/-}$ was completely supressed with LPS treatment and no additional synergy with atorvastatin could be observed. It is currently not fully understood why IL-18$^{-/-}$ and IL-18r$^{-/-}$ displayed differences in LPS sensitivity, but this result does align with published reports showing that IL-18 is an important cytokine for maintaining immune homeostasis. It is possible that the IL18 receptor has a broader role or additional ligands beyond the IL-18 cytokine. Deletion of IL-18 is sufficient to promote insulin resistance in diet induced obesity, contrary to the restoration of insulin sensitivity reported when IL-1β$^{-/-}$ mice are subjected to diet induced obesity[254–257]. One mechanism proposed linking IL-18 to insulin sensitivity is through the activation of AMPK[256]. IL-18 has been shown to activate AMPK. Activation of AMPK can inhibit the inflammasome through modulation of mitophagy and ER stress and limit inflammation[258–260]. Further investigation of the increased LPS sensitivity in IL-18r$^{-/-}$ resulting in insulin inhibition and linking to AMPK activity should be of interest.

4.3.2 Functional outcomes of statin-induced insulin resistance in adipose tissue

NLRP3 inflammasome derived IL-1β has been implicated in obesity driven insulin resistance[31,138]. IL-1β can reduce insulin sensitivity in adipocytes, cause decreased insulin-stimulated glucose uptake, and inhibit insulin-stimulated lipogenesis[43,261,262]. However, it was not known how NLRP3 inflammasome derived IL-1β due to statin exposure altered adipocyte function. Consistent with our data showing statins induce IL-1β secretion and insulin resistance, we found atorvastatin impaired insulin-stimulated lipogenesis in both white adipose tissue and in a cell-autonomous manner in 3T3-L1 adipocytes. However, we did not see differences in basal or stimulated lipolysis or a difference in glucose uptake in 3T3-L1 adipocytes. It is not yet clear how statin-mediated impaired lipogenesis is linked to changes in glycemia and risk of new onset T2D.

Fluvastatin supplementation of low-fat (i.e. chow diet) fed mice showed no worsening of glycemia or glucose tolerance/control. Interestingly, fluvastatin supplementation of genetically obese (ob/ob) mice worsened glucose intolerance and impaired insulin-stimulated glucose disposal in white adipose tissue, indicating insulin resistance within this tissue. These results may be associated with the NLRP3 priming triggers

during obesity in ob/ob mice. It is possible that *in-vivo* effects of statins require a priming signal for the inflammasome, but we have not identified the priming signal required in our experiments. Ob/ob mice have ten-fold higher circulated endotoxemia, and diet induced obesity increases components of the inflammasome[52,138]. The observed difference *in vitro* and in statin-induced glucose intolerance in ob/ob mice may suggest the requirement cross-talk between multiple cell types. Further it could also require exposure and alterations to both mature and preadipocytes. Measuring circulating free FA and insulin-stimulated lipogenesis in the statin supplemented ob/ob model is warranted in the future in order to know if statin-suppression of insulin-stimulated lipogenesis occurs in vivo. Our results suggest that impaired adipocyte lipogenesis underpins the relationship between lipids and glucose during statin treatment because higher blood triglycerides are a known risk factor predicting statin-induced diabetes[263]. Furthermore, those that have more diabetic risk factors when starting statin therapy, defined by higher BMI, elevated circulating triglycerides, increased fasting blood glucose and HbA1c, are those at greater risk of statin-new onset diabetes[264]. Understanding the connection between lower lipogenesis and statin-mediated risk of new onset T2D is warranted.

4.3.3 mTORC1 may mediate statin-induced IL-1β insulin-resistance in adipose tissue

We showed that statin-induced insulin resistance in adipose tissue is dependent on IL-1β. The IL-1β signalling cascade involves activation of MAPK proteins and these proteins can inhibit insulin signalling[61,265–268]. We hypothesized that either JNK, p38 or ERK would mediate statin-induced insulin resistance, but our results suggest this is not the case. Beyond activation seen with LPS, only p38 showed a marked increase in activation with the addition of atorvastatin. However, this increase persisted in the absence of IL-1β and with the treatment of the isoprenoid GGPP, suggesting that p38 activation it is not required for prenylation-dependent and IL-1β-mediated adipose insulin resistance caused by statins. These findings are in contrast to those showing in immune cells, p38 is activated by fluvastatin but not when GGPP is added back[269]. This difference could be explained by the cell type or dose of statin used. Previous reports used 10 μM fluvastatin *in-vitro* on immune cells compared to our results using 1 μM atorvastatin *ex-vivo* on adipose tissue. Our model could further test p38 by using an inhibitor to see if this is sufficient to mitigate statin-induced insulin resistance, but this is unlikely.

There are alternative routes of crosstalk between statins and IL-1β with inhibition of insulin signalling. Inducible nitric oxide synthase (iNOS) is a known mediator of insulin resistance and can serine phosphorylate IRS-1[216,217]. In muscle, IL-1β can activate iNOS directly, potentially providing a link between statin-induced IL-1β and iNOS-mediated insulin resistance[270]. Our findings showed that blocking iNOS with a chemical compound did not mediate statin-induced IL-1β suppression of insulin action in adipose tissue. We did not follow-up on these results with more careful dose response experiments or testing other ways to block iNOS because another pathway (i.e. mTOR) showed a promising link between statins and insulin resistance. There is also evidence to suggest that PTEN mediates statin-induce insulin resistance[271]. Rodents fed an obesogenic diet supplemented with rosuvastatin showed impaired glucose tolerance and increased PTEN protein in muscle. Heterozygous $PTEN^{+/-}$ mice were protected from rosuvastatin-induced glucose intolerance. This is an interesting series of results that are in agreement with our data showing that statin supplementation can worsen glucose control in ob/ob mice. We measured the amount of PTEN expression in LPS primed adipose tissue treated with atorvastatin and supplemented with GGPP. We saw no changes and no correlation between PTEN protein levels and statin treatment. There may be differences in mice (*in vivo)* during prolonged

statin exposure, compared to our *ex vivo* adipose tissue model, which is treated with a single long-term exposure. However, our findings suggest, PTEN is not sufficient to mediate statin-induced insulin-resistance in our adipose tissue model.

Finally, we tested if mTORC1 mediated statin-induced insulin resistance. Importantly, IL-1β can activate mTORC1[272]. Activation of mTORC1 can directly and indirectly inhibit insulin signalling through serine phosphorylation of IRS1[218,273]. Activation of mTORC1 phosphorylates p70s6k which can inhibit IRS-1 by serine phosphorylation at Ser270, Ser307 and Ser1101[274]. Rapamycin is an inhibitor of mTOR and at low doses (1-10 nM) predominately inhibits the mTORC1 complex, while mTORC2 activity is largely unaffected[221]. Only high dose (>100 nM), prolonged exposure of rapamycin has been shown to inhibit mTORC2 in cancer cells[275]. This is important as our marker of insulin signalling is the mTORC2 phosphorylation site Ser473 on Akt/PKB. Our results show that a low dose of 20 nM rapamycin prevents statin-induced insulin resistance in adipose tissue explants. Rapamycin alone did not alter insulin signalling suggesting the selected dose did not inhibit mTORC2. This suggests that mTORC1 may mediate statin-induced IL-1β insulin resistance. However, assessment of phosphorylation of p70s6k showed no increase with atorvastatin treatment of primed adipose tissue and phosphorylation of

p70s6k was not lower with the addition of GGPP. This suggests either mTORC1 directly inhibits IRS-1 through Ser636 phosphorylation or there is transient activation of p70s6k. Measuring phosphorylated p70s6k at multiple timepoints following atorvastatin treatment in adipose tissue is warranted. Further, assaying IRS-1 serine phosphorylation at both mTORC1 and s6k specific sites will reveal if either of these mediate the effects of statin-induced insulin resistance. Last, genetic deletion of mTORC1 in 3T3-L1 adipocytes with the dominate-negative mTORC1 adenovirus will provide further evidence this effect is mTORC1 dependent[276].

4.4 Statin lowering of protein prenylation activates the NLRP3 inflammasome

The statin-mediated cellular processes that elicit activation of the inflammasome and IL-1β secretion in adipose tissue are not clear. Inhibition of the mevalonate pathway lowers cholesterol, but also reduces several other key metabolites such as cholesterol derivatives, ubiquinone, and isoprenoids, which modify over a hundred proteins. Our objective was to test what metabolite was sufficient to cause IL-1β secretion from immune cells and test the components of the mevalonate pathway that are involved in statin-mediated insulin resistance in adipocytes. It was clear that providing isoprenoids required for protein prenylation was the only

intervention that restored insulin signalling during statin exposure in adipose tissue. Providing cholesterol or its derivatives did not restore insulin signalling during statin exposure. In fact, LDL cholesterol exposure caused insulin resistance in a separate manner to mechanisms involving NLRP3 inflammasome.

4.4.1 Statins reduce protein prenylation and cause mitochondrial ROS production to activate the inflammasome

Statin-induced insulin resistance in adipose tissue requires IL-1β. Our results show that reduction in isoprenoids is sufficient to cause IL-1β secretion in BMDM from C57Bl/6J mice. Addition of GGPP to primed BMDMs treated with fluvastatin reduced IL-1β secretion. This aligns with similar findings in other immune cells showing statin-reduction of isoprenoid is sufficient to increase IL-1β , since inhibition of GGTase-I with GGTI-298 promotes IL-1β secretion[172,277]. Recent evidence from Skinner *et al* demonstrated a similar connection between lower isoprenoids and NLRP3 activation in THP-1 and human peripheral blood mononuclear (PBMC)[277]. This effect was independent of the Pyrin inflammasome and priming of the inflammasome with the TLR2 antagonist Pam3CSK4 was sufficient. Interestingly, both TLR4 and TLR2 priming agents were able to stimulate IL-1β release from mevalonate-kinase-deficient (MKD) patient derived monocytes in an NLRP3-dependent manner. MKD patients have a

genetic defect in mevalonate kinase and have limited mevalonate pathway production, parallel to statin suppression of the mevalonate pathway.

It is not yet understood how decreased protein prenylation can drive NLRP3 inflammasome activation. Statins can alter mitochondrial metabolism[278–280]. Generation of mitochondrial reactive oxygen species (mtROS), altered mitochondrial membrane potential and decreased intracellular ATP levels have been observed with different statins in muscle cells. Dysfunctional mitochondria can produce ROS or release oxidized mitochondrial DNA (mtDNA), which have both been shown to activate the inflammasome[71]. Our data shows treatment with a general and mitochondrial ROS scavenger decreases statin-induced IL-1β secretion from macrophages.

The mevalonate pathway produces important metabolites for many metabolic pathways. How a reduction in the mevalonate pathways can trigger the inflammasome through mtROS is of interest. A potential explanation for statins alteration of mitochondrial metabolism is through reduction of ubiquinone in the electron transport chain of mitochondria. The source of ubiquinone in a cell is from the cholesterol synthesis pathway. Inhibition of HMG-CoA reductase lowers production of ubiquinone. Ubiquinone is used to shuttle electrons through the electron transport chain in mitochondria. However, attempts to restore

mitochondrial function with cell-permeable CoQ10 supplementation in the presence of statin have been limited in muscle cells[279]. Though ubiquinone can be synthesized from geranylgeranyl pyrophosphate, suggesting that supplementation experiments are restoring cellular ubiquinone, experiments using radiolabeled GGPP show that all exogenously provided prenylation substrates are directed towards protein prenylation[281]. Any addition of GGPP or FPP to supplement mevalonate pathway inhibition, is incorporated exclusively into the protein prenylation pathway, suggesting our findings and others are mediated by a reduction in a prenylated protein. A recent publication demonstrated disruption of glycolysis through inhibition of GAPDH and α-enolase can cause NLRP3 inflammasome activation by altering mitochondrial redox homeostasis[282]. NLRP3 activation was shown when macrophages were exposed to the inhibitor GB111, which inhibits GAPDH and α-enolase, which are key enzymes of glycolysis. Despite the presence of the glycolysis inhibitor, addition of pyruvate, and succinate restored mitochondrial homeostasis preventing inflammasome activation in macrophages. Intriguingly, statins can disrupt glycolysis[280,283]. In HepG2 cells treated with 10 μM fluvastatin, a reduction in glycolysis was observed. Fluvastatin also caused an increase in Pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4) mRNA (messenger ribonucleic acid), which can inhibit pyruvate dehydrogenase

(PDG), the key enzyme in decarboxylation of pyruvate to acetyl-coA that feeds into the TCA cycle[283]. Addition of GGPP reduced PDK4, however glycolysis rate with GGPP treatment was not reported in HepG2 cells. Testing if statin-mediated inflammasome dependent insulin resistance disrupts glycolysis in adipocytes is warranted.

Connecting mevalonate pathway metabolite changes to statin-induced insulin resistance was a key goal of this thesis. We sought to determine whether statins impaired insulin action by lowering cholesterol or another mevalonate pathway metabolite. We found that LDL cholesterol and 25-HC were dispensable for statin-mediated insulin resistance in adipose tissue. This was an important finding as lower 25-HC can mediate caspase-1 activation[224]. Further, no difference in cellular cholesterol was observed in 3T3-L1 adipocytes. Others have shown that statins can attenuate ligand-induced NLRP3 or pyrin inflammasome responses in macrophages or monocytes[239,284,285]. Interestingly, stimulation of the NLRP3 inflammasome with the classical activator cholesterol crystals, was significantly reduced in the presence of simvastatin, although LPS and simvastatin alone caused inflammasome activation and IL-1β secretion[285]. Oxidized LDL particles can also cause inflammasome activation upon uptake into macrophages[286]. Possibly through modulation of cellular cholesterol, and reduction of circulating LDL, statins may limit excessive

inflammasome activation at atherosclerotic lesions, and explain one aspect of their benefit in reduction of inflammation in atherosclerosis. A common theme in this thesis is that cell type is a key determinant of how statins engage specific inflammasomes. Many of the previous results are based on the effects of statins in immune cells. However, chronic low-grade NLRP3 inflammasome activation by statins may in adipose tissue be positioned to influence adipocyte metabolism, particularly lipogenesis.

Our results did show that isoprenoids required for protein prenylation were sufficient to prevent statin-mediated defects in insulin signalling in adipocytes. This is important because co-administration of statin and isoprenoids may mitigate adipose tissue side effects, but not interfere with lipid and cholesterol-lowering benefits of statins. Determination of the prenylated proteins that alter inflammasome activation and insulin resistance is an important future goal. Targeting specific prenylation events may be superior to widespread reversal of isoprenoid lowering that could mitigate longevity and cardiac benefits of statins shown in Drosophila[287].

4.5 Limitations and future application

4.5.1 Limitation of current work

The work in this thesis utilized cell culture and animal models to test our hypothesis. There are several limitations to these approaches. Our mouse

model of statin-induced insulin resistance utilized genetically obese mice (ob/ob). Our gavage treatment began around 14 weeks of age and progressed till ~21 weeks of age till we observed glucose intolerance. Ob/ob mice experience a profound degree of hyperglycemia in early age, which is overcome by a large expansion in pancreatic β-cells around 16-18 weeks of age[288]. Though our adipose tissue glucose disposal finding suggests statins cause adipose tissue insulin resistance, it is possible that statin treatment may have impacted the well known β-cell expansion that is known to occur in ob/ob mice at the ages that coincide with the statin exposure. Statins can affect β-cells metabolism and mitochondria, and in some doses *in vitro*, cause apoptosis[289,290]. Further, IL-1β and inflammasome activation can cause β-cell death and may be an interesting link to investigate in future *in-vivo* statin supplementation experiments[86].

Our results suggest that statins engage the inflammasome in adipose tissue to cause insulin resistance. Taken together with the findings from others, it is evident that both tissue and cell type is very important in dictating the effects and effectors of statins. Our findings and others show that NLRP3 is required for statin-induced IL-1β, however there is also findings showing a role for the pyrin inflammasome in immune cells[239,277]. We do not know if immune cell or adipocyte NLRP3 or IL-1β is

required for statin-mediated insulin resistance in adipose tissue or worse glucose control in ob/ob mice. Future experiments using statin treatment of chimeric mice (with hematopoietic cell deletion) or adipocyte-specific deletions for NLRP3 and IL-1β could address this cell type issue.

We found that statin-induced insulin resistance was associated with decreased insulin-stimulated lipogenesis and *in vivo* glucose intolerance. Our experiments have not yet connected these two metabolic features of metabolic syndrome. An important future goal is to link these responses to the requirement of prenylation or IL-1β upon statin exposure. Although we showed statin decreased lipogenesis in 3T3-L1 adipocytes is isoprenoid dependent, further testing is needed to link the inflammasome and IL-1β specifically in adipose tissue. Testing the requirement for either IL-1β or the NLRP3 during in vivo statin exposure in mice should be done to link statin-induced glucose intolerance and impaired lipogenesis in adipose tissue through activation of this inflammasome. Finally, determining how to increase GGPP-related isoprenoids that are required for protein prenylation in vivo or better yet defining the specific prenylated protein during statin exposure in vivo is positioned to mitigate glycemic and lipogenesis side effects of statins without compromising the cholesterol lowering effects of statins, which should be tested in vivo.

4.5.2 Future application

The findings in this thesis provide important insight to how statins may cause insulin resistance and increase the risk of new onset diabetes. The NLRP3 inflammasome and importantly, IL-1β mediates statin-induced insulin resistance in adipose tissue. This knowledge provides an important point for intervention to improve the statin drug class.

Further, our results show that statins can limit adipose lipogenesis and alter lipid handling. This appears relevant to statin intolerance and diminishing returns of increased statin dose on triglyceride/lipid lowering. Statin engagement of an NLRP3/caspase-1/IL-1β response may limit the effectiveness of statins to lower blood triglycerides independent of cholesterol lowering. Targeting NLRP3/caspase-1/IL-1β may allow enhanced blood lipid lowering at a given statin dose and/or mitigate side effects at a statin dose that achieves lipid-lowering goals.

Chapter Five: References

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