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OBESITY-LINKED INSULIN RESISTANCE, INFLAMMATION, AND OMEGA-3 FATTY ACIDS: EXPLORING THE ANTI-DIABETIC POTENTIAL OF NOVEL OMEGA-3 DERIVED PRO-RESOLUTION MEDIATORS.

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Résumé

L'objectif principal des études présentées dans cette thèse était d'étudier les effets métaboliques et anti-inflammatoires des acides gras oméga-3 et leurs dérivés bioactifs, nommés résolvines et protectines, dans le contexte d'obésité et d'insulino-résistance. Afin d'atteindre ce but, nous avons utilisé une nouvelle lignée de souris transgénique, *fat-1,* qui nous permet d'augmenter les niveaux d'acides gras oméga-3 sans modifier la diète expérimentale.

Dans la première étude, nous avons démontré que le rétablissement des acides gras oméga-3 dans les souris obèses nourries avec une diète riche en gras, pouvait augmenter la synthèse des dérivés bioactifs des acides gras oméga-3, notamment les protectines, dans le tissu adipeux et le muscle squelettique. Ceci était associé à une meilleure capacité à résoudre une réponse inflammatoire aigüe et à une diminution de l'inflammation dans le tissu adipeux. De plus, les souris *fat-1* obèses ont démontré une meilleure sensibilité à l'insuline et une plus grande tolérance au glucose. Tout cela avec un gain de poids et accrétion de graisse équivalents à leurs homologues sauvages.

Dans la seconde étude, nous avons analysé les effets métaboliques et anti-inflammatoires de l'administration de la protectine DX dans des macrophages *in vitro* ainsi que dans les souris *in vivo*. En plus des activités anti-inflammatoires anticipées, nous avons pu observer que la protectine DX possède une activité antidiabétique unique qui pourrait être expliquée par une sécrétion d'interleukine-6 impliquée dans l'inhibition de l'expression des enzymes de la gluconéogenèse dans le foie.

Dans la troisième étude, nous avons effectué une analyse comparative par biopuces du tissu adipeux des souris *fat-1* obèses et des souris sauvages. Cette étude nous a permis de révéler cinq voies ciblées par des acides gras oméga-3 dans le tissu adipeux. Nous avons pu valider certaines cibles par PCR quantitatif et par histologie.

L'ensemble de nos études démontre le rôle déterminant des acides gras oméga-3 et leurs dérivés, notamment les protectines, dans l'homéostasie métabolique. De plus, nous avons été en mesure d'identifier la protectine DX comme un nouvel agent thérapeutique qui possède un important potentiel pour le traitement du diabète de type 2 et de l'obésité.

Abstract

The primary objective of this thesis was to evaluate the metabolic and anti-inflammatory functions of omega-3 fatty acids and their bioactive derivatives namely, the resolvins and protectins, in the context of obesity and insulin resistance. To achieve this objective we employed a novel line of transgenic mice, the *fat-1* mice, which allowed us to raise endogenous levels of omega-3 fatty acids without altering the experimental diet.

In the first study we demonstrated that transgenic restoration of omega-3 fatty acids in high fat fed obese *fat-1* mice raises the synthesis of their bioactive derivatives, namely the protectins, in adipose tissue and skeletal muscle. This was associated with an improved capacity to resolve an acute inflammatory response and reduced inflammation in adipose tissue as well as improved systemic insulin sensitivity and glucose tolerance. Importantly, these effects occurred in the absence of any changes in weight gain or fat accretion.

In the second study, we directly evaluated the anti-inflammatory and metabolic effects of protectin DX in palmitate-treated macrophages *in vitro* as well as in lipid-infused mice *in vivo*. In addition to the anticipated anti-inflammatory actions, here we unraveled a unique glucoregulatory activity of protectin DX. We found that protectin DX administration lowers fasting glycemia and improves insulin sensitivity by promoting IL-6 dependent suppression of gluconeogenic enzymes in the liver.

In the third study we performed Affymetrix microarray in adipose tissue from high fat fed *fat-1* transgenic mice and their wild type counterparts. This study allowed us to establish five key pathways that are regulated by omega-3 fatty acids in adipose tissue. Importantly, we were able to validate some of these pathways using real-time RT-PCR and histological analysis.

Together these works highlight the important contribution that omega-3 fatty acids and their bioactive derivatives, namely the protectins, make to the maintenance of metabolic homeostasis. Importantly, through our study of omega-3 fatty acids and

their metabolites, we were able to identify protectin DX as a novel molecule with exciting therapeutic potential for the treatment of obesity and type 2 Diabetes.

Foreword

The work contained in *Chapter I* of this thesis was published in the December issue of *Diabetes* in 2010. It is presented as an author-created, uncopyedited version of the original article. The American Diabetes Association (ADA), publisher of *Diabetes*, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version is available in *Diabetes* in print and online at http://diabetes.diabetesjournals.org. As the first author of this manuscript, I was responsible for planning and carrying out all animal studies, I generated the data contained in figures 2-4 and also prepared the tissues for the lipidomics analysis presented in figure 1. I also conducted all statistical analysis of the data, prepared the figures, and wrote the manuscript.

Chapter II contains a manuscript in preparation. I am the first author of this article. I planned all experiments and am responsible for most of the data contained in figures 1-6 as well as Table 2 and supplementary figures 1 and 2. The hyperinsulinemic-euglycemic clamp experiments presented in figure 2A-E and table 1 were performed by Alexandre Charbonneau whereas those presented in figure 5 were performed by me in collaboration with Philippe St-Pierre. In addition to generating the data I also conducted all statistical analysis, prepared the figures, and wrote the manuscript.

Chapter III also comprises a manuscript in preparation. I am the first author of this work. I extracted the RNA for the affymetrix microarray that was performed at the McGill University and Génome Québec Innovation Center in Montréal and performed the analysis of the microarray data set using FlexArray 1.6.1 and GenMAPP/MAPPFinder 2.0 software packages. I also performed the real-time RT-PCR and adipocyte size profiling as well as the cell culture studies in 3T3-L1 adipocytes. In addition to generating the data I also conducted all statistical analysis, prepared the figures, and wrote the manuscript.

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Last but not least, to my beautiful wife, I don't think I can ever thank you enough for all your understanding, support, encouragement and love. Having you by my side has made the disappointments easier to take and the achievements much more enjoyable.

For Martine and Sydney

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Abbreviations

AA	arachidonic acid
ACC	acetyl Co-A carboxylase
AEA	anandamide/*N*-arachidonylethanolamine
2-AG	2-arachidonoylglycerol
AGPI	acides gras polyinsaturés
AKT/PKB	acute transforming retrovirus thymoma/protein kinase B
ALA	α-linolenic acid
AMPK	adenosine monophosphate protein kinase
aPKC	atypical protein kinase C
APP	acute-phase protein
APS	adaptor protein containing PH and SH-2 domains
ASA	acetylsalicylic acid
A-SMase	acid-sphingomyelinase
AT	aspirin triggered
BAD	Bcl-2 antagonist of cell death
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CCL2/MCP-1	chemokine(C-C)motif ligand 2/monocyte chemotactic protein-1
CLS	crown like structure
COX-2	cyclooxygenase-2
CREB	cAMP response element binding
CRP	C-reactive protein
CVD	cardiovascular disease
DAMP	damage associated molecular pattern
DHA	docosahexaenoic acid
4EBP-1	eukaryotic initiation factor 4E binding protein-1
Enpp1	ectonucleotide pyrophosphatase phosphodiesterase 1
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
FA	fatty acid
FBP-ase	fructose-1-6-biphosphatase
F1C	*fat-1* chow
F1HF	*fat-1* high fat
FOXO-1	Forkhead BOX class-O winged helix transcription factor-1
GAB-1	GRB2-associated binding protein
GFP	green fluorescent protein
GLP-1	glucagon like peptide-1
G6Pase	glucose-6-phosphatase
GPCR	G-protein coupled receptor
GSK-3	glycogen synthase kinase-3
HDL	high-density lipoprotein

HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HF	high fat
HIE	hyperinsulinemic-euglycemic
HIF-1α	hypoxia inducible factor-1α
Hmgcr	HMG Co-A reductase
HNF4α	hepatocyte nuclear factor 4α
Hp	hydroperoxy
HSL	hormone sensitive lipase
IFN	interferon
Iκβ	Inhibitor of κβ
IKK	Iκβ kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IR	insulin resistance
IRS	insulin receptor substrate
JAK	Janus activated kinase
JIP-1	JNK interaction protein-1
JNK	c-Jun N-terminal kinase
LA	linoleic acid
LC	long chain
LTB_4	leukotriene B_4
LIRKO	liver specific insulin receptor knockout
LOX	lipoxygenase
LPS	lipopolysaccharide
LxA_4	lipoxin A_4
LXR	liver X receptors
MaR	Maresin
MCSF	monocyte colony stimulating factor
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MS	metabolic syndrome
mTOR	mammalian target of rapamycin
NFκβ	nuclear factor κβ
NGT	normal glucose tolerance
NK	natural killer
NO	nitric oxide
PAMP	pathogen associated molecular pattern
PD1	protectin D1
PDE-3B	phosphodiesterase-3B
PDK1/2	phosphoinositide-dependant kinase-1/2
PDX	protectin DX
PEPCK	phosphoenolpyruvate carboxykinase
PGC1	peroxisome proliferator-activated receptor-γ co-activator-1
PI3-K	phosphoinositide 3-Kinase
$PI(4,5)P_2$	phosphatidylinositol-4,5-bi-phosphate
$PI(3,4,5)P_3$	phosphatidylinositol-3,4,5-tri-phosphate

PKA	protein kinase A
PLA_2	phospholipase A_2
PMN	polymorphonuclear leukocytes
PPARγ	peroxisome proliferator activated receptor gamma
Ptprf	protein tyrosine phosphatase, receptor type F
PUFA	polyunsaturated fatty acids
RA	receptor antagonist
RANTES	regulated upon activation, normal T-cell expressed and secreted
RMA	robust multi-array average
RT-PCR	reverse transcription polymerase chain reaction
Rv	Resolvin
SAA	serum amyloid A
SH-2	src-Homology-2
SIK2	salt induced kinase 2
SIRPs	signal regulatory proteins
SOCS	suppressor of cytokine signaling
SREBP	sterol regulatory element binding proteins
STAT-3	signal transducer and activator of transcription 3
T2DM	type 2 diabetes mellitus
Tg	transgenic
TGF-β	transforming growth factor beta
TH	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TORC2	transducer of regulated CREB activity-2
T-regs	T-regulatory T-cells
TRPV1	Transient receptor potential vanilloid type-1
TZDs	thiazolidinediones
UCP-1	uncoupling protein 1
VEGF	vascular endothelial growth factor
WT	wild type
WTC	wild type chow
WTHF	wild type high fat

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Introduction

1-Type 2 Diabetes: A modern pandemic

Type 2 Diabetes Mellitus (T2DM) has grown to become one of the greatest threats to human health afflicting modern society. Presently it is estimated that ~ 150 million people worldwide suffer from T2DM and its associated complications and this number is predicted to escalate to ~ 300 million people by the year 2025 (1). As a heterogeneous disease state multiple risk factors contribute to the likelihood of a person developing T2DM. These include, but are not limited to, a family history of T2DM (2), impaired glucose tolerance (3), prior occurrence of gestational diabetes mellitus (4), body fat accumulation (particularly in visceral adipose depots) (5), physical inactivity (6), and poor diet (7). Of these factors, those that are lifestyle related are considered to be profoundly responsible for the pandemic emergence of this disease throughout society. Indeed, the parallel rise in the global prevalence and severity of obesity which in most part developed as a product of environmental and lifestyle changes towards physical inactivity and nutritional oversupply is believed to underlie the advent of T2DM as an epic health problem (8).

T2DM manifests as a combination of peripheral and hepatic insulin resistance alongside pancreatic beta cell dysfunction (9). In the diabetic state, the pancreas no longer possesses the capacity to support the elevated insulin requirements for maintaining euglycemia and hyperglycemia develops. In most cases of T2DM, insulin resistance precedes beta cell dysfunction and the progression towards impaired glucose tolerance (10). Devoid of beta cell compensation for peripheral insulin resistance, initial impediments in insulin signal transduction would directly lead to an ever worsening hyperglycemia resulting from reduced postprandial inhibition of hepatic glucose output and diminished glucose uptake by skeletal muscle and other peripheral tissues. However, superior insulin secretions from the compensating islets ensure that normal glucose tolerance (NGT) is maintained for some time, albeit in the presence of deteriorating hyperinsulinemia. Due to this highly plastic nature of the pancreatic beta cells the progression from insulin

resistance to T2DM may be delayed for up to 20 years (11). Indeed, it is only when the pancreas finally fails to compensate for the waning metabolic response to insulin that T2DM develops (12). Thus insulin resistance is considered to be a key primary defect in the pathogenic continuum of T2DM.

2-Insulin action

Insulin contributes to the maintenance of glucose and lipid homeostasis via coordinated pleiotropic actions in multiple target tissues, including liver (13), skeletal muscle (14), adipose tissue (14), pancreas (15), brain (16) and the vasculature (17). Insulin resistance is said to occur when insulin signal transduction in these tissues is impaired. To enhance understanding of the mechanisms underlying the development of insulin resistance, the outcomes and key elements of insulin signal transduction will be described for three important peripheral metabolic tissues: skeletal muscle, liver and adipose tissue.

2.1 Skeletal muscle

Skeletal muscle is responsible for approximately 80% of insulin stimulated glucose disposal (18). Thus, inhibition of insulin signaling in this tissue has a major impact on whole body glucose homeostasis. The insulin receptor can be found at the skeletal muscle sarcolemma, is tetrameric, and contains two extracellular alpha and two transmembranous beta-subunits. Insulin binding to the extracellular alpha-subunits of the insulin receptor, induces a conformational change in the receptor via a cam-dependent mechanism (19). This allows the opposing transmembane spanning beta-subunits to approach one another and enables the regulatory activation loop of each beta-subunit to come into contact with and become phosphorylated on three sites by the catalytic domain of the other. This trans-autophosphorylation of tyrosine sites on the beta-subunits activates the intrinsic receptor tyrosine kinases and also provides docking sites for the various intracellular src-Homology-2 (SH-2) domain containing proteins that serve as insulin receptor substrates. To date those identified include the appropriately named insulin receptor substrate (IRS) proteins, as well as GAB-1, Shc, APS, p60, SIRPs, and c-CBL (20).

Since the signaling intermediates downstream of the IRS proteins are responsible for insulin dependent glucose transport, glycogen synthesis, protein anabolism, and transcriptional regulation of target genes, the following description of insulin signaling will be focused primarily on this branch of the pathway. Interestingly, the roles of the different IRS isoforms appear to vary among insulin target tissues. The generation of knockout models (21) and mice carrying combined heterozygous mutations (22) of the insulin receptor with IRS-1 and/or IRS-2 has provided important insight in this area. Importantly, genetic deletion of IRS-1 in mice was found to be associated with impaired insulin action in skeletal muscle but not liver where expression of IRS-2 was found to be more abundant (21). Moreover, combined heterozygous mutation of the insulin receptor with IRS-1 produced severe skeletal muscle insulin resistance and mild hepatic insulin resistance whereas combined heterozygous mutation of the insulin receptor with IRS-2 resulted in mild skeletal muscle insulin resistance alongside severe impairment of hepatic insulin signaling (22). Together these data suggest that IRS-1 plays a predominant role in peripheral insulin target tissues whereas the activity of IRS-2 appears to be more important in liver.

Both the IRS proteins contain approximately 22 potential tyrosine phosphorylation sites which, once phosphorylated by the receptor kinase, serve as docking sites for the SH-2 domain containing p85 subunit of phosphoinositide 3-Kinase (PI3-K) (23). Once activated by p85 binding to IRS, the p110 catalytic subunit of PI3-K phosphorylates the D3 position on the inositol ring of the plasma membrane lipid, phosphatidylinositol-4,5-bi-phosphate, ($PI(4,5)P_2$) converting it to phosphatidylinositol-3,4,5-tri-phosphate, ($PI(3,4,5)P_3$) (24). The newly formed $PI(3,4,5)P_3$ then recruits pleckstrin homology domain containing proteins to the plasma membrane, namely the serine-threonine kinases, acute transforming retrovirus thymoma, (AKT) and phosphoinositide-dependant kinase-1/2, (PDK1/2) (25). Once localized at the plasma membrane, $PI(3,4,5)P_3$ bound PDK-1/2 phosphorylates nearby AKT (26) thereby stimulating its catalytic activity. It is noteworthy that $PI(3,4,5)P_3$ bound PDK-1/2 also activates atypical Protein Kinase C (aPKC) and p70S6-Kinase (27-28). Activated AKT and aPKC then stimulate

GLUT-4 glucose transporter translocation from intracellular vesicular pools to the sarcolemma by an incompletely resolved mechanism (29). AKT also inhibits Glycogen Synthase Kinase-3 (GSK-3) activity (30), induces nuclear extrusion of the transcriptional regulator Forkhead BOX class-O winged helix transcription factor-1, (FOXO1) (31), activates the mammalian target of rapamycin, (mTOR) (32), and inhibits the pro-apoptotic protein BAD from binding to Bcl-2 (33). These activities result in enhanced glucose entry into the cell, elevated glycogen synthesis, (a rate limiting step in skeletal muscle glucose metabolism), inhibition of the transcription of apoptosis related genes such as Fas ligand, and phosphorylation of the translation factors p70S6-Kinase and the eukaryotic initiation factor 4E binding protein-1, (4EBP-1), that are involved in the translational regulation of protein synthesis, and in the case of p70S6-Kinase, feedback inhibition of insulin signaling (see figure 1).

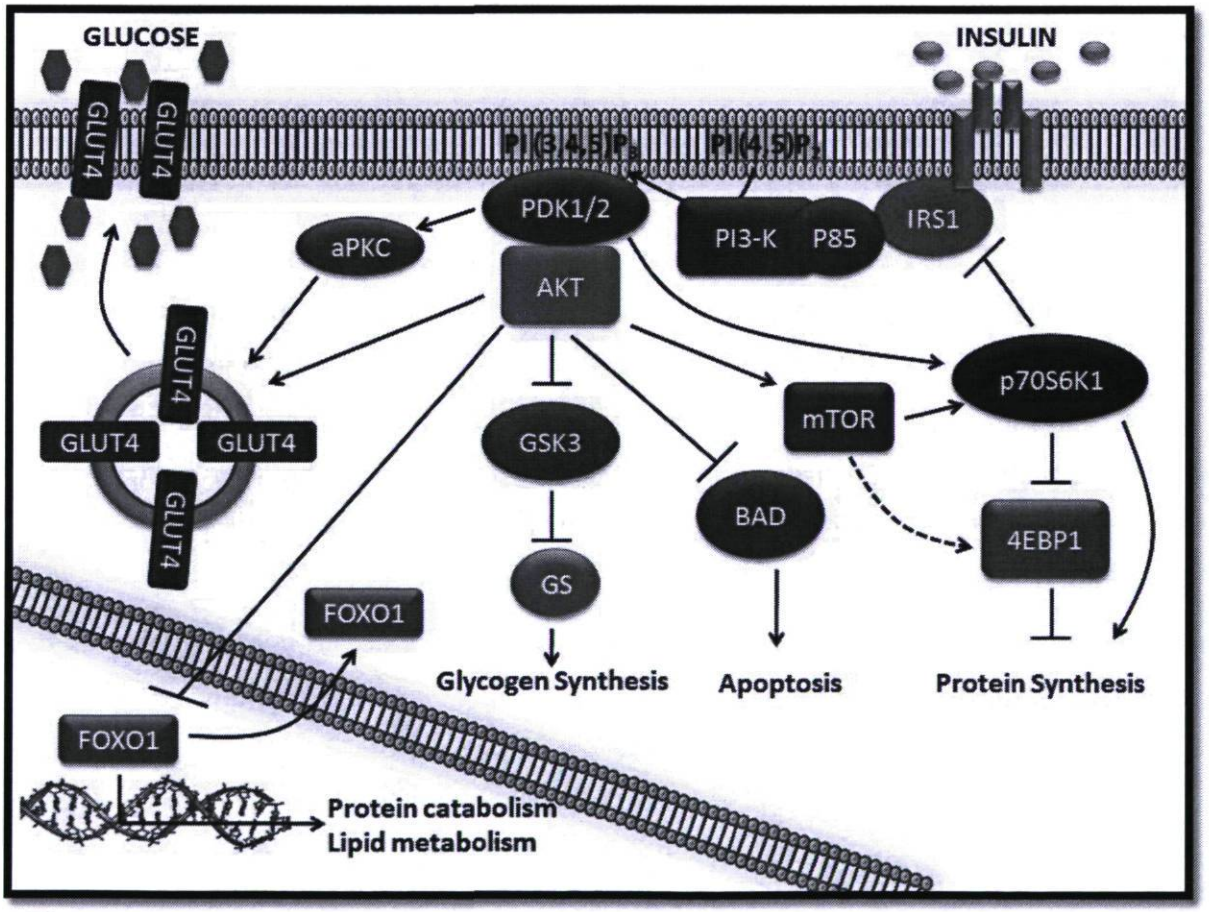

Figure 1. Simplified schema of insulin signaling via IRS in skeletal muscle.

2.2 Liver

The liver is a major regulator of energy homeostasis. It is the first site of carbohydrate and protein passage following digestion and such metabolic priority via the portal circulation allows the liver to act as an energy sensor producing, storing, or oxidizing carbohydrates, fats, and proteins when necessary. The liver also has priority to insulin and glucagon released from the pancreas and maintains glucose homeostasis by coordinating glucose output and storage as signaled by these key glucoregulatory hormones among others.

Glucagon signaling in the liver is cyclic AMP (cAMP)/protein kinase A (PKA) dependent and results in enhanced glucose output through a combination of gluconeogenesis and glycogenolysis (34). Briefly, activation of PKA by glucagon promotes phosphorylation of cAMP response element binding (CREB) and association of CREB and CREB binding protein (CBP) with transducer of regulated CREB activity-2 (TORC2) in the promoter region of the transcription factor peroxisome proliferator-activated receptor-γ co-activator-1(PGC1) leading to its expression (See figure 2)(35-39). PGC1 once expressed then associates with hepatocyte nuclear factor 4α (HNF4α) and FOXO1 to promote the expression of key enzymes involved in hepatic gluconeogenesis and glycogenolysis including glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), and fructose-1-6-biphosphatase (FBP-ase) (40-45). Insulin signaling in liver counteracts these actions of glucagon on hepatic glucose output and enhances glucose storage as glycogen. Insulin induces this response both indirectly, by reducing gluconeogenic substrate release from peripheral tissues (46-48) and by inhibiting glucagon secretion from the pancreatic alpha cells (49), and directly by acting on the liver itself (50-51). The existence of a gut- brain- liver axis regulating hepatic glucose metabolism has also been described (52). The regulation of endogenous glucose production by this mechanism appears to be insulin independent and involves lipid sensing in the upper intestine which promotes the activation of a neuronal network from gut to brain to liver that effectively lowers hepatic glucose output (52-54).

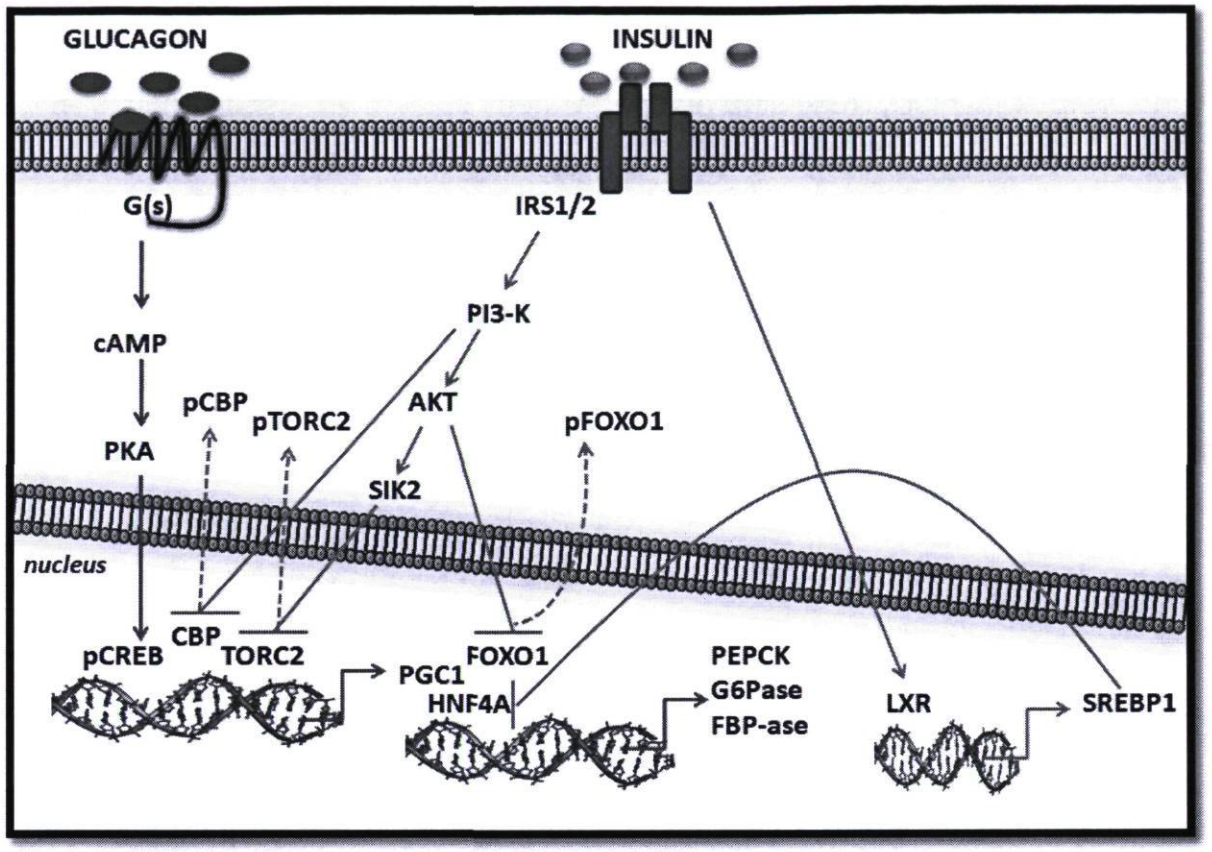

Figure 2. Simplified schema of the regulation of hepatic glucose production

Insulin binding to its receptor on the surface of hepatocytes results in activation of a similar signaling cascade to that presented for skeletal muscle in figure 1. However, hepatic insulin signaling relies on a dynamic functional relay between both IRS1 and IRS2 wherein IRS2 is responsible for insulin actions in liver during fasting and immediately after refeeding and IRS1 is the predominant isoform in the postprandial period (55). Insulin signaling in liver interferes with glucagon induced cAMP signaling in multiple ways. Firstly, insulin blunts glucagon mediated cAMP release (34; 56). Insulin also promotes the nuclear extrusion of the CREB co-factor CBP via a phosphorylation dependent mechanism (57). Furthermore, insulin stimulation results in AKT mediated phosphorylation and activation of salt induced kinase 2 (SIK2) which phosphorylates and promotes the degradation of TORC2 another key cofactor for CREB dependent PGC1 expression (58) (see figure 2). Moreover, insulin signaling through IRS/AKT in liver also induces the nuclear extrusion of the transcription factor FOXO1 (59) and thereby inhibits the actions of

FOXO1 and its transcriptional co-activator PGC1 (40) that are primarily responsible for increasing the expression of the aforementioned gluconeogenic/glycogenolytic enzymes. Finally, insulin promotes the transcriptional activity of liver X receptors (LXR) which in turn induce the expression of sterol regulatory element binding proteins (SREBP) (60). SREBP expression is thought to negatively regulate the transcriptional activity of PGC1 in liver by binding with HNF4α and preventing its interaction with PCG1 (61). In addition to their influence on hepatic glucose output, SREBP and LXR also play a major role in insulin mediated lipogenesis in the liver by coordinating the expression of a lipogenic program comprising enzymes responsible for biosynthesis and receptor mediated fatty acid and cholesterol transport (62).

Without these key actions of insulin in liver, liver specific insulin receptor knockout (LIRKO) mice display severe hepatic insulin resistance that is characterized by elevated hepatic glucose output in both the fasting and postprandial state and impaired insulin clearance (63). Interestingly, after two months the liver of these mice presented normal morphology with no sign of steatosis but by 6 months displayed moderately increased lipid storage, drastically reduced glycogen content and enlarged mitochondria (63). LIRKO mice have since been found to be particularly susceptible to gall stone formation (64) and the development of dyslipidemia and atherosclerosis (65). Importantly, LIRKO mice progress rapidly from an initial state of hepatic to full systemic insulin resistance (63), suggesting that loss of insulin action in liver can have a spillover effect on other key insulin target tissues. Together these findings underscore the importance of hepatic insulin action for the maintenance of systemic metabolic homeostasis.

2.3 Adipose tissue

For many years the adipose tissue was primarily thought of as a storage depot for excess energy; however, the relatively recent discoveries of multiple adipocyte derived signaling molecules termed adipokines has changed this way of thinking. Indeed, it has become evident with these advances that the adipose tissue itself is an endocrine organ that plays a major role in the regulation of energy homeostasis

via cross talk with other metabolically significant tissues (i.e. brain, liver, and muscle) (66). Like most endocrine organs adipose tissue is also subject to neuroendocrine input and regulation. In fact, multiple neural and hormonal signals are integrated in adipose tissue to maintain systemic energy homeostasis. In energy demanding situations such as exercise or infection, hormonal (e.g. catecholamines, glucagon etc.) and neural signals (e.g. sympathetic input) combine to enhance lipolysis and the liberation of stored triglycerides from adipose tissue via a cAMP/PKA dependant mechanism that results in the up-regulation of hormone sensitive lipase (HSL) activity (67-68). In contrast, in times of energy excess such as immediately following a meal, insulin signaling in adipose tissue enhances carbohydrate and lipid storage via enhanced glucose uptake and the up-regulation of lipoprotein lipase activity (LPL) (69-70). Insulin also inhibits free fatty acid output via the phosphorylation and activation of cAMP specific phosphodiesterase-3B (PDE-3B) (71-73) which decreases cAMP levels and inhibits the cAMP/PKA dependant up-regulation of HSL activity. This results in reduced liberation of stored triglycerides from adipose tissue and enhanced uptake of free fatty acids from the plasma. Furthermore, insulin has been shown to increase the expression and phosphorylation of the nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ) (74-75). PPARγ is a major transcriptional regulator of the adipogenic program that promotes pre-adipocyte differentiation, storage of fatty acids, and modulates the expression of multiple adipokines (76).

Accordingly, insulin resistance in adipose tissue results in reduced lipid incorporation which may lead to excess storage of triglycerides in other metabolically important tissues such as skeletal muscle and liver. Enhanced triglyceride storage in these tissues (i.e. ectopic fat) is a hallmark of the insulin resistance syndrome and is hypothesized to be a cause of systemic insulin resistance (77-80). However, somewhat interestingly, the adipose specific insulin receptor knockout model does not display altered glucose homeostasis or develop systemic insulin resistance (81), indicating that there is most likely another mechanism involved in the development of systemic insulin resistance.

3- Inflammation-induced insulin resistance

It is now widely accepted that inflammation is a key component of the etiology of obesity-linked insulin resistance, leading the way to T2DM and cardiovascular disease (CVD) (82-83). Over the past decade, genetic and pharmacological invalidation of multiple inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and c-Jun N-terminal kinase (JNK), in metabolic tissues has proved to be an effective means of preventing the development and progression of insulin resistance in mice (84-86). Furthermore, metabolic targets for the prevention and treatment of insulin resistance such as adiponectin, AMP activated protein kinase (AMPK) and PPARγ have also been shown to counter inflammation (87-89). Recent findings suggest that the origin of inflammation in obesity involves immune cell recruitment to adipose tissue (90-97); however, the primary event which promotes this phenomenon remains unknown. The next section will describe the chronic low grade inflammation present in obesity, discuss the immunological response to adipocyte hypertrophy, and provide a detailed description of the current state of understanding on how inflammation develops alongside an expanding adipose tissue mass. Finally molecular mechanisms by which inflammation may promote insulin resistance will be described.

3.1 Obesity: A chronic low-grade inflammatory state

It is well established that obesity is a chronic inflammatory disorder (83). Obesity-linked type 2 diabetes is associated with a cytokine-mediated acute-phase response or stress response (98-101) alongside the characteristic formation of inflammatory aggregates in metabolic tissues (90-91; 93-96; 102-106). Interestingly, levels of C-reactive protein (CRP), an acute-phase response protein and a sensitive marker of low-grade inflammation, are associated with higher adiposity in children (101) and young adults who are healthy (100) and are independently related to insulin sensitivity in subjects who are nondiabetic (107). These findings confirm that an early onset, low-level systemic inflammation exists in persons who are overweight or obese. This chronic inflammatory state is in line with observations of elevated plasma levels of interleukin (IL)-6 and tumor necrosis

factor (TNF)α (100) and of overexpression of TNFα, IL-1β, IL-6, and interferon (IFN)-γ in adipose tissues of humans and animals exhibiting obesity (84; 108). Interestingly an ever-increasing number of molecules that are best known for their roles in immunity are now being considered as key modulators of energy metabolism in insulin target cells (82-83) whereas a growing number of adipose-specific molecules termed adipokines, including leptin, resistin, and adiponectin, now appear to modulate inflammation (109-112). The various immune factors influenced by obesity are listed in table 1.

Table 1. Immune factors influenced by obesity

Molecule	Class	Physiological Function	Obesity Effect
Adiponectin	Adipokine	Suppresses macrophage function, NK cell cytotoxicity, and myelomonocytic proliferation. Induces antiinflammatory cytokine production and insulin sensitization (110; 113-115).	↓ plasma [ ] (116)
Adipsin	Adipokine	Analogue of Human Complement Factor D (117)	↑ blood [ ] (118)
CRP	APP	Cytokine production, complement activation, Phagocytosis, antigenic/apoptotic cell clearance (119)	↑ serum [ ] (99)
Ghrelin	Peptide hormone	Negative regulator of proinflammatory cytokine production, orexigenic, stimulates growth hormone release, and neurogenesis (120)	↓ plasma [ ] (121)
Haptoglobin	APP	Prevents iron loss and superoxide production, and stimulates angiogenesis (122)	↑ serum [ ] (123)
IFN-γ	Cytokine	Proinflammatory, activates innate immune system, enhances antigen presentation and iNOS expression, regulates TH1/TH2 balance, and controls cellular proliferation and apoptosis (124)	↑ [ ] adipose tissue (84)
IL-1β	Cytokine	Proinflammatory, induces COX-2, PLA_2, & iNOS (125)	↑ serum [ ] (126)
IL-1Ra	Cytokine	Antiinflammatory competitively inhibits IL-1 (125)	↑ serum [ ] (127)
IL-6	Cytokine	Activates acute phase response, stimulates lymphocytes and hematopoietic colony formation (128)	↑ serum [ ] (129)
IL-8	Chemokine	Neutrophil recruitment and activation (130)	↑ plasma [ ] (131)
IL-10	Cytokine	Antiinflammatory, prevents IFN-γ, IL-1β, TNFα, IL-8, IL-12, and NO production by macrophages, monocytes, and TH1 cells (132)	↑ serum [ ] in obesity ↓ serum [ ] with MS (133)

Leptin	Adipokine	Central regulation of energy balance, regulation of puberty and reproduction, functions in hematopoiesis and chemotaxis, modulates adaptive immune response, induces synthesis of pro-inflammatory cytokines, NO, and eicosanoids (134)	↑ serum [ ] (135)
LTB_4	Eicosanoid	Pro-inflammatory, promotes MCP-1, TNFα and IL-6 secretion from adipose tissue and promotes HSL expression in adipocytes (136)	↑ [ ] adipose tissue (136-137)
MCP-1	Chemokine	Induces monocyte, basophil, NK cell, and T-cell chemotaxis and IL-4, IL-5, IFN-γ production (138-139)	↑ plasma [ ] (140)
M-CSF	Cytokine	Primary regulator of mononuclear phagocyte survival, proliferation, differentiation, and function also mediates adipose tissue growth (141)	↑ adipose tissue expression (142)
MIF	Cytokine	Proinflammatory, enhances phagocytosis, induces TNFα, IL-8, and IL-12, inhibits apoptosis, and antagonizes the effects of glucocorticoids (143)	↑ plasma [ ] (144)
RANTES	Chemokine	Induces leukocyte chemotaxis (145)	↑ serum [ ] (146)
Resistin	Adipokine	Proinflammatory, induces TNFα, IL-1β, IL-6, and IL-12, diabetogenic (112)	↑ serum [ ] (147)
SAA	APP	Promotes monocyte and T lymphocyte chemotaxis and adhesion, induces expression of extracellular matrix degrading metalloproteinases (148)	↑ plasma [ ] (149)
TGFβ	Cytokine	Inhibits lymphoid and myeloid cell proliferation, Induces differentiation of myeloid dendritic cells, suppresses tumor formation (150)	↑ plasma [ ] (151)
TNFα	Cytokine	Proinflammatory, activates neutrophils and platelets, enhances macrophage and NK cell phagocytosis, stimulates immune system (152)	↑ plasma [ ] (108)
Visfatin	Adipokine	Proinflammatory, B cell growth factor, inhibits neutrophil apoptosis, promotes smooth muscle cell maturation, possesses insulin mimetic activity (153)	↑ plasma [ ] (154)
5-HETE	Eicosanoid	Pro-inflammatory, stimulates MCP-1, IL-6 and IL-12 expression in adipocytes (137)	↑ [ ] adipose tissue (137)
12-HETE	Eicosanoid	Pro-inflammatory, stimulates TNFα, MCP-1, IL-6 and IL-12 expression in adipose tissue (137; 155)	↑ [ ] adipose tissue (137; 155)

APP, acute-phase protein; COX-2, cyclooxygenase-2; LTB_4, leukotriene B_4; MCSF, monocyte colony stimulating factor; MIF, macrophage migration inhibitory factor; MS, metabolic syndrome; NK, natural killer; NO, nitric oxide; PLA_2, phospholipase A_2; RA, receptor antagonist; RANTES, regulated upon activation, normal T-cell expressed, and secreted; SAA, serum amyloid A; TGF-b, transforming growth factor beta; TH, T helper; HETE, hydroxyeicosatetraenoic acid; [ ], concentration; ↓, decrease; ↑, increase.

3.2 Immune cell accumulation in adipose tissue

3.2.1 Adipose tissue macrophages

In 2003, *The Journal of Clinical Investigation* simultaneously published two reports (90-91) that transformed our understanding of the primary pathogenic changes taking place in the visceral adipose tissue during obesity. These revelations clearly showed that obesity is characterized by progressive macrophage infiltration into adipose tissue that positively correlates with body mass index (BMI) and adipocyte size. Importantly, the infiltrating macrophages were shown to be responsible for almost all of the local expression of TNFα, IL-6 and iNOS in adipose tissue (90-91). These studies added depth and clarity to the discovery of Hotamisligil et al (108) a decade earlier which demonstrated the presence of pathogenic low-grade inflammation in obesity. Although it has become well accepted that inflammation is a major player in the development of insulin resistance and metabolic disease in obesity, prior to these findings (90-91), the cellular source of adipose inflammation remained a mystery. It is now known that adipocyte death and adipose tissue remodeling underlie the recruitment of bone marrow derived macrophages to visceral adipose tissue (156-157) where macrophages characteristically form inflammatory aggregates around dead or dying adipocytes in an arrangement termed *'crown like structures'* (156). Chemokines such as chemokine (C-C) motif ligand 2/monocyte chemotactic protein-1 (CCL2/MCP-1) and osteopontin are believed to be critical for the recruitment of macrophages to adipose tissue (158-159). Importantly, blocking the expression of either of these chemokines blunted macrophage infiltration into adipose tissue of obese mice and prevented the development of insulin resistance without altering fat mass. Conversely, overexpression of CCL2/MCP-1 in the adipose tissue of lean mice resulted in macrophage accrual and the development of insulin resistance in the absence of obesity (158).

The work of Lumeng et al (160) has added another dimension to our understanding of adipose tissue macrophages by revealing that in contrast to those found in adipose tissue of lean animals, the vast majority of macrophages present in the

adipose tissue of obese mice are polarized towards a so-called *M1* classically activated pro-inflammatory state. This subset of macrophages is characterized by elevated expression of the cell surface antigen CD11c as well as classic pro-inflammatory genes such as TNFα and iNOS. In contrast, the predominant macrophage population in adipose tissue of lean mice displays an *M2* or alternatively activated profile that is associated with higher expression of arginase-1 and IL-10 (160). This characterization of adipose tissue macrophages was not surprising since the earlier work of Weisberg et al (161) had shown that it is the notoriously pro-inflammatory $CCR2^{+}$ subset of circulating monocytes that are recruited to adipose tissue with obesity (161-162). Accordingly, the work of Lumeng et al (160) confirmed that adipose tissue macrophages from obese CCR2 knock-out mice express M2 markers to a similar degree as lean mice (160).

The discovery that the adipose tissue macrophage population consists of pro and anti-inflammatory subsets has led to the suggestion that it may be possible to limit adipose tissue inflammation in obesity by promoting M2 skewing. On this note, it has been revealed that cellular metabolic pathways play a key role in the determination of the macrophage inflammatory phenotype. Indeed, the M1 phenotype is associated with glycolysis while the M2 phenotype is closely linked to oxidative metabolism (163-164). Importantly, the transcriptional co-activator PGC1β, that promotes the expression of oxidative enzymes, was identified as a transcriptional switch that underlies IL-4 mediated alternate macrophage activation (164). Activation of the nuclear receptors PPARγ and PPARδ has also been shown to promote the switching of classically activated macrophages towards an alternately activated M2 phenotype (89; 165). Interestingly, it is now known that the insulin sensitizing actions of thiazolidinediones (TZDs) are significantly reduced in mice lacking macrophage PPARγ (166-167). These findings suggest that M2 skewing of adipose tissue macrophages may be a viable therapeutic strategy for the prevention of obesity-linked inflammation and insulin resistance.

3.2.2 Neutrophils

Following the discovery of adipose tissue macrophages, flow cytometry based profiling of the stromal vascular fraction of visceral adipose tissue has identified other types of immune cells such as neutrophils and T-cells that are also modulated during the course of obesity (92-93; 95-96). Interestingly, it appears that adipose tissue inflammation in obesity resembles a classical inflammatory response in that neutrophil recruitment to adipose tissue is transient and precedes macrophage accumulation in this tissue (92). However, the precise role of neutrophils in promoting adipose inflammation remains unclear since the authors of this work did not employ a model that would deplete or inactivate neutrophil recruitment to adipose tissue. Despite this shortfall, it has long been well appreciated that neutrophil recruitment in other classical inflammatory settings serves as a key primer for monocyte recruitment by promoting a local milieu which favors monocyte adhesion, extravasation and activation (168). It is conceivable that neutrophil recruitment would play a similar role in adipose tissue inflammation. Thus studies to determine whether this is the case are highly warranted.

In addition to being recruited to adipose tissue in the very early stages of obesity, (within 3 to 7 days of the commencement of a high fat diet) (92), circulating neutrophils are also found to be chronically activated in blood of obese individuals (169). Interestingly, studies in lean rats have shown that circulating neutrophils are activated within the first 3-4 hours directly following a high fat meal (170). These data suggest that neutrophils likely play an important early role linking excess nutrient intake to an immune response. However further study in this area is clearly required.

3.2.3 T-cells

In contrast to neutrophils, the regulation of adipose tissue inflammation by different T-cell populations appears rather well defined. In 2009, three simultaneous reports in *Nature Medicine* (93; 95-96) provided complementary discoveries revealing that obesity-related modulation of multiple classes of T-cells in adipose tissue plays a primordial role in adipose tissue inflammation and insulin resistance. The work of

Nishimura et al (93) centered primarily on the role of $CD8^+$ T-cells in obesity. The $CD8^+$ subset of T-cells, otherwise referred to as cytotoxic T-cells, are known to predominantly produce IFNγ and IL-2, cytokines that promote a classical type 1 inflammatory response (171). Nishimura et al (93) report that numbers of $CD8^+$ T-cells are increased in adipose tissue with obesity and that this increase precedes macrophage recruitment to adipose tissue occurring as early as 2 weeks after the commencement of a high fat diet. Complementary immunohistochemistry revealed that $CD8^+$ T-cells localize alongside macrophages within crown like structures. Importantly, $CD8^+$ T-cell depletion in adipose tissue of obese mice or neutralization of $CD8^+$ T-cells with anti-CD8 antibody blunted macrophage recruitment to adipose tissue. These data suggest that the expansion of the $CD8^+$ T-cell population is a key step in the recruitment of macrophages to adipose tissue. Interestingly, using co-culture experiments the authors showed that adipocytes from obese animals promote $CD8^+$ T-cell proliferation. Furthermore, they showed that an interaction between adipocytes and CD8+ T-cells is required to promote monocyte differentiation to macrophages. Indeed, neither adipocytes nor $CD8^+$ T-cells alone possessed the ability to promote monocyte differentiation to macrophages. However, when in the presence of both adipocytes and $CD8^+$ T-cells, monocytes differentiated into macrophages and this differentiation was enhanced significantly when adipocytes were derived from obese animals. Moreover, additional co-culture experiments revealed that activated $CD8^+$ T-cells secrete the chemokines MCP-1, MCP-3 and RANTES and promote macrophage migration. Finally, Nishimura et al (93) showed that activated $CD8^+$ T-cells stimulate macrophage expression of TNFα and this effect was greatest when using $CD8^+$ T-cells isolated from the adipose tissue of obese animals. Taken together this work describes a primary role for $CD8^+$ T-cells in the development of adipose tissue inflammation wherein expanding adipocytes stimulate $CD8^+$ T-cell proliferation and activation which provokes monocyte recruitment, differentiation and activation in adipose tissue.

In contrast to the work of Nishimura et al (93) which focused primarily on the role of $CD8^+$ T-cells the work of Winer et al (95) centered on the obesity-related modulation of $CD4^+$ T-cells in adipose tissue. $CD4^+$ T-cells can be divided into

multiple subsets which include the pro-inflammatory IFNγ expressing Th1 T-cells and IL-17 expressing Th17 T-cells as well as the anti-inflammatory $GATA3^+$ Th2 T-cells and $Foxp3^+$ T-regulatory cells (T-regs) that are known to express high levels of anti-inflammatory cytokines IL-4 and IL-10. This work showed that $CD4^+$ T-cells are present in visceral adipose tissue and also localize to crown like structures alongside macrophages. Importantly, the ratio of Th1 to T-regs and Th2 T-cells in visceral adipose tissue increased with increasing adiposity as a result of expanding Th1 numbers in the face of static T-regs and Th2 T-cell populations. This Th1 expansion in visceral adipose tissue with obesity appears to be antigen driven since the Th1 cells in adipose tissue of obese mice display a severely biased T-cell receptor Vα repertoire. However, the antigen/s responsible remain to be identified. Remarkably, experimental strategies that restored the balance between Th1 to T-regs or Th1 and Th2 cells reduced inflammation and restored insulin sensitivity in obese mice. Moreover, improving the Th2 population also had an effect on adiposity. Together these data suggest that unidentified obesity-related adipose tissue antigens promote the specific expansion of the Th1 population of $CD4^+$ T-cells in adipose tissue resulting in an inflammatory bias in the adipose tissue $CD4^+$ T-cell population which leads to the development of adipose tissue inflammation and insulin resistance.

These findings were complemented by the work of Feuerer et al (96) which showed that lean adipose tissue contains a unique population of T-regs that express extremely high levels of the anti-inflammatory cytokine IL-10. This population was shown to decline in adipose tissue with high fat feeding. Importantly, widespread depletion of T-regs in lean mice led to increased expression of the inflammatory mediators TNFα, IL-6, SAA, MCP-1 and RANTES in adipose tissue and increased plasma insulin indicative of the induction of insulin resistance. Conversely, *in situ* expansion of T-regs in obese mice fed a high fat diet for 15 weeks significantly reduced plasma glucose and increased adipose expression of IL-10. Furthermore, *in vitro* experiments showed that IL-10 can blunt TNFα mediated expression of the inflammatory mediators RANTES, SAA-3 and IL-6 in adipocytes. These data suggest that T-regs likely play the important role of

repressing inflammation in lean adipose tissue and when their numbers are reduced in obesity the brake on adipose inflammation is released. Interestingly, samples taken from omental (visceral) and subcutaneous adipose tissue of obese individuals revealed higher presence of T-regs in the subcutaneous adipose depot. This difference may explain in part the lesser inflammatory nature of the subcutaneous adipose depot.

In addition to these three key reports, Ohmura et al (94) recently described a role for pro-inflammatory natural killer (NK) $CD4^+$ T-cells in adipose tissue inflammation. Here they showed that adipose macrophage infiltration and glucose intolerance is greatly reduced in high fat fed mice lacking NK T-cells. Alternatively, when NK T-cells were activated in high fat fed wild-type mice by the administration of α-galactosylceramide glucose intolerance as well as adipose macrophage infiltration and cytokine expression were exacerbated. These data fit nicely with the overall T-cell picture which suggests that during the course of obesity a coordinated increase of pro-inflammatory subsets (e.g. $CD8^+$, Th1, and NK T-cells) with diminished or static populations of anti-inflammatory T-cells (e.g. T-regs and Th2 T-cells) in adipose tissue promotes the onset of adipose inflammation that is characterized by macrophage infiltration and pro-inflammatory cytokine and chemokine expression.

3.2.4 Eosinophils

Recent evidence suggests that eosinophils may also be found in adipose tissue where they play a similar role to T-regs suppressing inflammation (172). Since IL-4 and IL-13 are known to be key to maintaining an alternatively activated macrophage population in healthy adipose tissue of lean mice the authors of this work searched for IL-4 expressing cells in adipose tissue using IL-4 reporter mice that contain a green fluorescent protein (GFP) construct downstream of the endogenous IL-4 gene. In this experiment Wu et al (172) found that a resident eosinophil population accounts for 90% of all IL-4 expressing cells in adipose tissue with basophils, T-regs and Th2 T-cells accounting for the remaining 10%. Eosinophils were shown to make up 4-5% of total adipose stromal vascular cells however these numbers declined in adipose tissue with increasing adiposity. In

accordance with a regulatory role for eosinophils in adipose tissue inflammation, eosinophil deficient mice displayed similar numbers of total adipose tissue macrophages however there was a significant reduction in the alternatively activated M2 population. Importantly, this could be reversed via reconstitution of eosinophils with wild-type but not IL-4/IL-3 double knock out bone marrow. These data suggest that eosinophil derived IL-4/IL-13 signaling is a key regulator of the macrophage inflammatory phenotype in adipose tissue. Interestingly, high fat diet studies employing mice with either depleted or enhanced eosinophil populations also revealed an important role for adipose eosinophils in the regulation of adipose mass. Indeed, whereas eosinophil deficient (ΔdblGATA) mice displayed elevated adiposity, hypereosinophilic IL-5 transgenic (Tg) mice had perigonadal fat pads about half the size of their wild-type counterparts. Infection of high fat fed mice, with *Nippostrongylus brasiliensis,* a migratory helminth also increased eosinophil numbers in adipose tissue and reduced alternatively activated adipose macrophages and adipose mass supporting the findings in IL-5 Tg mice. Remarkably these changes were maintained for 35 days post infection despite the helminth being cleared after 8 days. Glucose tolerance and insulin sensitivity was positively correlated in all studies with adipose eosinophil numbers however this is not surprising since experimental manipulation of eosinophils clearly influenced adipose mass. These data suggest that eosinophils play a key role in adipose biology not only by supplying the signal which maintains adipose tissue macrophages in an alternatively activated state but also by limiting adipose tissue expansion. Future studies are warranted to determine whether the eosinophil effect on adipose tissue mass are directly related to macrophage polarization or a separate direct metabolic action of eosinophils and eosinophil secreted factors on adipocyte lipogenesis, angiogenesis, energy expenditure or satiety signaling among other possibilities.

3.2.5 Mast cells

Mast cells like eosinophils are commonly associated with allergic inflammation; however, in contrast to eosinophils mast cell numbers appear to be increased in adipose tissue with obesity. In a letter to *Nature Medicine* Liu et al (173) revealed

that mast cells are increased in adipose tissue of obese humans and animals where they associate with microvessels. Interestingly, $Kit^{W\text{-}sh/W\text{-}sh}$ mice that are mast cell deficient as well as mice treated with the mast cell stabilizers disodium cromoglycate (DSCG) or Kitotifen displayed reduced weight gain that was related to lower visceral and subcutaneous adiposity. The anti-obesity effect of mast cell stabilizers was also present in mice that were rendered obese by 12 weeks of high fat feeding prior to stabilizer administration suggesting that a key role of mast cells in adipose tissue is to facilitate adipose tissue expansion. The anti-obesity phenotype of mast cell deficient/inactive mice was associated with an elevated basal metabolic rate that was linked to higher uncoupling protein 1 (UCP-1) expression in brown fat as well as reduced mast cell associated protease activity and angiogenesis in adipose tissue. These data suggest that adipose tissue mast cells co-ordinate basal energy expenditure with adipose tissue expansion. Indeed, since extracellular matrix proteolysis plays an important role in promoting angiogenesis by releasing proangiogenic peptides the authors proposed that mast cell associated protease activity was required for adipose tissue angiogenesis and tissue expansion. Importantly, they confirmed that reconstituting the mast cell population of $Kit^{W\text{-}sh/W\text{-}sh}$ mice with bone marrow derived mast cells from wild type and TNFα deficient mice but not IL-6 and IFNγ deficient mice enhanced cathepsin activity and promoted body weight recovery in these mice. Complementary *in vitro* experiments revealed that exposure to bone marrow derived mast cells from wild type and TNFα deficient mice but not IL-6 and IFNγ deficient mice stimulates cathepsin activity in cultured 3T3-L1 adipocytes. Taken together these data suggest that mast cell derived IL-6 and IFNγ but not TNFα stimulate adipocyte derived cathepsin activity which is central to adipose tissue angiogenesis and expansion. Despite these interesting findings studies in mice that overexpress cathepsin in adipose tissue crossed with $Kit^{W\text{-}sh/W\text{-}sh}$ mice or treated with mast cell stabilizers are warranted to confirm that mast cell dependent proteolysis is the major mechanism by which mast cells regulate obesity.

3.2.6 B-cells

In addition to these studies on macrophages, neutrophils, T-cells, eosinophils and mast cells a remarkable report in *Nature Medicine* has very recently described in great detail the role of B-cells in the etiology of adipose tissue inflammation (174). Herein B-cells were shown to accumulate in visceral adipose tissue after only 4 weeks of high fat feeding and this was associated with an increase in IgG and IgM antibodies, especially IgG2c, which was increased 3 fold in adipose tissue of obese mice. Importantly IgG and IgM were shown to be localized to crown like structures at the interface of multinucleate macrophages and dying adipocytes. Suggesting that antibodies are likely employed for dead cell clearance. Spleen IgG secretion was also increased and this was associated with an increase in serum IgG2c supporting the existence of a specific humoral immune response to obesity. Importantly, high fat fed B-cell null mice were as obese as their wild type counterparts yet displayed reduced fasting glycemia alongside improved glucose tolerance and insulin sensitivity. Reconstituting B-cells from obese mice but not lean mice reversed these effects. With regards to adipose inflammation, high fat fed B-cell null mice displayed a reduced inflammatory phenotype that was characterized by a decrease in M1 macrophages, decreased expression of IFNγ and TNFα in the stromal vascular fraction, and reduced IFNγ expressing $CD8^+$ T-cells. These data suggest that B-cells play a role in activating rather than recruiting macrophages and $CD8^+$ T-cells in adipose tissue. Accordingly, B-cell reconstitution in lymphocyte null mice had no detrimental effects on adipose inflammation or insulin sensitivity indicating that B-cells require the actions of other immune cells to exert their effects. On this note, the observation that B-cell reconstitution in major histocompatibility complex I (MHCI) or MHCII null mice had no influence on inflammation or glucose metabolism revealed that B-cell modulation of T-cells in adipose tissue occurs via antigen presentation in a MHC dependant manner. Furthermore, IgG transfer from obese mice worsened insulin sensitivity in obese mice but not lean mice and IgG was found to be localized at the crown like structures. These data imply that IgG require the presence of an obesity-related antigen to exert their effects. As for B-cell reconstitution, IgG increased TNFα and

M1 macrophage polarization and it was shown that the Fc region of the IgG was critical for these effects on adipose tissue macrophages. Finally, the authors examined circulating IgG from lean and obese individuals and found that 122 IgG segregate with insulin resistance. Notably, those at the top of the list were reactive for intracellular proteins such as RNA polymerase and Golgi proteins. These data strongly suggest that there is an auto-immune response specific to obesity that involves a humoral response to intracellular proteins released from dead adipocytes. This likely also explains the severely biased T-cell receptor Vα repertoire described for Th1 T-cells.

3.3 What leads to adipocyte death?

While it has now become clear that adipocyte death plays a central role in the immune response present in adipose tissue during obesity the precise cause of this phenomenon remains unknown. Two plausible candidates that have been proposed in the literature are endoplasmic reticulum (ER) stress and hypoxia. Those proponents of the hypoxia theory suggest that as adipocytes and adipose tissue expand they reach a critical size where hypoxia ensues leading to cell death and the induction of signals for angiogenesis and adipose tissue remodeling (175). Accordingly multiple reports have shown reduced oxygen tension in adipose tissue of obese animals and induction of hypoxia related molecular signals such as hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) (175-178). Furthermore, unsurprisingly, experimental hypoxia has been shown to cause an inflammatory response in cultured adipocytes and macrophages and to also induce adipocyte apoptosis and necrosis (176; 178). The work of Strissel et al (157) supports this theory showing that macrophage infiltration in obesity occurs in a cyclical manner that is timed to a complete tissue remodeling phenomenon where the large cells are replaced by multiple smaller adipocytes.

Alternatively, the ER stress theory suggests that the endoplasmic reticulum of expanding adipocytes may be overwhelmed by the higher protein and lipid synthesis demands required to meet the changes in tissue architecture and increased secretory profiles (179-180). Indeed, ER stress is known to occur when

changes in cellular metabolism result in the accumulation of un/misfolded proteins in the ER leading to an unfolded protein response. In mammals, this involves the activation of NF-κβ and JNK pathways and the transcription of inflammatory genes such as those encoding the pro-inflammatory cytokines. Such a response if left unresolved, as in the case of increasing obesity, may lead to apoptosis via JNK mediated expression of the Fas death receptor (181-182). Work by Ozcan and colleagues (183) first linked ER stress to obesity and the development of insulin resistance. They demonstrated the presence of ER stress in peripheral tissues during obesity and that the genetic induction of ER stress caused insulin resistance in the absence of obesity. Furthermore, a follow-up study showed that use of chemical chaperones effectively reduces ER stress in obese mice and improves insulin sensitivity (184). However, these studies did not describe whether ER stress is causally linked to adipocyte cell death or immune cell recruitment in obese animals. In support of a role for ER stress in adipocyte death, ER stress is known to be involved in beta cell apoptosis in the pancreas of hyperinsulinemic mice (185-186). It is thus plausible that ER stress may play a similar role in the adipocyte which like the pancreatic beta cell has higher secretory demands in obese mice.

3.4 Pattern recognition receptors: An additional source of inflammation in obesity

Although present evidence based on immune cell localization in adipose tissue (156) and antibody/antigen profiles (174) seem to suggest that adipocyte cell death is the primordial signal for the induction of obesity-linked inflammation, an additional source of inflammation has also been established for obesity. Indeed, Toll-like receptors (TLR) 2 and 4, which are an important piece of the innate immune system and serve as endogenous sensors for pathogen associated molecular patterns (PAMPs), have been found to be prominently activated in obese humans and animals (187-192). TLR can be found on the cell surface of sentinel immune cells, as well as epithelial and endothelial cells (193-195). Recent cell culture studies also suggest that TLR may be present and intact on the surface of adipocytes (196), skeletal myotubes (197) and hepatocytes (198). Importantly,

genetic invalidation of either TLR-2 or 4 was found to be sufficient to prevent obesity-linked inflammation and insulin resistance (189-190; 192; 199-200).

Two mechanisms have been proposed to explain the higher activation of TLR in obesity. Firstly, saturated fatty acids have been suggested to act as endogenous ligands for TLR-2 and 4 (191; 197; 199). However, this mechanism is presently subject to debate as there is some evidence to suggest that metabolites of saturated fatty acids such as ceramides rather than the saturated fatty acids themselves are the actual ligands of TLR (201-202). Nevertheless there appears to be clear evidence *in vivo* that lipid infusion leads to activation of TLR (191). Furthermore, a lipid dependent mechanism underlying obesity-linked inflammation is in line with a recent report from Kosteli et al (203) which shows that fat cell lipolysis directly promotes macrophage recruitment to adipose tissue where macrophages appear to buffer extracellular lipid. However the involvement of TLR in lipolysis mediated macrophage recruitment was not studied in this work. Further effort is warranted in this area to determine whether lipid mediated activation of TLR is an underlying cause or simply an exacerbating factor by which adipose tissue lipolysis propels obesity-linked inflammation.

In addition to fatty acid mediated activation of TLR in obesity it has also been proposed that gut microbiota may promote obesity-linked inflammation via a TLR dependent mechanism. Indeed, it is now known that both human and murine obesity is characterized by alterations in the gut microbiome that promotes increased energy harvest and inflammation (204-206). The obesity-related gut microbiome has been shown to contain higher levels of Gram-negative bacteria which contain the endogenous TLR ligand, lipopolysaccharide (LPS), in their cell wall (205). Importantly, high fat feeding was also found to strongly increase intestinal permeability by reducing the expression of genes coding for proteins of the tight junctions (206). These two factors appear to combine to raise circulating LPS by two to three fold in obese mice, a state termed metabolic endotoxemia (205). Importantly, transfer of gut microbiota from obese mice to lean mice induces obesity, inflammation and insulin resistance and this can be prevented by genetic

deletion of the TLR co-receptor CD14 (205-206). Together these data support a role for TLR in obesity-linked inflammation and likely provide a mechanism for the inflammation witnessed in tissues other than adipose, such as liver and skeletal muscle, where cell death may be less important. However, it is noteworthy that TLR are also likely involved in the primary immune response to adipocyte cell death since damage associated molecular patterns (DAMPs) such as intracellular proteins are important endogenous ligands of TLR (207).

3.5 Immune cell recruitment in other metabolic tissues

Although most studies on leukocyte recruitment in obesity are adipocentric some studies have also identified leukocyte homing and accrual in other important metabolic tissues in obesity, such as liver and skeletal muscle. Indeed, greater numbers of macrophages have been found between skeletal muscle fibers in obese humans (105) and skeletal muscle of obese animals is also found to be characterized by streaks of adipose tissue which also contains higher levels of macrophages (90). Furthermore, elevated numbers of hepatic neutrophils, macrophages/Kupffer cells, CD4 and CD8 T-cells as well as B cells have been described in obesity-related fatty liver (102-104; 106). These data suggest that immune cell recruitment is likely fundamentally linked to the changes in metabolic homeostasis that occur with obesity and that immune cells recruited to tissues other than adipose also contribute to the development of systemic inflammation in obesity. Importantly, adipose tissue inflammation appears to precede immune cell recruitment to liver (102). However, further studies are warranted to determine the precise sequence of immune cell recruitment to each of these important metabolic tissues.

In contrast to adipose tissue, the mechanism responsible for immune cell recruitment in skeletal muscle and liver has not been studied in great detail; however, it is noteworthy that neutrophils and macrophages in fatty liver have been reported to encapsulate steatotic hepatocytes in aggregates that resemble adipose tissue crown like structures (104). These data suggest that a cell death related mechanism might also contribute to hepatic leukocyte recruitment in obesity.

Alternatively, antibiotic treatment which reduces circulating LPS was also shown to effectively reduce leukocyte accumulation in liver of obese mice (106). These data imply a role for metabolic endotoxemia and TLR in obesity-related liver inflammation. Future studies are clearly required to elucidate the precise mechanisms responsible for immune cell accrual in skeletal muscle and liver with obesity.

3.6 The evolution of obesity-linked systemic inflammation

In parallel with adipose tissue expansion in obesity what begins as local inflammation induced by leukocyte infiltration and activation spreads into other tissues and eventually evolves into a chronic low grade systemic inflammatory state. By the time that insulin resistance has developed in obese individuals inflammatory signaling pathways have already been activated in most tissues throughout the body and vascular damage has likely begun (208). Current evidence supports a model wherein leukocyte infiltration and activation in adipose tissue leads to the development of pathogenic global inflammation by means of a vicious pro-inflammatory cycle. In essence, the primary invading leukocytes in the adipose tissue produce pro-inflammatory factors including cytokines, chemokines, eicosanoids, antibodies and reactive oxygen and nitrogen species that induce insulin resistance and promote pro-inflammatory adipokine, chemokine, cytokine and eicosanoid production as well as lipolysis and possibly cell death in the surrounding adipocytes. This in turn stimulates further leukocyte infiltration and activation, greater production of antibodies and pro-inflammatory mediators, increased circulating lipids, lipid deposition in other tissues and the initiation of the acute phase response.

The acute phase response induced by pro-inflammatory cytokines such as TNFα, involves a series of reactions in sites distal to the inflammatory foci that promote the neutralization of the inflammatory agent(s) (209). Upon the induction of the acute phase response hepatic protein synthesis switches to major plasma elements required for proper immune function such as complement factors, cytokines, coagulation proteins, metal-binding proteins, proteinase inhibitors and

major acute phase reactants such as C-reactive protein (CRP) and serum amyloid A (SAA) (210). These factors further the spread of inflammation in obesity and are involved in the pathogenesis of obesity-related cardio-metabolic complications (211). Indeed, while seen to be necessary for the resolution of acute cases of inflammation, such as bacterial infection, the acute phase response appears to be harmful when sustained over prolonged periods of time, such as in obesity-linked chronic low grade inflammation, where circulating concentrations of acute phase reactants such as CRP correlate positively with both markers of adiposity and cardio-metabolic disease risk (98). Thus it may be said that the chronic feed-forward production of pro-inflammatory cytokines, chemokines, adipokines, eicosanoids, antibodies and acute phase reactants mediates the spread of inflammation in obesity (Figure 3).

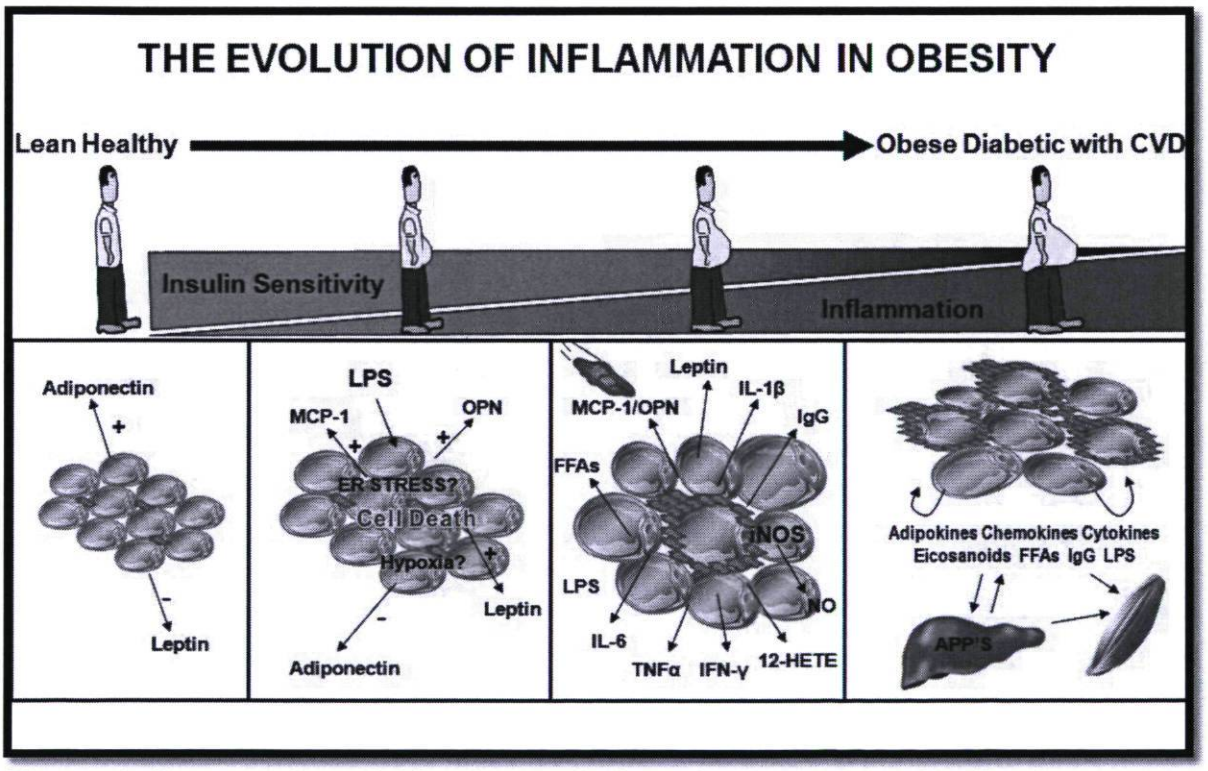

Figure 3. Proposed model for the evolution of inflammation in obesity

3.7 Mechanisms linking inflammation to insulin resistance in obesity

Several lines of evidence support a causal link between inflammation and the development of insulin resistance. Indeed, acute inflammatory stimuli (e.g. LPS) and also some pro-inflammatory cytokines, adipokines, and chemokines (e.g. TNFα, IL-1β, IFN-γ, resistin, and MCP-1) overexpressed in obesity are known to promote insulin resistance (84; 108; 147; 212-213). We are now aware that this pathogenic effect of inflammation is mediated by the activation of protein kinases, lipid mediators, and transcriptional pathways in insulin target tissues that may impair insulin signal transduction in sites both proximal and distal to the insulin receptor. It is noteworthy that some of these pathways, particularly the kinase and lipid-mediated, are also involved in free fatty acid and amino acid-induced insulin resistance (214-216). The potential mechanisms by which these pathways promote the development of inflammation-induced insulin resistance will be discussed in the following sections.

3.7.1 The Lipid Mediators

3.7.1.1 The sphingomyelin pathway

The sphingomyelin pathway is a lipid signaling pathway that is initiated upon activation of sphingomyelinases by various stress signals (217). The activated sphingomyelinases cleave the membrane phospholipid sphingomyelin forming ceramide and phosphorylcholine. The newly formed ceramide is metabolically active and functions as a second messenger. In humans, skeletal muscle ceramide content is inversely related to insulin sensitivity (218), and substantial evidence suggests that the pro-inflammatory cytokines TNFα, IL-1β, and IFN-γ, which are all elevated in obesity, employ the sphingomyelin pathway to effect signal transduction in target tissues (219-220). Furthermore, enzyme mediated ceramide catabolism was recently revealed to underlie the beneficial actions of the insulin sensitizing adipokine adiponectin (221).

In line with these data, mounting evidence suggests that the ceramide pathway is necessary for the induction of inflammation-induced insulin resistance. Indeed, TNFα promotes activation of acid-sphingomyelinase (A-SMase) and the formation of ceramides that when introduced to 32D cells in culture increase IRS-1 serine phosphorylation and subsequently impair the insulin receptor kinase (222-223). Other studies suggest that ceramides also act downstream of IRS-1 (224), causing insulin resistance either by blocking Akt/PKB recruitment to the plasma membrane (225-226), by stimulating protein phosphatase 2A-induced dephosphorylation of Akt (227-228), or by promoting the transcriptional repression of the GLUT4 glucose transporter (229-230) (see Figure 4).

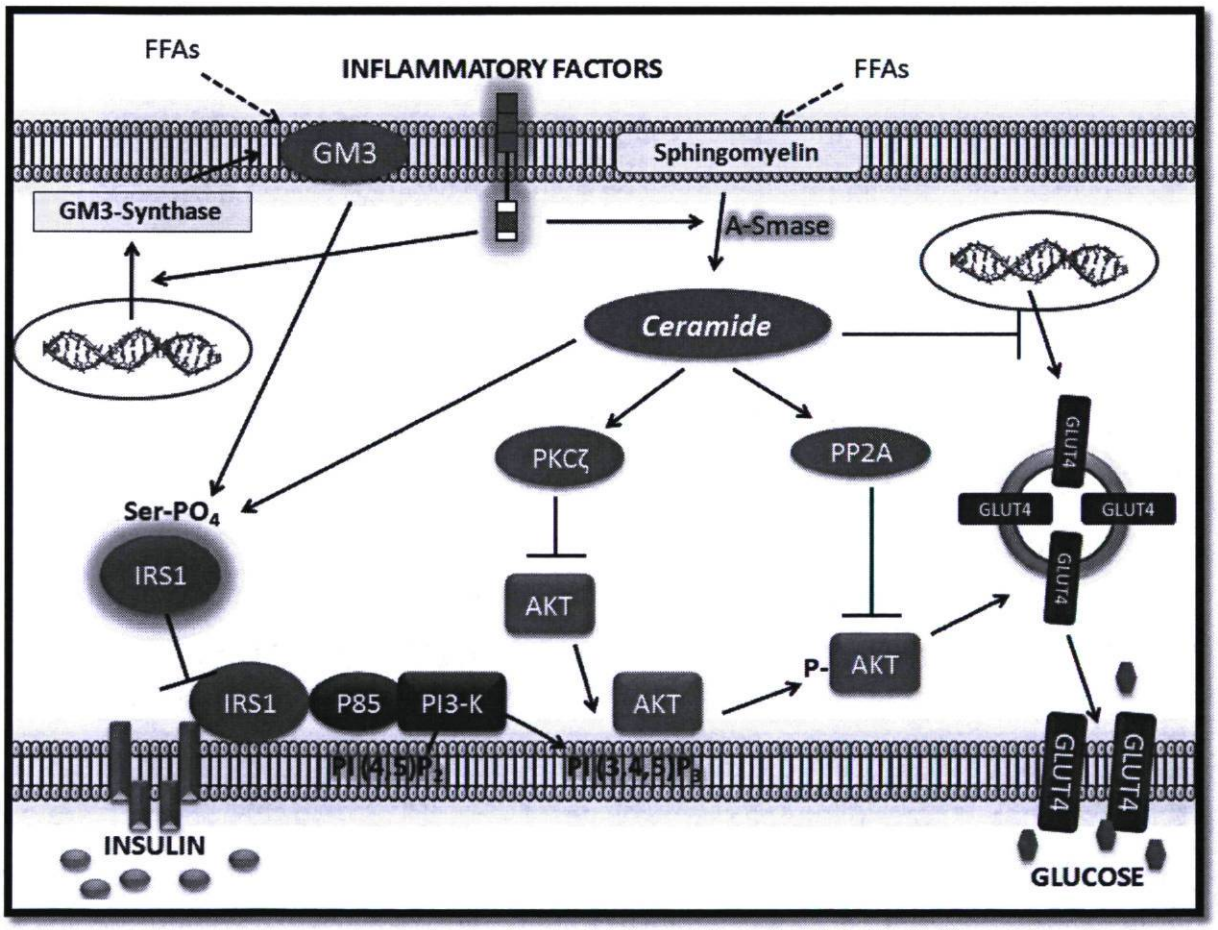

Figure 4. The lipid mediators of insulin resistance

It is noteworthy that generation of ceramide may also lead to the activation of other stress signaling pathways including JNK and PKCζ (231-232). It is therefore likely that these pathways act downstream to directly mediate ceramide induced-insulin

resistance. Indeed, ceramide induced PKCζ-mediated phosphorylation of Akt on the PH domain appears to be responsible for impaired Akt recruitment to the membrane (233). It is likely that a similar indirect mechanism involving the activation of stress pathways is also responsible for the ceramide-induced serine phosphorylation of IRS-1 but this remains to be demonstrated. Nevertheless, substantial support exists for the role of this lipid derivative in inflammation-induced insulin resistance.

3.7.1.2 Gangliosides

Gangliosides are another class of lipids that have been proposed to mediate the insulin desensitizing effect of cytokines. Gangliosides are acidic glycosphingolipids that are distributed alongside sphingomyelin and cholesterol within the cell membrane in functionally important microdomains known as lipid rafts (234). Heightened ganglioside content has been observed in the adipose tissue of two animal models of obesity (235) and studies using genetic models of ganglioside upregulation (236), and depletion (237), support a role for Gangliosides in insulin-resistance *in vivo*. Importantly, TNFα administration was shown to enhance cellular ganglioside GM3 content by raising the expression of GM3 synthase and this response was seen to be necessary for the insulin-desensitizing effect of TNFα (238). Together these data suggest that Gangliosides are involved in inflammation-induced insulin resistance; however, more work is required to determine the precise molecular mechanisms involved. It shall be interesting to see whether gangliosides, like ceramides, interact with other stress signaling pathways to exert their effects (see Figure 4).

3.7.2 The Protein Kinase Mediators

3.7.2.1 The MAP kinases

It is well known that pro-inflammatory cytokines activate MAP kinases in several cell types. The classical MAP kinase pathways include p42/44 (ERK-1/2), p38, and the c-jun N-terminal kinase (JNK). TNFα has been reported to activate each of these pathways (239-242), and the latter was reported to interact with IRS-1 and

increase its phosphorylation on serine 307 (243). Ser307 (Ser312 in humans) is located near the phophotyrosine-binding (PTB) domain in IRS-1 and is known to be phosphorylated by several mechanisms, including insulin-stimulated kinases and TNFα-activated kinases like JNK1 (242-243). Phosphorylation of Ser307 by JNK1 disrupts the interaction between the catalytic domain of the insulin receptor and the PTB domain of IRS-1, resulting in reduced insulin stimulation of downstream effectors such as PI-3kinase (243).

In accordance with this mechanism, JNK activation is elevated in insulin target tissues in obesity and the absence of JNK, induced by genetic ablation, confers resistance to the development of obesity and enhances insulin signaling (86). Furthermore, pharmacological inhibition of JNK using the cell permeable JNK inhibitory peptide improves insulin sensitivity and glucose tolerance in diabetic mice (85) and hepatic expression of a dominant negative JNK decreases the expression of gluconeogenic enzymes and reduces hepatic glucose output (244). The presence of JNK interaction protein-1 (JIP-1) is essential for the induction of JNK induced insulin resistance since JIP-1 acts as a scaffolding protein facilitating the interaction between JNK and its upstream signaling partners MAP kinase kinase-4 and 7 (245). Interestingly, JNK was shown to be required for both the production of TNFα and also its lipolytic effect in adipose tissue (240; 246). These data support a role for JNK in the development of obesity-linked inflammation and inflammation-induced insulin resistance. It will be interesting to see in the future whether this role is ceramide-dependent.

Although the role of the other members of the MAP kinase family has been less studied they also appear to play a part in inflammation-induced insulin resistance. Indeed, TNFα-induced insulin resistance is also blocked by PD98059, an inhibitor of MEK, the upstream activator of p42/44, (239; 242). Furthermore, p38 MAP kinase is rapidly activated in adipocytes upon exposure to TNFα and IL-1, and adenovirus-mediated overexpression of p38 down-regulates GLUT4 expression in these cells (241). Thus the MAP kinase family appears to play a prominent role in inflammation-mediated insulin resistance. However, further research is necessary

to elucidate the precise means and extent to which each member is involved (see figure 5).

3.7.2.2 The mTOR/S6K1 nutrient sensing pathway

First discovered as a target of the immunosuppressive drug rapamycin, mTOR is known to integrate signals arising from nutrients as well as growth factors. mTOR and its downstream effector S6K1 both possess serine/threonine kinase activity and activation of the mTOR/S6K1 pathway by insulin and amino acids is well known to inhibit insulin action by increasing phosphorylation of IRS-1 on multiple serines (215; 247-250). The mTOR pathway has also been implicated in cytokine-induced insulin resistance since both TNF-α and IFN-γ can activate mTOR/S6K1 and cause inhibitory serine phosphorylation of IRS-1 (251-253). mTOR/S6K1 activation is also necessary for endotoxin-induced production of inflammatory mediators in macrophages (254). Importantly, TNFα infusion in healthy humans induces insulin resistance in skeletal muscle in association with increased activation of S6K1 and elevated serine phosphorylation of IRS-1 (255). These data support the involvement of the mTOR/S6K1 pathway in inflammation-induced insulin resistance (see Figure 5).

3.7.2.3 Janus Activated Kinase (JAK)

Jaks act as tyrosine kinases for ligated receptors that lack intrinsic kinase activity. Accordingly Jaks are involved in early signal transduction for a wide variety of polypeptides which include leptin, TNFα, most interleukins, and IFN-γ (256). In inflammatory signaling, MAP kinases and also transcriptional mediators of inflammation-induced insulin resistance are often downstream of Jak suggesting that this protein may be involved in inflammation-induced insulin resistance. In line with this rationale, Jak2 is more highly expressed in obese insulin-resistant animals (257-259), and recent findings suggest that Jak2 may directly interact with insulin signaling intermediates and impair insulin signaling by repressing Akt Ser473 phosphorylation in insulin-resistant muscle cells (260). Jak2 was also found to partially mediate ceramide-induced defects in insulin signal transduction. Thus Jaks appear as potential mediators of inflammation-induced insulin resistance.

However, the extent of their contribution in obesity remains to be fully elucidated (see Figure 5).

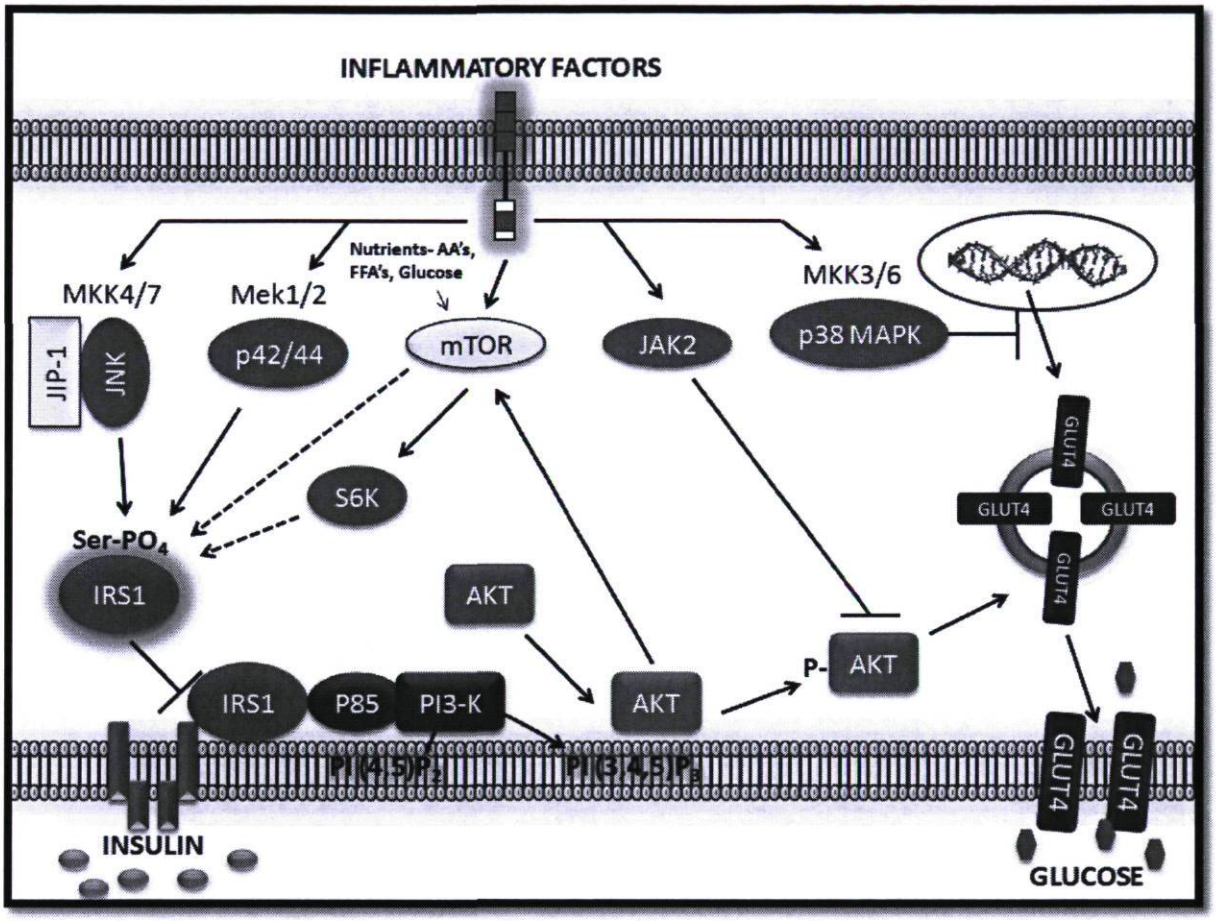

Figure 5. The protein kinase mediators of insulin resistance

3.7.3 The Transcriptional Mediators

3.7.3.1 The IκB kinase (IKK)-NF*k*B pathway

NF-κβ , a collective name for inducible dimeric transcription factors of the Rel family of DNA-binding proteins, is found in essentially all cell types and is involved in the activation of a large number of genes in response to infection and inflammation (256). The subcellular location of NF-κβ is controlled by a family of inhibitory proteins, termed IκBs, which bind NF-κβ and mask its nuclear localization signal, thereby preventing nuclear uptake. Exposure of cells to pro-inflammatory adipokines, cytokines, and endotoxins leads to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of IκB, which frees NF-κβ to

translocate to the nucleus where it regulates gene transcription. The multi subunit IκB kinase (IKK) responsible for IκB phosphorylation is the point of convergence for most NF-κβ -activating cytokines.

Yuan and colleagues (257) first suggested that IKK was a potential link between inflammation and obesity-linked insulin resistance. Using both heterozygous deletion of IKKβ (IKKβ +/-) and pharmacological inhibition of IKK via high dose salicylate treatment they were able to improve insulin sensitivity in rodent models of obesity. Interestingly, myeloid specific ablation of IKKβ was subsequently found to prevent the development of insulin resistance in all insulin target tissues (258). While LIKK mice that contain a constitutively active form of IKKβ in their liver, displayed enhanced hepatic cytokine production that was linked to the development of global insulin resistance (259). Together these studies suggest that IKK likely promotes insulin resistance via the function of its downstream transcription factor NF-κβ which controls both cytokine production and the expression of inflammatory signaling mediators such as iNOS (see Figure 6). However, it is noteworthy that IKK has also been shown to interact with and promote serine phosphorylation of IRS1 therefore IKK should also be considered as a kinase mediator of insulin resistance (260). Future studies wherein IKK is constitutively active but NF-κβ is lacking its transcriptional activity would be very important for delineating the precise contribution that each of these potential mechanisms make to insulin resistance.

3.7.3.2 Inducible nitric oxide synthase

The radical gas nitric oxide (NO) is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). The expression and activity of the inducible isoform of NOS (iNOS) is synergistically stimulated by bacterial endotoxins and also inflammatory cytokines. When induced, this NOS isotype generates NO at a much higher rate and for longer periods of time than constitutive NOS enzymes (nNOS and eNOS) (261). The pathways regulating iNOS expression seem to vary in different cells and species, but it is widely recognized that the IKK pathway regulates iNOS transcription through multiple NF-κβ binding sites on the iNOS

promoter (262). Other important transcription factors for iNOS induction are IRF-1, STAT1α, AP-1 and C/EBP (262). There is also evidence that MAP kinases, and in particular, JNK increase iNOS transcription in macrophages (263-265). Interestingly the JNK pathway may also regulate iNOS mRNA stability (266) which is another major mechanism of iNOS induction by cytokines.

It was first proposed more than a decade ago that insulin resistance represents a deleterious effect of iNOS induction during systemic inflammation. Administration of the endotoxin lipopolysaccharide (LPS) in rats was seen to induce iNOS in muscle, liver, and adipose tissues (267-268). Furthermore, cytokines and LPS also induced iNOS expression in cultured muscle and adipose cells causing marked insulin resistance (267; 269) which was significantly abrogated by iNOS inhibition (269). Importantly, iNOS was subsequently found to be more highly expressed in fat and skeletal muscle tissues of dietary and genetic models of obesity as well as type 2 diabetic humans and high-fat fed obese mice lacking iNOS were protected from developing skeletal muscle insulin resistance (84; 270-274). These findings indicated that iNOS plays a key role in the pathogenesis of insulin resistance.

iNOS is thought to induce insulin resistance via NO and peroxynitrite ($ONOO^-$) mediated post translational modifications of insulin signaling proteins (275). Indeed, iNOS-derived NO in animal models has been shown to cause S-nitrosylation (or S-nitrosation) of the insulin receptor, IRS-1 and Akt resulting in reduced activation of PI 3-kinase and Akt (274; 276). In biological systems, NO also reacts with superoxide (O_2^-) to form the potent oxidant peroxynitrite ($ONOO^-$) which is known to produce nitration of tyrosine residues (277-278). Interestingly, increasing $ONOO^-$ levels in 3T3-L1 adipocytes with a NO/O_2^- donor (SIN-1) inhibited insulin-stimulated glucose transport and was associated with increased nitration of key tyrosine residues within IRS-1 as assessed by mass spectrometry (279). This was also found to occur *in vivo* in a model of lipid-induced insulin resistance where lipid infusion was found to increase tyrosine nitration of IRS1 and Akt in muscle and liver *in vivo* (280) (see Figure 6). These data suggest that iNOS mediated nitrosative modifications underlie iNOS-induced insulin resistance.

3.7.3.3 The suppressors of cytokine signaling (SOCS)

The SOCS family of proteins, as the name suggests, play an important role in the negative feedback control of cytokine-activated pathways (281). SOCS expression is rapidly upregulated in response to several cytokines (TNFα, IL-1β, IL-6, and IFN-γ) and also adipokines (leptin, resistin) as a means of asserting feedback inhibition on inflammatory signaling. Emanuelli and colleagues (282) first demonstrated that SOCS might be involved in the development of inflammation-induced insulin resistance when they reported the TNFα-dependent overexpression of SOCS-3 in the adipose tissue of obese mice. Since then SOCS overexpression has been reported in the skeletal muscle of diabetic patients (283), and also in experimental models of obesity and LPS-induced insulin resistance, with the expression of SOCS-1 and -3 being greatest in liver, followed by muscle and adipose tissue (284). In accordance with this data, SOCS-3 appears to play a major pathogenic role in the liver as a mediator of hepatic insulin resistance (285) and hepatic steatosis (286). It is also noteworthy that the hepatitis C-mediated downregulation of liver IRS-1 and 2 occurs via a SOCS-dependent mechanism (287). On another interesting note, the cytokine-mediated upregulation of SOCS proteins in obesity may also impact on the hypothalamic regulation of metabolism, as SOCS-3 deficiency in the brain has been shown to improve leptin sensitivity and to confer resistance against the development of diet-induced obesity (288). Furthermore, SOCS-3 is also recognized as a mediator of resistin-induced insulin resistance in adipocytes (289).

The molecular action of SOCS on insulin resistance is thought to involve the obstruction of several components of the insulin signaling cascade (see Figure 6). Indeed, SOCS contain an SH2-domain allowing interaction with phosphotyrosines within the insulin receptor leading to downstream inhibition of IRS-1 tyrosine phosphorylation (282; 290-292), reduced association with the p85 regulatory element of PI-3 kinase (282), and impaired insulin-dependent activation of ERK-1/2 and Akt/PKB (292). In addition, evidence gathered from SOCS overexpression models also suggests that SOCS-1 and -3 may impair insulin signaling by targeting IRS-1 and IRS-2 for ubiquitin-mediated proteasomal degradation (293). However,

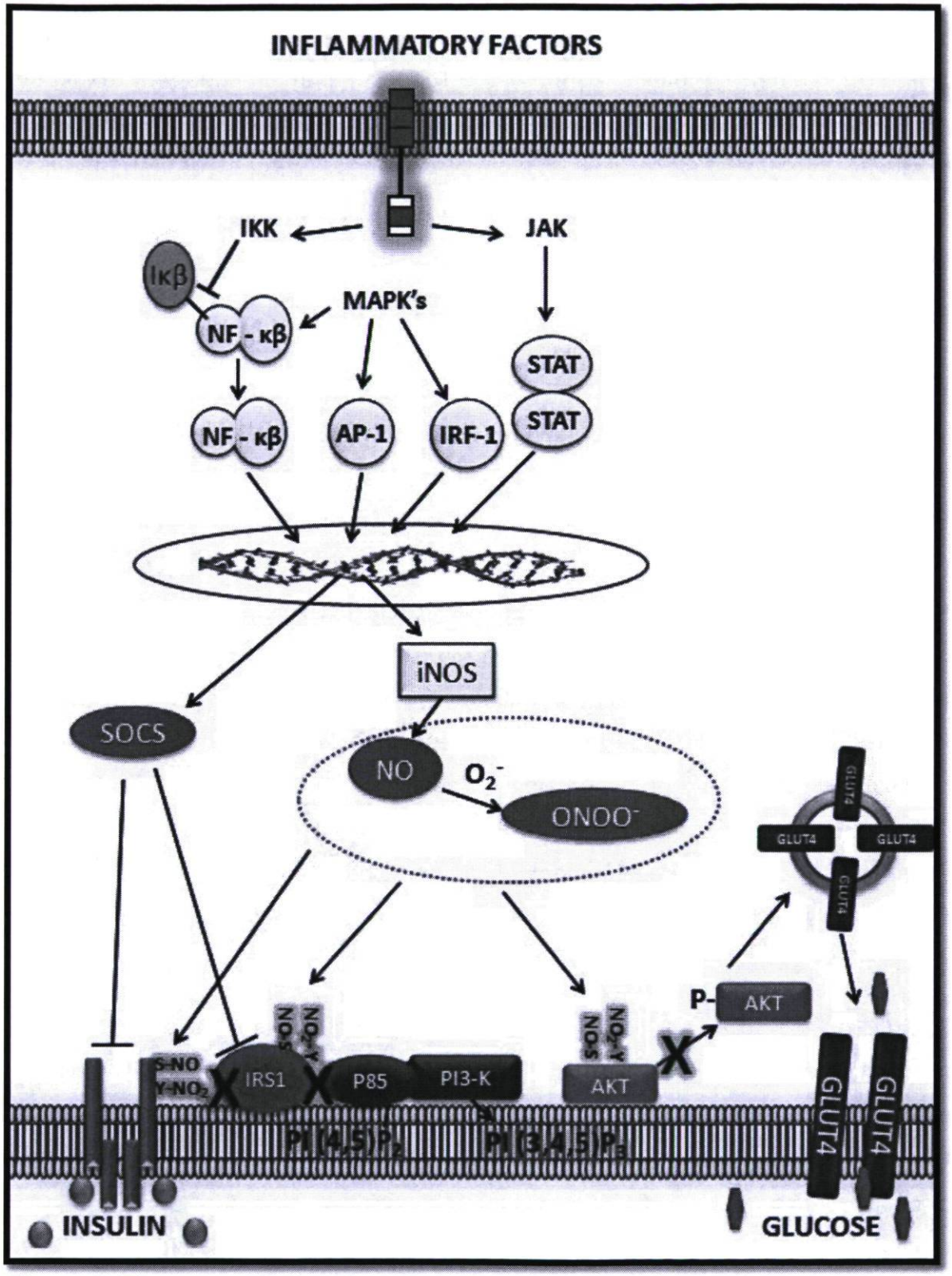

Figure 6. The transcriptional mediators of insulin resistance

the physiological relevance of this mechanism is subject to debate, as the degree of SOCS-3 induction does not always correlate with the degradation of IRS-1 and IRS-2 (294). In any case, the inflammation-mediated induction of SOCS molecules can be seen to have a profound impact on hepatic function in particular but also on other insulin target tissues including the brain.

In summary, inflammatory mediators such as adipokines and cytokines may employ various mechanisms including lipid derivatives, serine/threonine kinases, and transcriptional pathways to inhibit insulin signaling. The influence of each mechanism on insulin signaling often varies between tissues and it is most likely that global insulin resistance occurs as a result of the combined actions these mechanisms rather than one in particular.

3.8 The interleukin-6 paradox

While the involvement of inflammation in the development of insulin resistance is rather well defined the role of the cytokine IL-6 remains highly controversial (295-298). It is a widely held viewpoint that IL-6 is a prominent pro-inflammatory mediator that instigates insulin resistance in a manner similar to TNFα during the course of obesity (295; 299-302). However, this dogma is the subject of current debate and has been challenged by multiple observations in the literature that suggest a contrary role for IL-6. Indeed, IL-6 has been shown to dampen inflammation by suppressing the expression of pro-inflammatory cytokines such as TNFα and IL-1β while promoting the secretion of anti-inflammatory factors such as IL-1 receptor antagonist and IL-10 (303-305). Furthermore, mice lacking the IL-6 receptor specifically in liver were recently shown to display elevated hepatic inflammation and impaired glucose metabolism (306). These data suggest that IL-6 might be better thought of as an anti- rather than pro-inflammatory mediator *in vivo* and that increased IL-6 in obesity might actually represent a consequence rather than a cause of obesity-related inflammation.

IL-6 has also been identified as a skeletal muscle derived cytokine or '*Myokine*' that is released from skeletal muscle in large quantities following exercise (307-308). Consequently, it has been suggested that muscle derived IL-6 might underlie

the beneficial effects of skeletal muscle contraction on glucose and lipid metabolism (309-312). In line with this hypothesis, administration of IL-6 has been reported to improve muscle glucose uptake and fatty acid turnover by stimulating AMPK activity (313-316). Furthermore, IL-6 has also been reported to play an important role in the insulin mediated regulation of hepatic gluconeogenesis via STAT-3 mediated suppression of the transcription of the gluconeogenic enzymes PEPCK and G6Pase (317-318). Moreover, adipose derived IL-6 was also recently shown to mediate the insulin sensitizing actions of adiponectin in liver (319). Importantly, the source of adipose IL-6 in this study was found to be the myeloid component thus these findings argue against a distinct role for immune cell derived versus muscle derived IL-6. Instead, these data suggest a model wherein IL-6 acts as an endogenous break on inflammation and an important mediator in the tissue cross talk regulating whole body energy metabolism. However further research is warranted to understand why IL-6 appears to act as a mediator of insulin resistance in some circumstances and a beneficial metabolic signal in others.

4 - Omega-3 fatty acids

Omega-3 (ω-3) fatty acids represent a class of polyunsaturated fatty acids (PUFA) that are characterized by the presence of a carbon-carbon double bond at the third carbon from the methyl end of the fatty acyl chain (320). They are considered essential fatty acids because they may not be synthesized *de novo* by vertebrates. However, endogenous elongase and desaturase enzymes can be employed to produce long chain ω-3 PUFA such as the 20 carbon eicosapentaenoic acid (EPA; 20:5, n-3) and 22 carbon docosahexaenoic acid (DHA; 22:6, n-3) from the 18 carbon parent ω-3 fatty acid α-linolenic acid (ALA; 18:3, n-3) (320). Linseed and canola oil are known to be good dietary sources of ALA (18:3, n-3) whereas marine oils are considered to be the best source of long chain ω-3 PUFA (321). It is noteworthy that most of the actions ascribed to ω-3 PUFA appear to be mediated by the long chain members, EPA and DHA.

When considering the bioactivities of ω-3 PUFA it is important to acknowledge the ω-6 class of fatty acids. Indeed, aside from the lack of a carbon-carbon bond at the

ω-3 position, which results in the first double bond being present at the sixth carbon from the terminal end of the fatty acyl chain, ω-6 PUFA are structurally identical to their ω-3 counterparts and thus may be metabolized by the same enzymes (320). However, despite this close structural similarity, these two classes of essential PUFA display distinct and often competing physiological functions. The major ω-6 PUFA include the parent 18 carbon linoleic acid (LA; 18:2, n-6) and the 20 carbon arachidonic acid (AA; 20:4, n-6). In the diet, LA can be found in most vegetable oils such as corn, safflower and sunflower oil (321). The structures of the major ω-3 and ω-6 PUFA are shown in Table 2.

Table 2. Nomenclature and structures of the major ω-3 and ω-6 PUFA

Common name	Systematic name	Chemical Structure
Linoleic acid LA (18:2, n-6)	*9Z,12Z-octadecadienoic acid*	
Arachidonic acid AA (20:4, n-6)	*5Z,8Z,11Z,14Z-eicosatetraenoic acid*	
α-linolenic acid ALA (18:3, n-3)	*9Z,12Z,15Z-octadecatrienoic acid*	
Eicosapentaenoic acid EPA (20:5, n-3)	*5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid*	
Docosahexaenoic acid DHA (22:6, n-3)	*4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid*	

Historically speaking, it is believed that the human diet contained a relatively balanced ratio of ω-6 and ω-3 PUFA up until the industrial and agricultural advancements of the 20th century (322). During this era, the widespread implementation of grain feeding for livestock and the development of a mechanized process that allowed the mass production of vegetable oil led to the rapid development of disequilibrium in the dietary supply of ω-6 and ω-3 fatty acids. These changes were so severe that in this relatively short period of time, the average dietary ω-6: ω-3 ratio vaulted from around 1:1 to ~25:1.

Dyerberg and Bang (323-324) were first to make the connection between this shift in PUFA consumption and poor human health in the late seventies when they performed an epidemiological study comparing the incidence of cardiovascular disease (CVD) related death in Caucasian Danes with that occurring in the Greenland Eskimos. Here they showed that the Greenland Eskimos, a population that has maintained a relatively balanced intake of ω-6 and ω-3 fatty acids over time, displayed markedly reduced incidence of CVD related death compared to the Danes. Following on from this study, numerous other pathologies have been linked to an elevated ω-6: ω-3 ratio. These include but are not limited to: cancer (325), Crohns disease (326), rheumatoid arthritis (327), and systemic lupus erythrematosus (328).

In light of these wide ranging associations with human pathologies, much effort has been exerted towards elucidating the mechanisms of action of ω-3 fatty acids *in vivo*. Thus far ω-3 fatty acids have been proposed to improve membrane fluidity, impede the formation of ω-6 derived bioactive lipids, activate endogenous nuclear and membrane bound receptors and serve as substrates for a novel genus of bioactive lipids termed resolution mediators. Each of these proposed mechanisms of ω-3 fatty acid action will be discussed in the following sections.

4.1 Membrane fluidity

One of the original mechanisms proposed to underlie the beneficial actions of ω-3 fatty acids was an increase in membrane fluidity. In brief, it was postulated that long chain ω-3 PUFA would simply replace shorter less unsaturated fatty acids in

the lipid bilayer and thereby automatically improve membrane fluidity leading to altered cellular function by virtue of effects on membrane bound signaling proteins and ion channels. In line with this hypothesis, ω-3 PUFA were found to integrate membrane phospholipids at the Sn-2 position replacing the less unsaturated LA (18:2, n-6) and AA (20:4, n-6) (329-331). However, membrane modeling studies have shown that increasing the unsaturation of a fatty acid component of membrane phospholipids beyond 3 double bonds leads to no additional improvement in actual membrane fluidity (332-333). This means effective gains in membrane fluidity achieved by administration of the ω-3 fatty acids EPA or DHA which have 5 and 6 double bonds respectively would be no greater than that achieved with the parent ω-3, ALA, which contains 3 double bonds or the ω-6 fatty acid, AA, which contains 4 double bonds and may also be integrated at the Sn-2 position of membrane phospholipids. Thus this simplistic model most likely does not explain the beneficial effects ω-3 versus ω-6 PUFA.

In contrast, the longest most unsaturated ω-3 PUFA, DHA, does appear to alter membrane dynamics in a specific manner, albeit via a more complex mechanism than first envisaged. Indeed, the structure of DHA has been found to be sterically incompatible with cholesterol (334). Accordingly, DHA but not EPA administration has been reported to modify both membrane cholesterol content and localization in the lipid bilayer (335-337). Moreover, DHA administration is found to promote the lateral segregation and alter the composition of cholesterol rich microdomains known as lipid rafts which serve as membrane platforms for multiple signaling events (335; 337). Such DHA-dependent actions have been associated with altered protein rearrangement and co-localization in these important membrane compartments leading to altered downstream events (335; 337-340). Although it is difficult to prove that the DHA dependent modification of membrane raft signaling events such as protein-protein interactions are the direct result of changes in the lipid microenvironment rather than downstream effects of altered lipid mediator signaling, the impact of DHA on membrane lipid raft dynamics should be considered when discussing the mechanisms of action of ω-3 PUFA.

4.2 ω-3 PUFA and the formation of ω-6 derived bioactive lipids

The earliest studies aimed at discerning the mechanisms underlying the cardio-protective effects of ω-3 PUFA initially revealed that the long chain ω-3 PUFA, EPA and DHA, competitively inhibit the formation of AA derived pro-inflammatory and pro-thrombotic eicosanoids that are synthesized in the 5-lipoxygenase (5-LOX) and cyclooxygenase (COX) pathways. Collectively, these studies showed that EPA competes with AA in the 5-LOX and COX pathways to generate less active or entirely inactive pentaene leukotrienes and triene prostanoids; while, DHA acts as a potent inhibitor of AA oxygenation in the COX pathway (341-346). These studies led to a general consensus that ω-3 PUFA exert most of their beneficial effects by blanketing the potent actions of their ω-6 counterparts (347). This mechanism of action is of particular interest for obesity and insulin resistance since 5-LOX activating protein and the AA derived product of the 5-lipoxygenase pathway LTB_4 were recently shown to promote obesity-related adipose tissue inflammation and lipolysis (136-137).

In addition to leukotrienes and prostanoids, the ω-6 PUFA AA is also metabolized to form other biologically important lipid mediators termed endocannabinoids (348). Indeed, the two most studied members of this family, anandamide/*N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), are AA metabolites. Importantly, endocannabinoid signaling plays an important role in the regulation of food intake (349) and elevated endocannabinoid tone seems to contribute to the peripheral metabolic derangements taking place in obesity (350). Although there are relatively few studies on the impact of ω-3 PUFA on the generation of ω-6 derived endocannabinoids in the literature, it appears that the formation of these AA derivatives may also be sensitive to dietary ω-3 content (351-353). Notably, a diet rich in DHA was shown to reduce concentrations of 2-AG in the brain (351) and the inclusion of fish or krill oil in a high fat diet reduced levels of both AEA and 2-AG in adipose tissue, skeletal muscle, liver, kidney and heart (352-353). These studies suggest that ω-3 PUFA might exert part of their beneficial effects by limiting the formation of endocannabinoids; however, the mechanism underlying this dietary regulation remains unknown.

4.3 ω-3 PUFA as ligands

Aside from their established role in eicosanoid formation and potential capacity to regulate the endocannabinoid system, long chain ω-3 PUFA have also been proposed to act as ligands for endogenous receptors. ω-3 PUFA were first identified as natural ligands for the PPAR nuclear receptor family in studies using reporter assays which showed that a wide array of fatty acids could activate these important nuclear receptors (354-355). These findings have been supported by x-ray crystallography and computational studies which suggest that the structures of long chain ω-3 PUFA possess affinity for the ligand binding domains of PPARs (356-357). Accordingly, multiple studies have since linked PPAR activation to the physiological outcomes of ω-3 PUFA administration *in vivo*. Indeed, the insulin sensitizing and lipid lowering actions of ω-3 PUFA were seen to be lost in mice lacking PPARα in liver (358). Furthermore, fish oil administration was found to raise circulating adiponectin in a dose and time dependent manner and this effect was inhibited by treatment with a chemical inhibitor of PPARγ, bisphenol-A-diglycidyl, but remained in PPARα null mice (359). These data suggest that activation of diverse PPARs also likely contributes to the beneficial actions of ω-3 PUFA.

In addition to their role as endogenous ligands for the PPAR nuclear receptor family long chain ω-3 PUFA have also been found to activate the G-protein coupled receptor (GPCR) GPR120. GPR120 was first identified as a receptor for long chain ω-3 PUFA by Hirasawa and colleagues (360), when they showed that long chain ω-3 PUFA in the gut promote release of the incretin hormone, glucagon like peptide-1 (GLP-1), by activating this orphan receptor. More recently, the work of Oh and colleagues (361) revealed that DHA and EPA exert their anti-inflammatory and insulin sensitizing effects by activating GPR120 in adipose tissue macrophages and adipocytes. Interestingly, GPR120 appears to compete with TLR's for intracellular signaling components namely TAB1, and thereby inhibits downstream inflammatory signaling. Importantly, the anti-inflammatory and insulin sensitizing effects of EPA and DHA were lost in GPR120 null mice. Future studies are warranted to determine whether ω-3 PUFA signaling via GPR120 is also

responsible for the anti-inflammatory effects of ω-3 PUFA in other tissues and in conditions where TLR signaling is less important.

4.4 ω-3 derived resolution mediators

Although it was originally thought that inflammation subsided when the production of pro-inflammatory lipid mediators waned, more recently, it has become apparent that lipid oxygenation pathways may also produce anti-inflammatory lipids that actively promote the return to homeostasis during inflammation (362). The work of Levy et al (363) elegantly described the existence of a so called 'resolution circuit' wherein first phase pro-inflammatory lipids, namely leukotrienes, signaled discrete changes in enzyme expression and activity that lead to the production of anti-inflammatory lipids derived from the same long chain ω-6 PUFA substrate. The latter product known as lipoxin A_4 (LXA_4) would then act to switch off the inflammatory response. Further screening of inflammatory exudates during the resolution phase of immune responses revealed that multiple families of novel lipid derived resolution mediators exist. Interestingly most were derived from long chain ω-3 PUFA (362; 364-365). The identification of these novel ω-3 derivatives and their biosynthetic pathways has greatly expanded our understanding of long chain ω-3 PUFA action. The major classes of ω-3 derived resolution mediators identified to date are the resolvins, protectins and maresins. The biosynthesis and mechanisms of action of these mediators will be described in the following sections.

4.4.1 ω-3 resolution mediator synthesis

Resolvin (Rv) which stands for 'resolution phase interaction products' is the term given to the first identified class of ω-3 derived resolution mediators. Rv's may be classified as either E-series derived from enzymatic oxidation of EPA (20:5 n-3) (Figure 7) or D-series derived from DHA (22:6 n-3) (Figure 8). The synthesis of E-series resolvins is a three step process (366). EPA is first converted to the hydroxy intermediate 18R-HEPE by COX-2 or cytochrome P450. It is important to note here that the enzymatic formation of hydroxy intermediates such as 18R-HEPE actually involves the initial formation of hydroperoxy (Hp) intermediaries (not depicted in

Figure 7) that are rapidly reduced via epoxidation and hydrolysis to the final hydroxy product. In this case, the 18R-HEPE is then transformed to a 5S,6R-epoxy intermediate by 5-LOX activity. Finally the 5S,6R-epoxy-18R-HEPE is converted to either RvE1 by LTA_4-hydrolase or reduced to form RvE2.

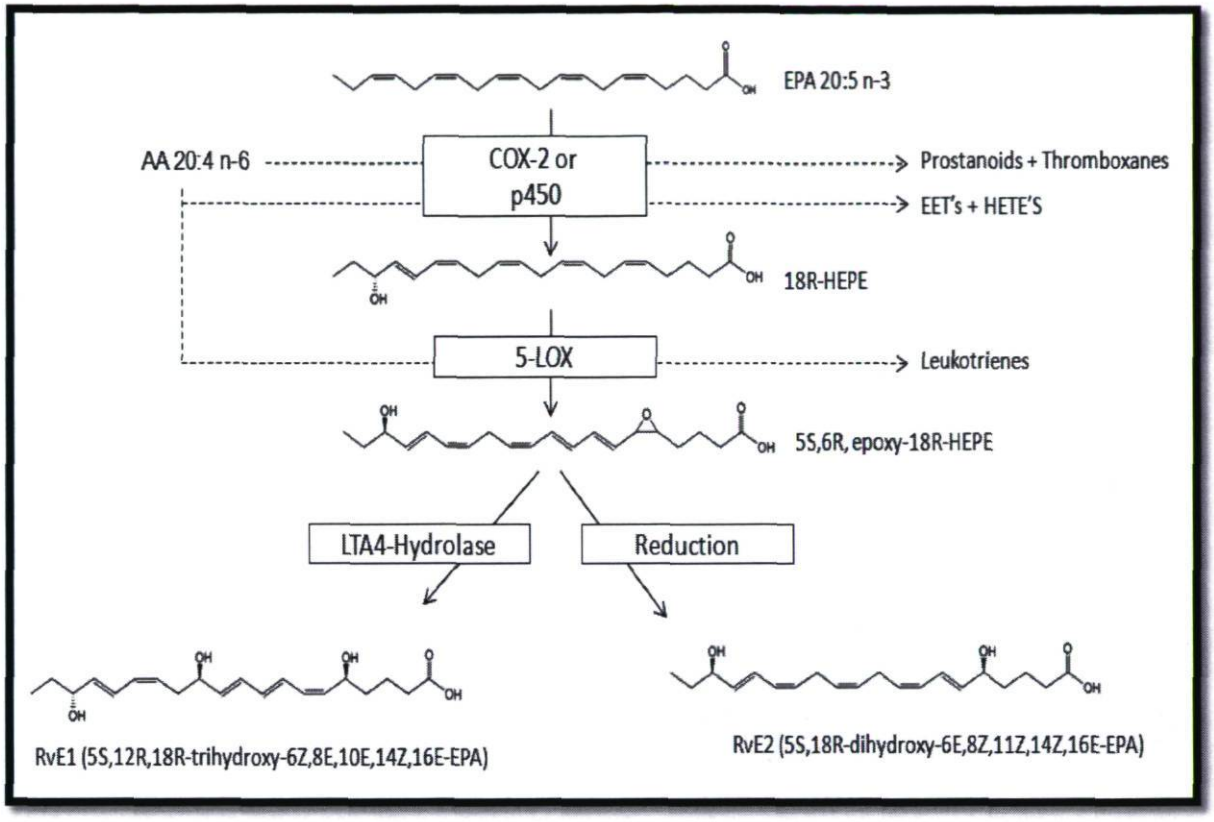

Figure 7. The E-Series Resolvins

D-series Rv's (RvD1, RvD2, RvD3 and RvD4) are also produced via sequential oxidation (367). However in this case the enzymes involved are 12/15-LOX and 5-LOX (Figure 8). Firstly, 12/15-LOX converts DHA to the hydroxy intermediate 17S-HDoHE, via the formation and reduction of 17S-HpDoHE (not depicted in Figure 8), a common step in the synthesis of all RvD's. Next 5-LOX inserts a hydroperoxy group at the 7 position of 17S-HDoHE (Figure 8). The 7S-Hp,17S-HDoHE is then converted to RvD1 and its epimer RvD2 via epoxy intermediates. 5-LOX interactions with 17S-HDoHE may also lead to the formation of an alternative hydroperoxy intermediate, 4S-Hp,17S-HDoHE, which results in the subsequent formation of RvD3 and epimeric RvD4 (Figure 8).

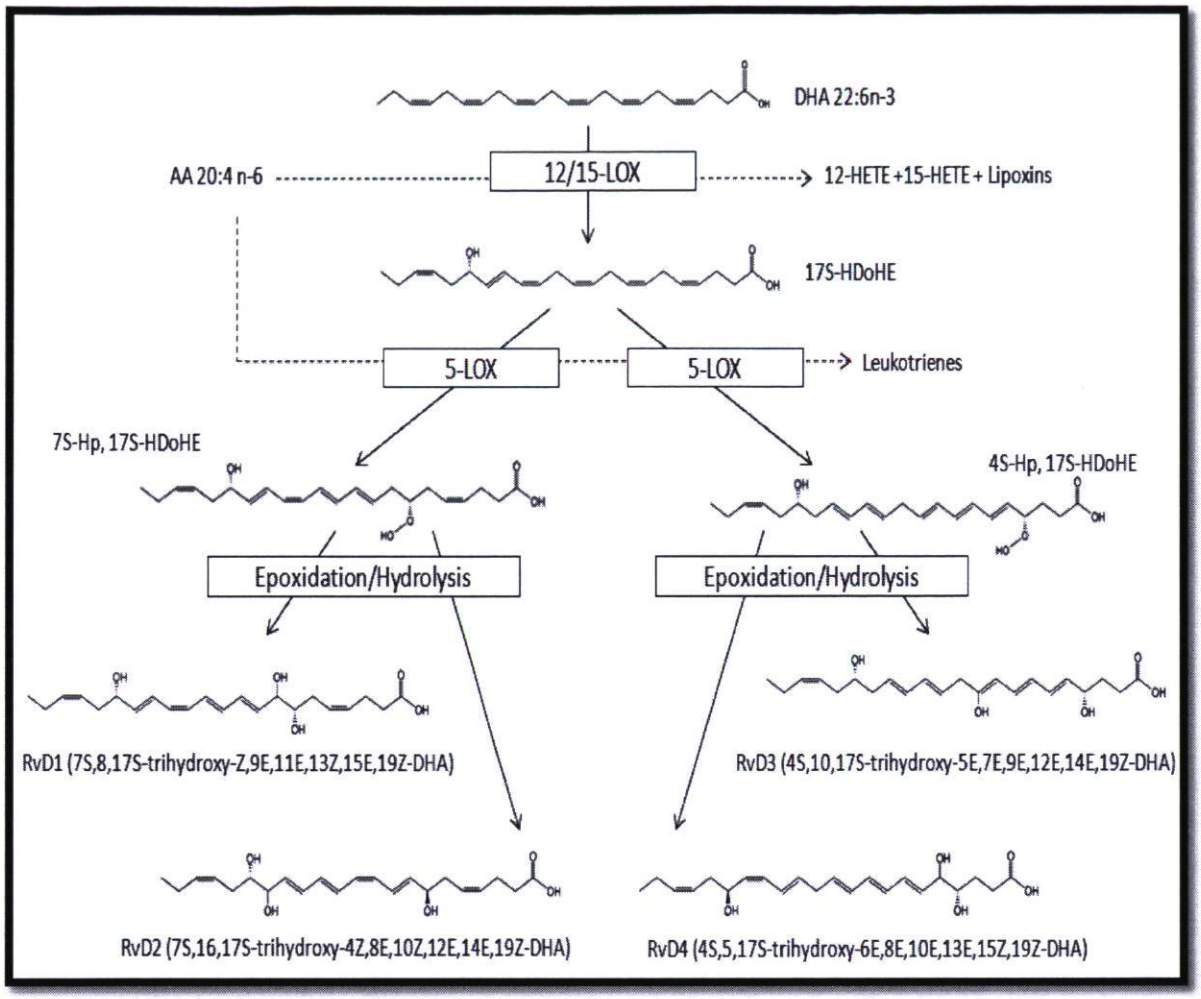

Figure 8. The D-Series Resolvins

Although the precise regulation is not completely understood, the proclivity of LOX enzymes to produce multiple regiospecific isomers from the same substrate, as is the case for 17S-HDoHE, is believed to reside in the fact that the LOX enzymatic pocket can accept fatty acid substrates in both the conventional (methyl end first) and inverse orientations (carboxy terminal first). Frameshift realignments are also thought to underlie the potential of a given fatty acid substrate to be transformed into multiple regiospecific isomers by the same enzyme (368-369).

Protectin D1 (PD1) is somewhat similar to the D-series Rv's in that it is derived from DHA and produced as a result of 12/15-LOX activity. However, the initial product in the PD1 biosynthetic route is the hydroperoxy intermediate of DHA, 17S-HpDoHE (370), which in this case is not reduced to 17S-HDoHE (Figure 9).

Instead, for PD1 synthesis, 17S-HpDoHE is first converted to a 16,17 epoxide and then to PD1. A natural 10S isomer of PD1 can also be produced via a double LOX reaction (370). This mediator holds similar or even greater activity to PD1 but, it is produced in much smaller quantities *in vivo* and is referred to as 10S,17S-DiHDoHE or PDX in the literature (371).

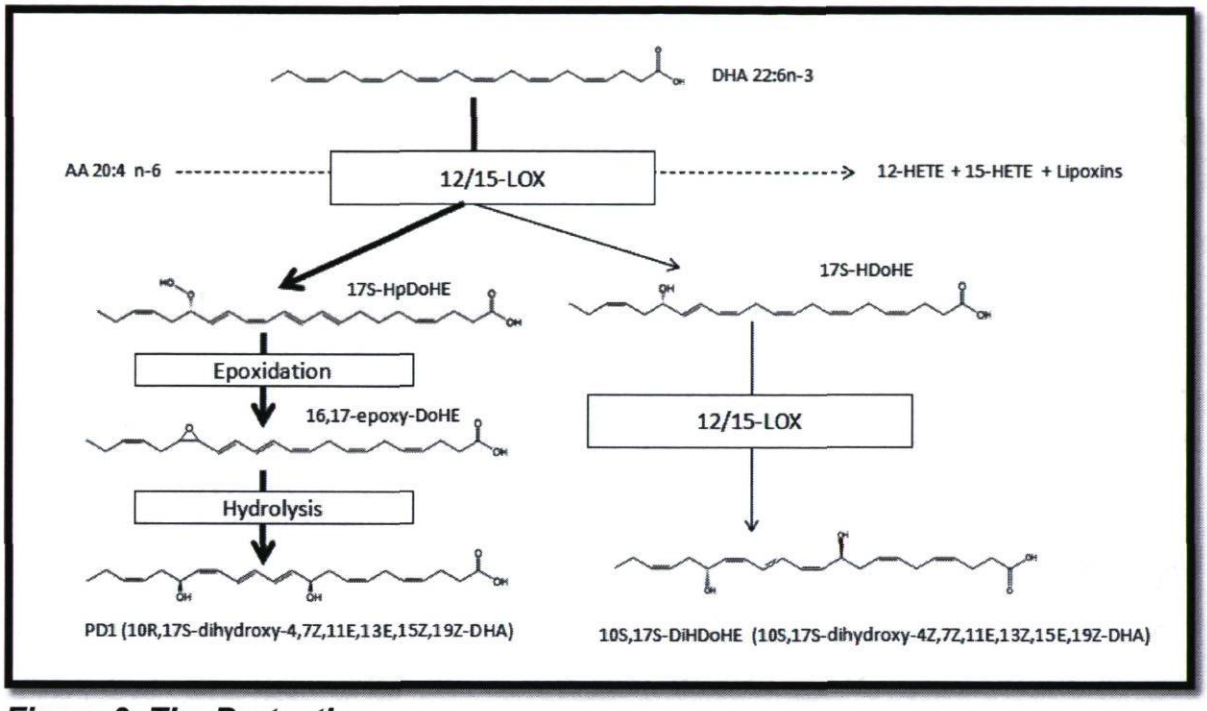

Figure 9. The Protectins

Finally, Maresins (MaR's) are the most recently identified family of ω-3 derived resolution mediators. This new family is of interest since it appears to be specifically produced by macrophages. The biosynthesis of MaR's is similar to PD1 in that it begins with the formation of a hydroperoxy-intermediate of DHA (Figure 10). In this case macrophage 12/15-LOX via 12-LOX rather than 15-LOX activity is thought to convert DHA to 14S-HpDoHE which is then converted to a 13,14-epoxide-containing intermediate that is enzymatically hydrolyzed to form MaR1 (372).

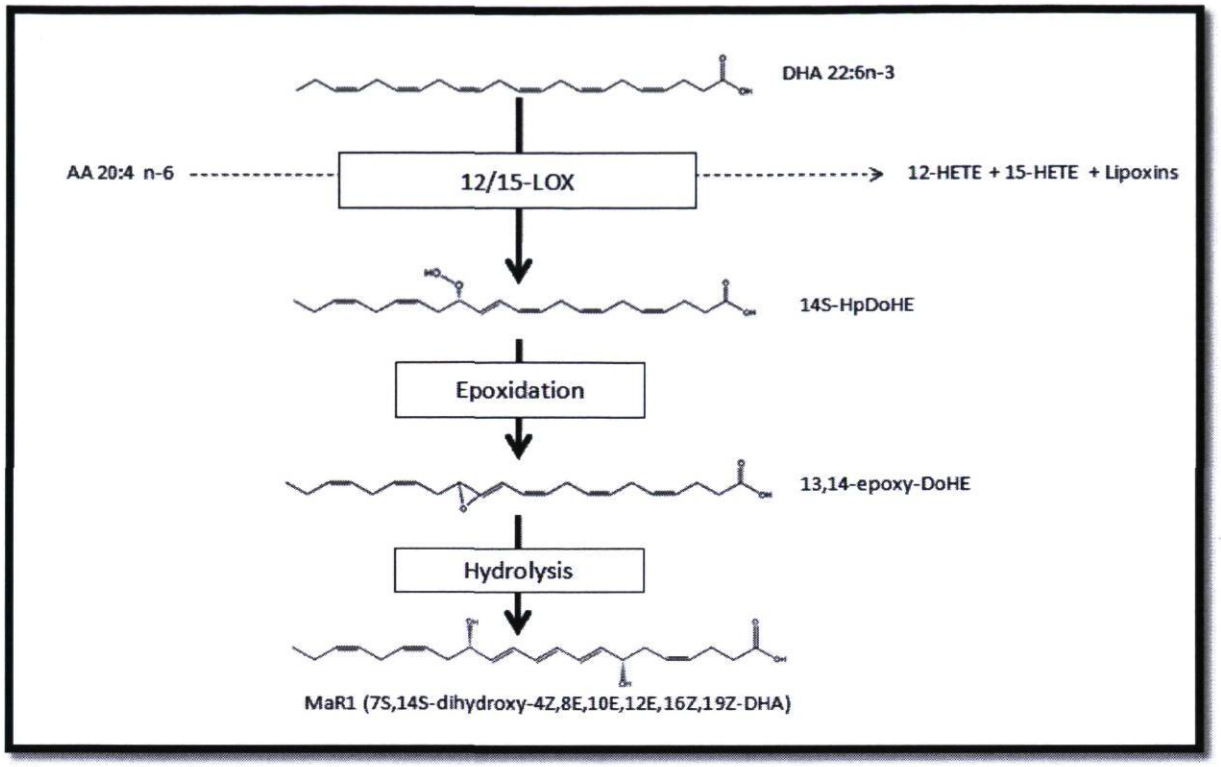

Figure 10. The Maresins

It is important to note that each of the enzymes involved in ω-3 derived resolution mediator synthesis are also responsible for the production of pro-inflammatory lipid mediators (Figures 7-10). A good example of this is 12/15-LOX which plays a role in RvD, PD1 and MaR1 formation but is also responsible for producing the inflammatory mediators, 12-HETE and 15-HETE, from AA. In accordance with this dual role in inflammation, the function of 12/15-LOX remains controversial in the context of atherosclerosis (373). Indeed, there is *in vivo* evidence for both a positive and a negative role of 12/15-LOX for this disease state (373). This likely explains discrepant reports in the literature showing that 12/15-LOX deficient mice on LDLr (374) or ApoE (375) knockout backgrounds are protected from atherosclerosis to the same extent as ApoE knockout mice overexpressing 12/15-LOX in macrophages (376). Although substrate availability has been proposed to be an important modulator of enzyme product formation underlying these phenotypes, the precise regulation of LOX substrate preference under different physiological conditions and in different tissues/cell types remains poorly understood. The inherent complexity of the regulation of these lipid biosynthetic

pathways thus argues against using conventional molecular genetic approaches such as enzyme overexpression or disruption for delineating the function and mechanism of action of ω-3 derived resolution mediators. Alternative more relevant approaches include increasing ω-3 availability, direct administration of individual resolution mediators, and interference with identified receptors and intermediates in the signal transduction pathways.

4.4.2 Mechanisms of action of ω-3 resolution mediators

In fitting with their role directing the catabasis of inflammation, resolvins, protectins and maresins have been shown to signal a reduction in leukocyte recruitment and blunt pro-inflammatory cytokine production as well as pro-inflammatory cytokine and lipid mediator signaling (372; 377-379). ω-3 resolution mediators have also been reported to promote the nonphlogistic clearance of apoptotic neutrophils and stimulate the clearance of chemokines in the inflammatory milieu by upregulating the expression of their receptors on apoptotic cells (378; 380). More recently, resolvins were also found to exert analgesic effects by inhibiting the activation of the transient receptor potential vanilloid type-1 (TRPV1) in sensory neurons (364). Accordingly, these novel compounds have proved to be effective for the prevention and treatment of a wide array of inflammatory disorders including experimentally induced colitis (381), asthma (382), periodontitis (383), and they also appear to underlie ω-3 mediated protection from pathological retinal angiogenesis (384).

RvE1 appears to exert its effects by acting as an agonist for the GPCR ChemR23 and an antagonist for the LTB_4 receptor BLT_1 (379); whereas, RvD1 appears to act via the LXA_4 GPCR ALX and the orphan GPCR GPR32 (385). Although, it is likely that each resolution mediator activates its own discrete receptor mediated signaling pathways, cell surface receptors for the other ω-3 resolution mediators remain to be identified. Future identification of receptors for each of these mediators will greatly facilitate advancements in this field.

4.4.3 ω-3 resolution mediators in obesity

Presently, there is very little known about the role of these important lipid mediators in metabolic tissues and their potential to mitigate obesity-linked inflammation and insulin resistance. Outside of the data presented in the following sections of this thesis only 3 other reports have been published in this area. Gonzaliz-Periz et al (386) first showed that treating genetically obese ob/ob mice with DHA raises the production of the DHA derived resolution mediators, PD1 and RvD1, in adipose tissue. RvE1 was not detected in this study; however, the authors revealed that the administration of RvE1 for 1 week improves hepatic steatosis in ob/ob mice by reducing both lipid storage and macrophage accumulation in liver. Although they did not study glucose metabolism directly the authors also suggested that RvE1 might improve insulin sensitivity since adipose mRNA expression of adiponectin, PPARγ, IRS-1 and GLUT-4 was increased. These data suggest that RvE1 likely has therapeutic potential for obesity related metabolic disorders; however, the mechanistic relevance of these findings with reference to the beneficial actions of ω-3 PUFA is somewhat questionable since RvE1 has yet to be detected in adipose, liver, or skeletal muscle.

In contrast, Hellman et al (387) administered the docosanoid mediator RvD1 to genetically obese db/db mice for 16 days. RvD1 treatment resulted in a modest improvement in glucose tolerance that was associated with improved insulin signaling to Akt in adipose but not liver or skeletal muscle. The improved insulin action in adipose tissue was associated with reduced macrophage accumulation and elevated expression of adiponectin. Interestingly, the authors showed that the RvD1 receptor ALX was expressed in both the stromal vascular fraction of adipose tissue as well as in isolated adipocytes. These data suggest that RvD1 likely underlies part of the beneficial actions of ω-3 PUFA in adipose tissue. However, further studies are required to profile the effects of RvD1 on inflammatory cytokine production and signaling in obesity since this was not examined in this study. Additional experiments wherein DHA is administered to high fat fed mice lacking the RvD1 receptor ALX would also add important insight on the role of RvD1 in the beneficial actions of ω-3 PUFA.

Finally, a very recent work by Flachs et al. (388) suggests that combining calorie restriction with administration of ω-3 PUFA synergistically raises the synthesis of Protectin D1 in adipose tissue. However, this finding is likely an artifact resulting from the calorie restriction mediated reduction in neutral lipid content in this tissue. Indeed, all lipids measured, including DHA and AA, were detected at higher levels in the caloric restriction group. This work therefore highlights the difficulties associated with studying lipid mediators in lipid laden tissues. However it is noteworthy that in accordance with our work (389) and that of Gonzalez-Periz et al (386) the authors of this study were also unable to detect RvE1 in adipose tissue or liver despite using a diet that contained EPA. These data suggest that RvE1 is likely not produced or is very rapidly metabolized in metabolic tissues. In contrast, PD1 was easily detected; however, thus far the effects of this lipid mediator on inflammation or glucose metabolism in metabolic tissues have not been directly examined. Further work on this mediator is certainly warranted.

4.5 ω-3 PUFA and T2DM

Not surprisingly, imbalanced dietary intake of ω-6 and ω-3 PUFA has also been linked to T2DM (390-392). Incorporation of ω-3 PUFA into the diet has been associated with improved insulin sensitivity (393-394), reduced macrophage accumulation in adipose tissue (361; 395-396), lower circulating triglycerides and cholesterol (397), adipose remodeling (398) and increased activity of brown fat (399-400). However, at this point the molecular underpinnings of these effects remain incompletely understood. The major reason for the lack of advancement on this front is that dietary supplementation of ω-3 PUFA in rodent diets often prevents weight gain, most likely attributable to a reduction in palatability of the supplemented diet (401-402). Importantly, the primary report of the anti-diabetic action of ω-3 PUFA in high fat fed rodents, published in *Science*, made no mention of body weight gain or visceral fat mass in the rats that were protected from obesity-linked insulin resistance (393). Thus it is not clear whether it is the lack of weight gain or the direct actions of ω-3 PUFA themselves that offer protection from obesity-linked insulin resistance and T2DM. As mentioned in the previous section, ω-3 PUFA might also influence feeding behavior through their impact on the

endocannabinoid system, but this remains to be tested. Therefore, innovative models that overcome the requirement for dietary manipulation are needed to help clarify whether or not ω-3 PUFA act directly to prevent obesity-linked inflammation and insulin resistance and which mechanisms are involved.

4.6 Solution: The *fat-1* transgenic mouse

The *fat-1* transgenic mouse has been genetically engineered to express the *fat-1* ω-3 fatty acid desaturase from *C. elegans* (403). This enzyme, not found in mammals, efficiently converts endogenous ω-6 to ω-3 PUFA such that in *fat-1* transgenic mice fed a diet rich in ω-6 and deficient in ω-3 PUFA the tissue ω-6: ω-3 ratio is approximately 1:1 compared to 50:1 in wild type animals. The *fat-1* transgenic mouse therefore represents the ideal model to study the effects of ω-3 PUFA in an environment that is not confronted by dietary issues.

5 - Objectives of the Thesis

The major objective of the thesis was to determine whether *fat-1* transgenic mice were protected from obesity-linked insulin resistance and to elucidate the mechanisms contributing to this protection with a particular emphasis on the role and mechanisms of action of novel ω-3 derived resolution mediators.

CHAPTER I

Transgenic Restoration of Long-Chain ω-3 Fatty Acids in Insulin Target Tissues Improves Resolution Capacity and Alleviates Obesity-Linked Inflammation and Insulin Resistance in High-Fat-Fed Mice

RÉSUMÉ

OBJECTIFS : Des médiateurs lipidiques dérivés des acides gras polyinsaturés (AGPI) ω-3 jouent un rôle majeur dans la résolution de l'inflammation. Nous avons cherché à déterminer si une déficience en AGPI ω-3 induite par une diète riche en gras pouvait compromettre la capacité des souris obèses à résoudre l'inflammation, contribuant ainsi au développement de l'inflammation et de l'insulino-résistance reliée à l'obésité.

PLAN DE RECHERCHE ET MÉTHODES: Nous avons utilisé l'expression transgénique de la *fat-1* ω-3 désaturase provenant de *C. elegans* pour restaurer de façon endogène les AGPI ω-3 dans des souris nourries avec une diète riche en gras. Après 8 semaines sur une diète soit faible ou riche en gras, des souris sauvages et transgéniques ont été soumises à des tests de tolérance au glucose et à l'insuline. De plus, un essai de résolution a été effectué et les tissus métaboliquement actifs ont été récoltés pour des analyses biochimiques.

RÉSULTATS: Nous avons démontré que le rétablissement des acides gras oméga-3 dans les souris obèses nourries avec une diète riche en gras, pouvait augmenter la synthèse des dérivés bioactifs des acides gras oméga-3, notamment les protectines, dans le tissu adipeux et le muscle squelettique. Ceci est associé à une meilleure capacité à résoudre une réponse inflammatoire aigüe et à une diminution de l'inflammation dans le tissu adipeux. De plus, les souris *fat-1* obèses ont démontré une meilleure sensibilité à l'insuline et une plus grande tolérance au glucose. Tout cela avec un gain de poids et accrétion de graisse équivalent à leurs homologues sauvages.

CONCLUSIONS: Nous concluons que la biosynthèse inefficace des médiateurs de résolution dérivés des AGPI ω-3 dans le muscle et le tissu adipeux contribue à l'entretien de l'inflammation chronique dans l'obésité et que ces nouveaux lipides offrent un potentiel intéressant pour le traitement de la résistance à l'insuline et du diabète.

Transgenic restoration of long chain ω-3 fatty acids in insulin target tissues improves resolution capacity and alleviates obesity-linked inflammation and insulin resistance in high fat-fed mice

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ABSTRACT

Objectives- The catabasis of inflammation is an active process directed by ω-3 derived pro-resolving lipid mediators. We aimed to determine whether high fat (HF) diet-induced ω-3 deficiency compromises the resolution capacity of obese mice and thereby contributes to obesity-linked inflammation and insulin resistance (IR).

Research design and methods- We employed transgenic expression of the *fat-1* ω-3 fatty acid (FA) desaturase from *C. elegans* to endogenously restore ω-3 FAs in HF-fed mice. After 8 weeks on HF or chow diets, wild type (WT) and *fat-1* transgenic mice were subjected to insulin and glucose tolerance tests and a resolution assay was performed. Metabolic tissues were then harvested for biochemical analyses.

Results- We report that the ω-3 docosanoid resolution mediator protectin D1 is lacking in muscle and adipose tissue of HF-fed WT mice. Accordingly, HF-fed WT mice have an impaired capacity to resolve an acute inflammatory response and display elevated adipose macrophage accrual and chemokine/cytokine expression. This is associated with IR and higher activation of iNOS and JNK in muscle and liver. These defects are reversed in HF-fed *fat-1* mice, in which the biosynthesis of this important ω-3 docosanoid resolution mediator is improved. Importantly transgenic restoration of ω-3 FAs prevented obesity-linked inflammation and IR in HF-fed mice without altering food intake, weight gain or adiposity.

Conclusions-We conclude that inefficient biosynthesis of ω-3 resolution mediators in muscle and adipose tissue contributes to the maintenance of chronic inflammation in obesity and that these novel lipids offer exciting potential for the treatment of IR and diabetes.

INTRODUCTION

Obesity is linked to chronic inflammation that plays a key role in the pathogenesis of insulin resistance (IR), leading the way to type 2 diabetes and cardiovascular disease (1,2). Efforts to understand this process have focused on identifying the many factors that may initiate and promote inflammation. We took an alternate approach with the view that pathological inflammation in obesity likely represents an impaired endogenous capacity to "switch off" or more precisely counter-regulate the natural immune response to adipose tissue expansion and lipid excess.

The newly identified genus of ω-3 derived lipid mediators termed resolvins and protectins have been shown to play an important role in the endogenous regulation of inflammation (3,4). Interestingly, dietary long chain (LC) ω-3 polyunsaturated fatty acid (PUFA) insufficiency has been linked to the incidence of chronic metabolic disorders, including type 2 diabetes and cardiovascular disease (5-7). It is thus conceivable that inefficient biosynthesis of ω-3 resolution mediators due to low substrate availability might inherently contribute to the development of obesity-linked inflammation.

González-Périz and colleagues recently showed that acute administration of ω-3 derived Resolvin E1 (RvE1) prevents hepatic steatosis in genetically obese mice (8). However, the other main resolution mediator Protectin D1 (PD1) remains to be investigated, and it is unknown whether HF-feeding *per se* actually restricts resolution mediator biosynthesis and whether this might alter the endogenous resolution capacity of obese mice. Furthermore, it is critical to determine whether ω-3 lipid mediators regulate key obesity-related inflammatory reactions such as macrophage accrual in adipose tissue or activation of inflammatory signaling molecules such as JNK and iNOS that play a role in the etiology of IR (1,2).

Unfortunately studying the effects of dietary ω-3 content in the context of high fat feeding has proven to be rather complicated since incorporation of ω-3 fatty acids (FAs) in rodent diets often prevents weight gain (9). As a result it is not clear whether it is the lack of weight gain or the ω-3 FAs themselves that offer the

protection from insulin resistance and T2DM and what mechanism underlies this protection. Therefore, innovative models that overcome the requirement for dietary manipulation are needed to help clarify whether or not ω-3 FAs act directly to prevent obesity-linked insulin resistance and which mechanisms are involved.

The *fat-1* transgenic mouse has been genetically engineered to ubiquitously express the *fat-1* ω-3 FA desaturase from *C. elegans* (10). This enzyme, not found in mammals, efficiently converts endogenous ω-6 to ω-3 FAs such that in *fat-1* mice fed a diet extremely rich in ω-6 and deficient in ω-3 the tissue ω-6:ω-3 ratio is ~1:1 compared to ~50:1 in wild type (WT) animals. The *fat-1* transgenic mouse therefore represents the ideal model to study the effects of ω:3 FAs in an environment that is not confronted by dietary issues.

Herein we show that HF-feeding WT mice results in diminished ω-3 docosanoid resolution mediator synthesis in muscle and adipose tissue and impaired resolution. Transgenic restoration of ω-3 FAs in HF-fed *fat-1* mice improved resolution capacity and prevented the development of obesity-linked inflammation and IR. These data uncover a new role for pro-resolving lipid mediators in the counter regulation of obesity-linked inflammation and its associated metabolic complications.

METHODS

Animal Studies

Hemizygous *fat-1*(+/-) mice (10) were bred with WT littermates at the Laval University hospital research centre. 6 week-old male mice were fed standard laboratory chow (diet-2018, Harlan Teklad) or HF diets (diet-9302, 55%Kcal from fat, Harlan Teklad) for 8 weeks. ITT's/GTT's were performed in week 7 in 6h fasted mice as previously described (11), and mice were sacrificed in week 8. At sacrifice a cohort of mice were used for the air-pouch resolution assay. Remaining mice were injected via tail vein with either insulin (3.8U/kg) or saline 5min prior to being euthanized. Tissues were rapidly excised and snap frozen in liquid nitrogen. Sections of liver and epididymal adipose were placed in 4% paraformaldehyde.

Animal procedures were approved and carried out in accordance with the Laval University and Canadian Councils for Animal Care.

Lipidomics

Fatty acid composition of phospholipid fractions was analyzed by GC as per (12). Briefly, lipids were extracted along with internal standards (C:15, Avanti Polar Lipids, Alabaster, AL, USA) in a chloroform–methanol (C-M) mixture (2: 1, by volume). Extracted lipids were then weighed and dissolved in a chloroform–methanol mixture (3: 1, by volume). Polar lipids (phospholipids, i.e. phosphatidylcholine, phosphatidyethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin) were separated by thin-layer chromatography (TLC; Silica Gel H, 250 µm, Analtech Inc, Newark, DE, USA) using an isopropyl-ether–acetic acid mixture (96: 4, by volume). Fractions were then recovered in individual glass tubes and direct transesterification was performed by adding acetyl chloride. Fatty acid methyl esters of phospholipids were analysed by gas chromatography using Hewlett-Packard 5890, series II (Hewlett-Packard, Toronto, Canada) equipped with a fused silica column (DB23; 30 m, 0.25 mm internal diameter, 0.25 µm film, Agilent Technologies, Mississauga, Canada), helium as carrier gas, a split ratio of 1: 72, a flow of 0.72 ml min^{-1}, and a coupled flame ionization detector (FID). The fatty acid methyl esters were identified by comparison with retention times of a Supelco 37 component FAME mix (Supelco Inc., Bellefonte, PA, USA) and by using one internal standard (C:15, Avanti Polar Lipids, Alabaster, AL, USA).

For LC-MS/MS, lipid mediators were extracted in the presence of deuterated internal standard (1ng LTB4-6,7,14,15; Biomol) by solid phase extraction using Sep-Pak C18 cartridges (Waters). A triple quadrupole linear ion trap mass spectrometer (4000Q-TRAP; Applied Biosystems) equipped with an Acquity UPLC BEH C18 column (1.7µm, 1.0×150mm; Waters) was used. MS/MS analyses were conducted in negative ion mode, and eicosanoids/docosanoids identified by multiple reaction monitoring using transitions for 17-HDoHE (343>245m/z), 18-HEPE (317>215m/z), protectin D1 (PD1) (359>153m/z) and RvE1 (349>195m/z).

Calibration curves (1-1000pg) and LC retention times for each compound were established with synthetic standards.

In vivo resolution assay

The air pouch resolution assay was performed as described by Levy et al (13). Dorsal air pouches were raised and maintained 6 and 3 days prior to the assay via subcutaneous injection of sterile air (5 and 3 ml respectively). On the day of the experiment, 10ng of recombinant murine TNFα (R&D Systems Inc.) in 100µl sterile PBS was injected into the pouch. At 0, 4 and 6.5 hours post injection mice were sacrificed and pouches washed 2 times with 1ml of sterile PBS to collect infiltrating polymorphonuclear leukocytes (PMN). PMN were then enumerated. Sterile PBS was used as a control for TNFα and did not stimulate PMN infiltration into the pouch (data not shown).

Histology

Adipose and liver sections were embedded, mounted and H&E staining of liver was performed by the University Laval microscopy facility. Immunohistochemistry detection of F4/80+ cells was performed as previously described (14).

Western blotting

Immunoblotting was performed in gastrocnemius muscle and liver as previously described (15). 50ug of protein was loaded onto a 7.5% acrylamide gel, subjected to SDS-PAGE then transferred onto nitrocellulose membranes. Membranes were then blocked and probed with the appropriate antibodies. Antibodies for p-AKT ser473, p-JNK thr183/tyr185, and Total JNK were obtained from Cell Signaling Technology (MA, USA). Antibodies for total AKT and iNOS were from Santa Cruz Biotechnology (CA, USA) and BD Transduction Laboratories™ (Canada) respectively.

Analytical methods

Plasma insulin levels were assessed by RIA (Linco, MI, USA). Chemokines and cytokines were quantified in 25µl of adipose tissue lysates (50ug of protein in PBS containing 1% NP-40) using a MILLIPLEX^{TM} MAP kit (Millipore).

Statistical Analysis

LC-MS/MS data were analyzed using students T-test, Air-pouch, ITT and GTT data were analyzed using two-way ANOVA. For all other data one-way ANOVA was used. Bonferonni was the post-hoc test. Results were considered significant when $P<0.05$.

RESULTS

HF-feeding reduces ω-3 availability for resolution mediator synthesis

We first examined the effect of HF-feeding on ω-3 bioavailability in metabolic tissues. The HF diet mimicked western diets in terms of ω-3 content with an ω-6:ω-3 ratio of ~18-1. After eight weeks, HF-fed wild-type (WT) mice displayed an elevated long chain (LC) ω-6:ω-3 ratio in skeletal muscle, liver and adipose tissue membranes compared to their chow-fed counterparts (Fig. 1A). Importantly, transgenic expression of the *fat-1* ω-3 FA desaturase that converts endogenous ω-6 to ω-3 FAs restored the membrane LC ω-6:ω-3 ratio of HF-fed *fat-1* mice to levels comparable to chow-fed mice (Fig. 1A).

Using LC-MS/MS to detect ω-3 lipid oxygenation products, we found evidence of both docosanoid and eicosanoid biosynthetic activity in metabolic tissues of HF-fed mice. 17-HDoHE and 18-HEPE, hydroxy-metabolites of docosahexaenoic and eicosanpentaenoic acid and biosynthetic markers of PD1 and RvE1 respectively, were readily detected in muscle, liver and adipose tissue (Fig. 1B-C). Interestingly, the docosanoid biosynthetic route appeared to have greater flux in these tissues, since 17-HDoHE was present in significantly higher concentrations than 18-HEPE, and PD1 was readily detected in all tissues while RvE1 was under the detection limit. Compared to HF-fed WT mice, HF-fed *fat-1* mice displayed increased flux

through the docosanoid biosynthetic route in muscle and adipose tissues, but not in liver (Fig. 1D). Indeed, 17-HDoHE was increased by approximately 215% in muscle and 138% in adipose tissue while PD1 was increased by approximately 176% in muscle and 201% in adipose tissue of *fat-1* mice compared to WT mice ($P<0.05$). These data suggest that *fat-1* mice display increased ω-3 bioavailability for pro-resolution mediator synthesis in these two key metabolic tissues.

HF-feeding impairs resolution

We hypothesized that the HF diet-induced deficit in ω-3 resolution mediator synthesis would impact endogenous counter-regulation of inflammation in WT mice. To test this we subjected mice to a dorsal air-pouch TNFα challenge, an established model of self resolving inflammation (13). The injection of 10ng of TNFα into the air-pouch stimulated an influx of polymorphonuclear leukocytes (PMN) that peaked at 4h and resolved completely 6.5h post challenge. In contrast to their chow-fed counterparts, HF-fed WT mice only resolved ~65% of infiltrating PMN by this time (Fig 2A-B). Restoration of ω-3 in HF-fed *fat-1* mice was sufficient to completely recover the deficit in resolution capacity ($P<0.001$). This is the first evidence that HF-diet induced ω-3 deficiency can impede the normal resolution of inflammation.

Transgenic restoration of ω-3 prevents adipose inflammation

To determine what role the HF diet-induced deficit in ω-3 docosanoid mediators plays in obesity-linked inflammation we examined macrophage accrual in adipose tissue. Since PD1 directs tissue phagocyte flux in inflammatory exudates (16) we hypothesized that transgenic restoration of this ω-3 resolution pathway would be sufficient to limit adipose macrophage accumulation in HF-fed mice. Immuno-histochemical staining for F4/80+ cells, revealed that HF-fed WT mice have abundant accumulation of macrophages in adipose tissue compared to their chow-fed counterparts (Fig. 2C-D). Furthermore, many of the F4/80+ cells in HF-fed WT fat clearly formed inflammatory crown like structures (CLS) around adipocytes (Fig. 2C-E), a hallmark of obesity-linked inflammation (14). In line with the improved

resolution capacity macrophage accrual and CLS formation were entirely prevented in adipose tissue of HF-fed transgenic mice.

To further characterize the impact of ω-3 resolution mediators on obesity-linked inflammation we also examined adipose chemokine and cytokine expression. We detected elevated concentrations of the pro-inflammatory chemokines CCL2/MCP-1 and CCL5/RANTES alongside the cytokines, IL-1β, IL-2 and IL-6 in HF-fed WT mice (Fig. 2F-J). Importantly, these key inflammatory factors were not significantly raised by HF-feeding in adipose of *fat-1* mice.

Transgenic restoration of ω-3 protects against obesity-linked IR and glucose intolerance

We next characterized whole-body insulin sensitivity to determine whether transgenic restoration of ω-3 also prevents the development of obesity-linked IR. Insulin sensitivity was markedly reduced in HF-fed WT mice, as illustrated by elevated fasting insulin levels and diminished glucose excursion during the insulin tolerance test (ITT) (Fig. 3A-D). Conversely, *fat-1* mice were protected from HF diet-induced IR as both fasting insulin values and ITT curves were similar to that observed for chow-fed mice.

Fat-1 mice were also partially protected from HF diet-induced glucose intolerance (Fig. 3E-F). The area under the glucose tolerance curves of HF-fed WT mice was increased compared to their chow-fed counterparts (Fig. 3F) however this parameter was not significantly different between HF-fed *fat-1* mice and their chow-fed controls. Importantly, the improved metabolic phenotype of HF-fed *fat-1* mice was not related to changes in food intake (data not shown), body weight gain (Fig. 3G) or adiposity (Fig. 3H). We also found no changes in hepatic lipid accretion as determined by liver weight (Fig. 3I) and histological examination of liver sections which showed similar accumulation of fat vesicles in both HF-fed WT and *fat-1* mice (Fig. 3J). Fasting plasma FFAs were not significantly influenced by 8 weeks of high fat feeding or transgenic restoration of ω-3 in our study (data not shown). Although not statistically significant, circulating adiponectin tended to be reduced

by ~30% in HF-fed WT mice compared to their chow fed counterparts (2150±638ng/ml vs 3345±768ng/ml respectively), this was not the case in HF-fed *fat-1* mice which displayed circulating levels of adiponectin that were comparable to chow-fed mice (3420±565ng/ml).

To understand the mechanism underlying the improved metabolic phenotype of HF-fed *fat-1* mice, we examined insulin signaling to Akt in muscle and liver. As expected, insulin stimulation induced robust phosphorylation of Akt on ser473 in muscle and liver of chow-fed WT mice but this response was impaired in their HF-fed counterparts (Fig. 4A-B). Remarkably, this defect was normalized in both muscle and liver of HF-fed *fat-1* mice and despite clear accumulation of ectopic lipid in the latter tissue.

We next examined whether the improved insulin action in metabolic tissues of HF-fed *fat-1* mice resulted from decreased inflammatory signaling. As expected, HF-feeding WT mice led to robust phosphorylation of JNK on thr183/tyr185 in both muscle and liver; however, this was not the case for HF-fed *fat-1* mice in either tissue (Fig. 4C-D). HF-feeding also resulted in significant iNOS induction in muscle of WT mice but not in HF-fed *fat-1* mice (Fig. 4E). These data suggest that prevention of HF diet-induced ω-3 deficiency and the maintenance of resolution capacity protects from the development of obesity-linked IR not only by limiting inflammation in the expanding adipose tissue but also by inhibiting two key inflammatory mediators of IR, JNK (17) and iNOS (11), in muscle and liver.

DISCUSSION

In the present study we took advantage of *fat-1* mice to investigate the role of endogenous ω-3 derived resolution mediators in key metabolic tissues in obesity. We found that the biosynthetic flux of the ω-3 docosanoid resolution pathway in muscle and adipose tissue is dependent on LC ω-3 PUFA bioavailability and that the pro-resolving lipid mediator, PD1 is lacking in normal mice chronically fed a typical western diet (ω-6:ω-3 ratio ~18-1). HF-fed obese mice exhibited an impaired capacity to resolve an acute inflammatory response to TNF-α and showed abundant macrophage infiltration in adipose tissue that was linked to heightened

chemokine and cytokine expression, a hallmark of obesity-linked inflammation (18,19). Remarkably, restoration of PD1 via *fat-1* transgenesis improved global resolution capacity and prevented adipose macrophage accrual in HF-fed *fat-1* mice. Accordingly, the expression levels of 5 key pro-inflammatory chemokines / cytokines were not found to be significantly elevated in adipose tissue of HF-fed *fat-1* mice compared their chow-fed controls. To the best of our knowledge this is the first demonstration that endogenous biosynthesis of ω-3 derived resolution mediators is associated with obesity-linked inflammation in metabolic tissues.

This is also the first report on the regulation of inflammation and insulin sensitivity in an animal model of obesity in which ω-3 status has been enhanced without confounding effects of dietary manipulation. Indeed, previous studies have documented that the anti-inflammatory and metabolic effects of dietary ω-3 supplementation were associated with concomitant reductions in either food intake, body weight gain, adiposity or liver fat accretion (9, 20, 21). However, we report herein that transgenic-based elevation of LC ω-3 PUFAs protects from obesity-linked IR without altering food intake, weight gain, or lipid deposition in adipose tissue or liver. We propose instead that endogenous LC ω-3 PUFAs exert their protective effect through the actions of their lipid oxygenation products which resolve inflammation and limit macrophage accrual in the expanding adipose tissue of obese mice.

The lack of effect of *fat-1* expression on hepatic lipid accretion seems at odds with the recent report that dietary supplementation of LC ω-3 PUFAs reversed hepatic steatosis in genetically obese *ob/ob* mice possibly through the action of the EPA derived eicosanoid RvE1 (8). However, this may be due in part to the different animal models used (i.e. diet induced *vs* genetic-based obesity), and the fact that we readily detected the docosanoid PD1 but not the eicosanoid RvE1 in the liver of our HF-fed *fat-1* mice. Although we saw no effect of *fat-1* transgenesis on resolution mediator synthesis in liver, our data suggests that transgenic restoration of ω-3 FA's dissociates insulin resistance from hepatic lipid deposition and this is likely due to inhibition of inflammatory signaling, as revealed by prevention of JNK

activation in liver of HF-fed F1 mice. This anti-inflammatory effect in liver may be the result of reduced inflammatory crosstalk from adipose tissue with fewer recruited macrophages or may represent the local actions of another class of bioactive ω-3 metabolite such as the newly discovered maresins (22) or EFOX (23) which should be the focus of future investigations.

Our data showing protection from obesity-linked insulin resistance in HF-fed *fat-1* mice in which the levels of LC ω-3 PUFAs have been restored are in line with epidemiological studies in humans which showed that native populations traditionally consuming high levels of LC ω-3 PUFAs display a lower prevalence of type 2 diabetes (24, 25). Interestingly, data from another clinical study suggests that the positive influence of LC ω-3 PUFA supplementation on insulin sensitivity is greater in obese populations that display an inflammatory phenotype (26). These data lend support to our findings which indicate that the anti-inflammatory actions of ω-3 derived resolution mediators in metabolic tissues are key to the positive impact of LC ω-3 PUFAs on insulin sensitivity. It will be interesting in future studies to examine whether direct administration of purified ω-3 derived resolution mediators is sufficient to prevent the development of insulin resistance in obese animals and which mediators carry the greatest anti-diabetic potential.

Although our work represents the first study into the effect of endogenously enhancing tissue ω-3 content on insulin sensitivity in the context of obesity, it is noteworthy that another group has recently developed an adipose specific *fat-1* transgenic line (AP-3 mice) to expressly study the influence of adipose ω-3 content on weight gain, insulin sensitivity and glucose tolerance in lean mice (27). It was found that 3-mo old male, but not female, AP-3 transgenic mice fed a high carbohydrate diet weigh slightly less than their WT littermates, although this could not be explained by changes in adiposity. Interestingly, while male AP-3 mice were more glucose tolerant than WT controls, female AP-3 mice exhibited glucose intolerance as compared to their WT littermates. Insulin sensitivity was not affected in either gender. These findings differ from those of our study in which we found that *fat-1* transgenesis had no impact on weight gain or glucose tolerance in lean

chow-fed animals. It is of interest that homozygous expression of the *fat-1* transgene in our mice does not influence viability (10) while homozygous expression in the AP-3 mice was found to be lethal (27).

In conclusion, we propose that endogenous LC ω-3 PUFAs exert their protective effects through their lipid oxygenation products which reduce macrophage accrual and inflammation in the expanding adipose tissue of obese mice. Our data further suggest that restoring LC ω-3 PUFAs also prevents obesity-linked IR by blunting lipid-induced JNK and iNOS activation in muscle and liver. Collectively our findings unravel a novel mechanism by which endogenous ω-3 FAs prevent the development of obesity-linked inflammation and IR. This work supports the use of LC ω-3 PUFAs for the prevention of insulin resistance and glucose intolerance in obese individuals.

AUTHOR CONTRIBUTIONS

PJW planned experiments, researched data, and wrote the manuscript. MA researched data and reviewed/edited manuscript. RT researched data. JXK helped plan experiments, contributed to discussion and reviewed/edited manuscript. AM planned and supervised experiments, wrote and edited manuscript.

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FIGURE LEGENDS

Figure 1. HF-feeding reduces ω-3 availability for resolution mediator synthesis

(A) 8 weeks of high fat (HF) feeding raised but *fat-1* (F1) transgenesis restored the LC ω-6:ω-3 ratio in membrane phospholipids of muscle, liver and epididymal adipose tissue. C, standard laboratory chow; AA, Arachidonic Acid (20:4 ω-6); EPA, Eicosapentaenoic Acid (20:5 ω-3); DPA, Docosapentaenoic Acid (22:5 ω-3); DHA, Docosahexaenoic Acid (22:6 ω-3); Data are mean ± SEM (n=3). ** P<0.01 vs WTC; *** P<0.001 vs WTC;† P<0.05 vs WTHF; †† P<0.01 vs WTHF **(B)** Comparison of ω-3 docosanoid and eicosanoid biosynthetic pathways by LC-MS/MS in muscle, liver and epididymal adipose tissue of HF-fed mice revealed that the docosanoid biosynthetic pathway has greater flux in metabolic tissues. Above left schematic diagram of docosanoid biosynthetic pathway showing the biosynthetic marker 17-HDoHE and PD1 (protectin D1; 10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid (28)) as well as the immediate PD1 precursor 17HpDoHE. At right the eicosanoid pathway showing 18-HEPE and RvE1 (Resolvin E1; 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-EPA (29)). ND not detected. Data are mean ± SEM (n=9-14). * P<0.05, **P<0.01, ***P<0.001 vs 17-HDoHE. **(C)** Representative LC-MS/MS spectra for 17-HDoHE, PD1 and 18-HEPE, retention times were 22.6, 18.2, and 20.6 minutes respectively **(D)** Comparison of ω-3 docosanoid and eicosanoid biosynthetic pathway activity by LC-MS/MS in muscle, liver and epididymal adipose tissue of HF-fed WT and *fat-1* (F1) mice reveals increased levels of docosanoid resolution mediator synthesis in muscle and adipose tissue of *fat-1* mice compared to WT mice. Data are mean ± SEM (n=6-10). * P<0.05 vs WTHF.

Figure 2. Transgenic restoration of ω-3 resolution mediators re-establishes resolution capacity and prevents adipose inflammation in HF-fed mice

(A) Clearance of inflammatory PMN infiltrates in dorsal air-pouches of HF-fed mice is impaired during the resolution phase of the *in vivo* resolution assay (n=5-6) **(B)** Percent infiltrate clearance 6.5h after TNFα injection was reduced by ~35% in

obese HF-fed WT mice, HF-fed *fat*-1 (F1) transgenic mice displayed normal infiltrate clearance(n=5-6) **(C)** Representative image of F4/80 immunohistochemistry in epididymal adipose tissue show mass accumulation of macrophage in HF-fed WT mice that is prevented in HF-fed transgenic mice . MØ, macrophage; CLS, crown like structure **(D)** Percent F4/80+ cells in epididymal adipose tissue (n=4-6) **(E)** Macrophages present in adipose HF-fed WT mice formed multiple CLS, the formation of these inflammatory macrophage aggregates was greatly reduced in HF-fed *fat-1* (F1) mice (Number of CLS per 100 adipocytes) **(F-J)** Chemokine and cytokine expression in epididymal adipose tissue was elevated by HF compared to chow-feeding in WT mice but these factors were not significantly raised by HF-feeding in *fat-1* (F1) mice (n=5-10). All data are mean ±SEM, * P<0.05, **P<0.01, ***P<0.001 vs WTC; † P<0.05, †† P<0.01, †††P<0.001 vs WTHF.

Figure 3. Transgenic restoration of LC ω-3 PUFA protects against obesity-linked IR and glucose intolerance

(A) HF diet-induced elevation of fasting plasma insulin was prevented by transgenic restoration of ω-3 derived resolution mediators (n=4-9) **(B)** Glycemic excursion from 1.5U/kg i.p. ITT was normalized in HF-fed *fat*-1 (F1) mice (n=8-12) **(C)** Glycemic excursion expressed as percent basal glycemia **(D)** Percent basal glycemia at T=15 min after insulin injection **(E)** Glycemic excursion from 1g/kg i.p. GTT expressed as percent basal glycemia (n=7-11) and **(F)** Area under the curve from GTT show that HF-fed *fat-1* (F1) mice are partially protected from glucose intolerance. **(G)** HF-fed *fat-1* (F1) mice develop similar obesity to WT mice. Weight gain (n=16-20) **(H)** Epididymal fat pad weight (n=9-14). **(I)** Liver weight (n=9-14) **(J)** Representative H&E stained liver sections showing similar accumulation of fat vesicles in both WTHF and F1HF mice. All data are mean ±SEM, ND not detected, * P<0.05, **P<0.01, ***P<0.001 vs respective chow-fed control; † P<0.05 vs WTHF

Figure 4. ω-3 resolution mediators improve insulin signaling by blunting JNK and iNOS in muscle and liver

Transgenic restoration of ω-3 resolution mediators improves insulin signaling to Akt in muscle and liver and blunts activation of JNK and iNOS in these tissues. **(A-E)** Immunoblots for pAKTser473, total AKT, pJNKthr183/tyr185, and total JNK in gastrocnemius muscle and liver, and iNOS in muscle (n=5-9). Quantification of densitometry analyses are shown below the representative gels. Lanes were run on the same gel but were non contiguous. All data are mean ±SEM, ND not detected, * P<0.05, **P<0.01, ***P<0.001 vs respective chow-fed control; † P<0.05 vs WTHF

Figure 1

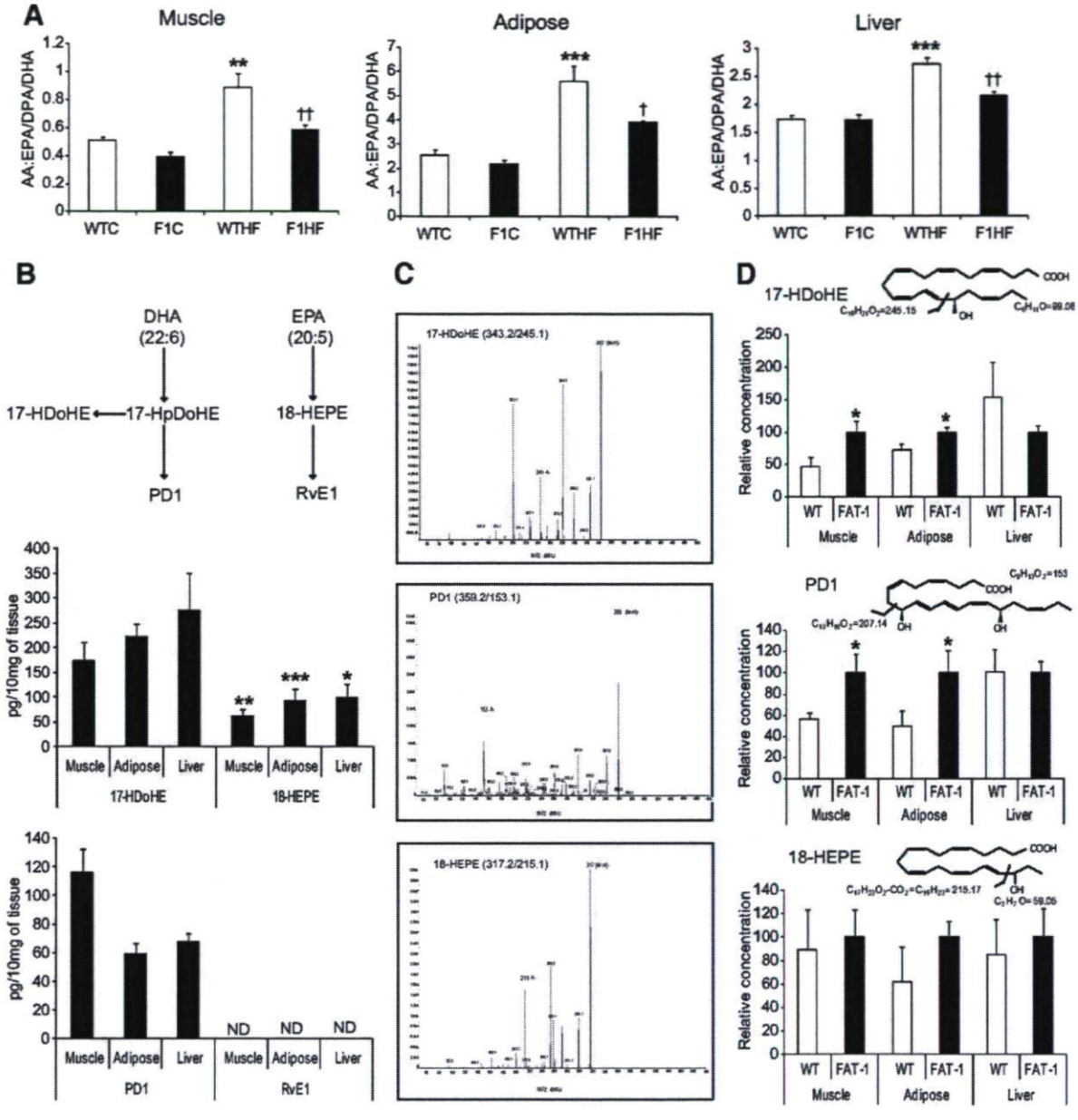

A
Muscle
Adipose
Liver
AA:EPA/DPA/DHA
WTC
F1C
WTHF
F1HF
B
DHA (22:6)
EPA (20:5)
17-HDoHE
17-HpDoHE
18-HEPE
PD1
RvE1
pg/10mg of tissue
Muscle
Adipose
Liver
17-HDoHE
18-HEPE
ND
C
17-HDoHE (343.2/245.1)
PD1 (359.2/153.1)
18-HEPE (317.2/215.1)
D
17-HDoHE
PD1
18-HEPE
Relative concentration
WT
FAT-1

Figure 2

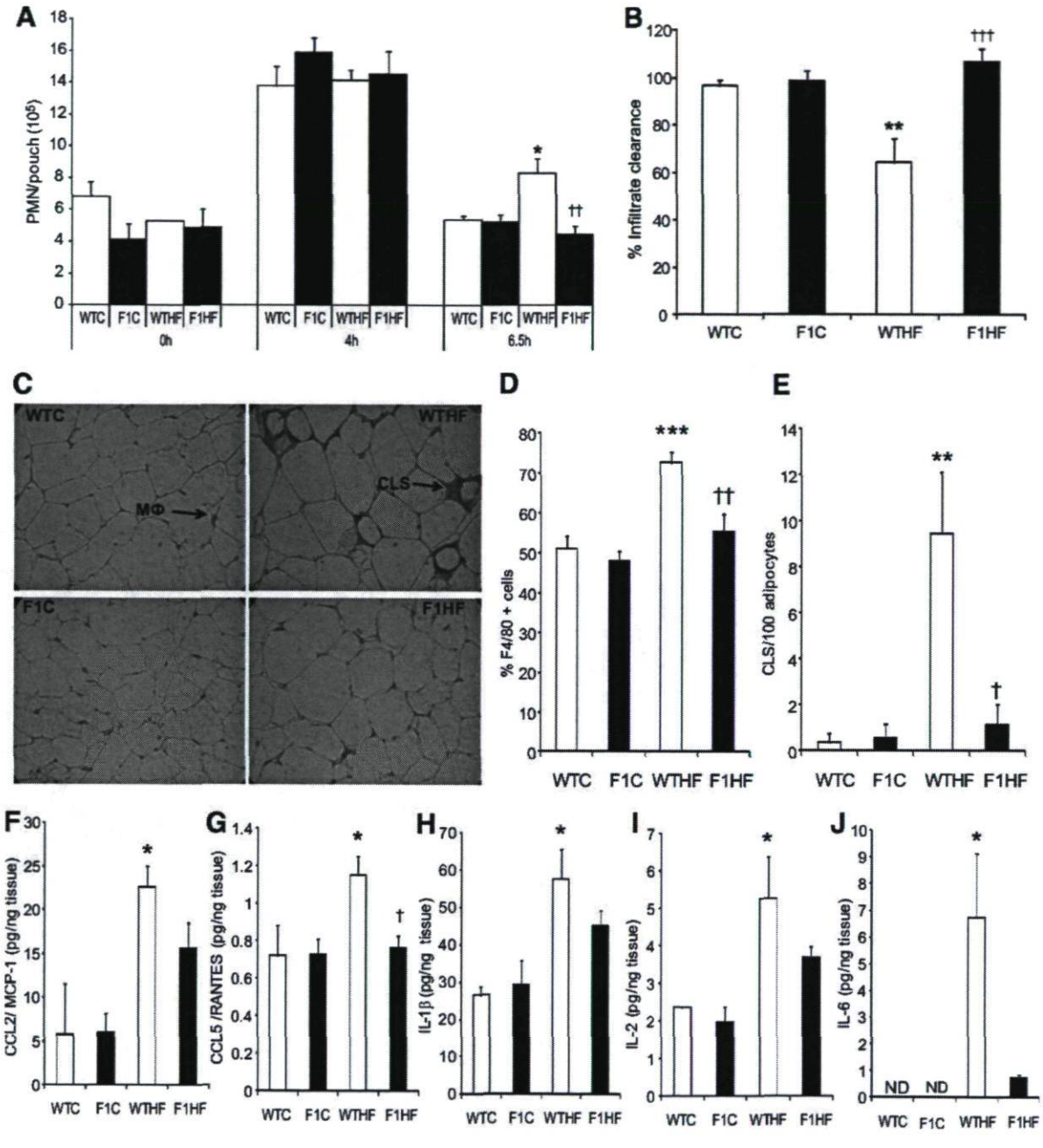

A
PMN/pouch (10⁶)
WTC F1C WTHF F1HF
0h
4h
6.5h
B
% Infiltrate clearance
C
WTC
WTHF
MΦ
CLS
F1C
F1HF
D
% F4/80 + cells
E
CLS/100 adipocytes
F
CCL2/ MCP-1 (pg/ng tissue)
G
CCL5 /RANTES (pg/ng tissue)
H
IL-1β (pg/ng tissue)
I
IL-2 (pg/ng tissue)
J
IL-6 (pg/ng tissue)
ND

Figure 3

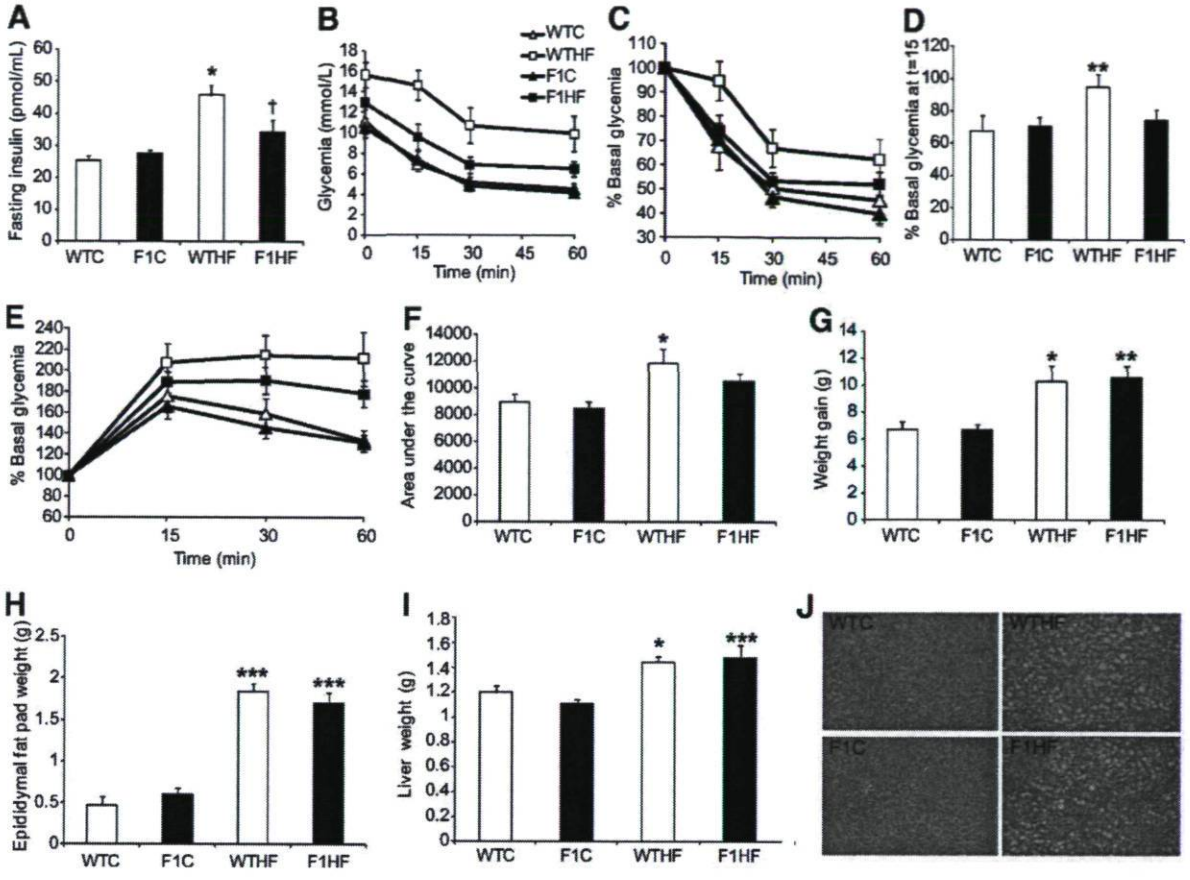
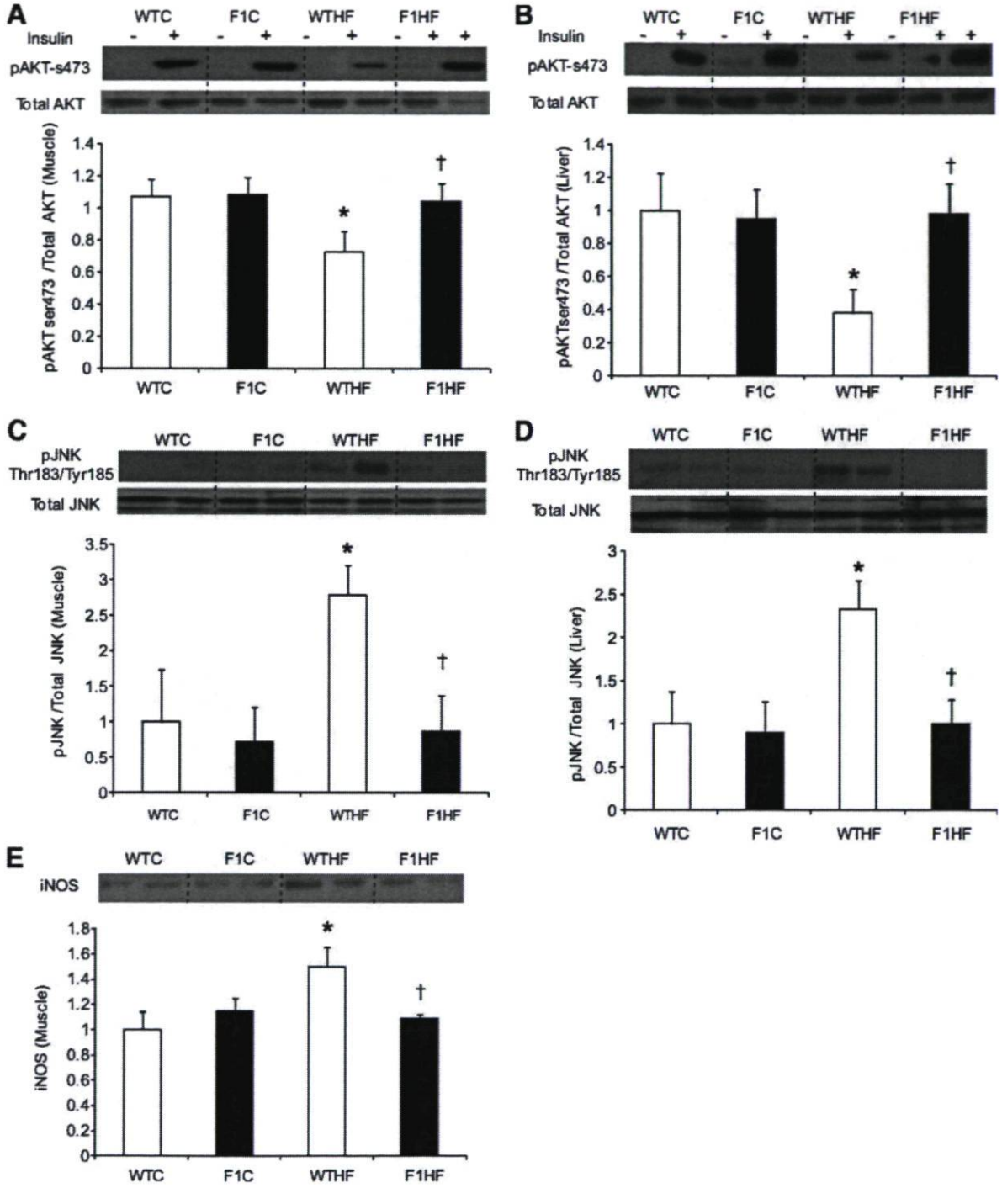

A
Fasting insulin (pmol/mL)
WTC
F1C
WTHF
F1HF
B
Glycemia (mmol/L)
Time (min)
WTC
WTHF
F1C
F1HF
C
% Basal glycemia
Time (min)
D
% Basal glycemia at t=15
E
% Basal glycemia
Time (min)
F
Area under the curve
G
Weight gain (g)
H
Epididymal fat pad weight (g)
I
Liver weight (g)
J
WTC
WTHF
F1C
F1HF

Figure 4


A
WTC
F1C
WTHF
F1HF
Insulin
- + - + - + - + +
pAKT-s473
Total AKT
pAKTser473 /Total AKT (Muscle)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
†
WTC
F1C
WTHF
F1HF
B
WTC
F1C
WTHF
F1HF
Insulin
- + - + - + - + +
pAKT-s473
Total AKT
pAKTser473 /Total AKT (Liver)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
†
WTC
F1C
WTHF
F1HF
C
WTC
F1C
WTHF
F1HF
pJNK
Thr183/Tyr185
Total JNK
pJNK /Total JNK (Muscle)
3.5
3
2.5
2
1.5
1
0.5
0
*
†
WTC
F1C
WTHF
F1HF
D
WTC
F1C
WTHF
F1HF
pJNK
Thr183/Tyr185
Total JNK
pJNK /Total JNK (Liver)
3
2.5
2
1.5
1
0.5
0
*
†
WTC
F1C
WTHF
F1HF
E
WTC
F1C
WTHF
F1HF
iNOS
iNOS (Muscle)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
†
WTC
F1C
WTHF
F1HF

CHAPTER II

The docosanoid resolution mediator, Protectin DX, employs the prototypic myokine, Interleukin-6, to regulate systemic glucose metabolism *in vivo.*

Résumé

Une baisse dans la biosynthèse des docosanoïdes dérivés des acides gras insaturés (AGPI) ω-3 dans les tissus métaboliques est associée au développement de l'inflammation et de l'insulino-résistance dans des souris obèses. Ici, nous avons analysé les effets métaboliques et anti-inflammatoires du docosanoïde, Protectine DX (PDX), dans des macrophages *in vitro* ainsi que dans les souris *in vivo*. En plus des activités anti-inflammatoires anticipées, nous avons pu observer que le PDX possède une activité antidiabétique. De façon inattendue, l'administration de PDX a stimulé une forte augmentation des concentrations circulantes d'IL-6. Cela était associé avec des niveaux élevés d'IL-6 dans le muscle squelettique mais pas dans le foie. Les niveaux élevés d'IL-6 étaient associés à une augmentation de l'activité d'AMPK et à une phosphorylation de l'ACC au muscle ainsi qu'à une meilleure inhibition de l'expression des enzymes de la gluconéogenèse dans le foie. Fait important, l'effet de la PDX sur le métabolisme du glucose était complètement absent chez les souris déficientes en IL-6. Ces données présentent PDX comme un nouveau régulateur du métabolisme du glucose, qui emploie l'IL-6 afin d'exercer ses effets bénéfiques.

The docosanoid resolution mediator, Protectin DX, employs the prototypic myokine, Interleukin-6, to regulate systemic glucose metabolism *in vivo*

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Running title: Protectin DX regulates glucose metabolism via IL-6

Summary

Reduced biosynthesis of omega-3 derived docosanoids in metabolic tissues is associated with inflammation and insulin resistance in high fat-fed mice. Here we studied the novel docosanoid resolution mediator, Protectin DX (PDX). Administration of PDX blunted lipid-induced inflammatory responses in palmitate treated macrophages as well as lipid-infused mice and prevented lipid-induced hepatic and peripheral insulin resistance *in vivo*. Unexpectedly, PDX administration stimulated a robust increase in circulating IL-6. This was associated with elevated skeletal muscle but not liver IL-6, and activation of muscle AMPK, ACC and hepatic STAT-3 as well as inhibition of PGC1α, PEPCK, and G6Pase expression in liver. PDX also enhanced glucose metabolism in saline-infused mice. Importantly, the effect of PDX on glucose metabolism in both saline and lipid-infused animals was completely abrogated in IL-6 knockout mice. These data present PDX as a novel regulator of glucose homeostasis that employs IL-6 crosstalk to exert its effects on glucose metabolism and insulin action in skeletal muscle and liver.

Introduction

It is now widely accepted that inflammation is a key component of the etiology of obesity-linked insulin resistance leading the way to type 2 diabetes mellitus (T2DM) (Wellen and Hotamisligil, 2005; White and Marette, 2008). Novel anti-inflammatory compounds are therefore of great interest to the field. The enzymatic oxidation of omega-3 (ω-3) polyunsaturated fatty acids (PUFA) yields multiple families of bioactive lipids, which include the resolvins, protectins and maresins (Norling and Serhan, 2010). These novel lipid mediators possess potent anti-inflammatory activity owing to their role in the active termination of endogenous inflammation; however, their therapeutic potential for the treatment of metabolic disorders such as insulin resistance remains to be fully explored.

Using *fat-1* transgenic mice that are characterized by elevated tissue ω-3 content (Kang et al., 2004), we recently demonstrated that greater endogenous biosynthesis of protectins in adipose tissue and skeletal muscle of high fat fed obese mice is associated with an improved global resolution capacity, reduced inflammation and protection from insulin resistance (White et al., 2010). These data suggest that protectins might carry important potential for alleviating insulin resistance and T2DM.

Protectin D1 (PD1), identified as 10R,17S-dihydroxy-docosa-4Z,7Z,11E, 13E,15Z,19Z-hexaenoic acid, is the most studied member of the protectin family (Serhan et al., 2006). PD1 is derived from 12/15-lipoxygenase mediated oxygenation of docosahexaenoic acid (DHA; 22:6 n-3) followed by epoxidation and reduction of the 17S-hydroperoxy DHA intermediate (Serhan et al., 2006). A natural stereoisomer of PD1, 10S,17S-dihydroxy-docosa-4Z,7Z,11E,13Z,15E,19Z-hexaenoic acid, first described as compound I (Serhan et al., 2006) and recently designated Protectin DX (PDX) (Chen et al., 2009) is also present in vivo. PDX is produced via sequential lipoxygenation of DHA and differs from PD1 with respect to the double bond geometry of carbons 13 and 15 as well as the position of the C-10 hydroxyl (Chen et al., 2009; Serhan et al., 2006). PDX is found alongside PD1 in murine inflammatory exudates and may also be produced by human neutrophils

exposed to DHA, albeit to a lesser extent than PD1 (Serhan et al., 2006). To the best of our knowledge the influence of protectins on insulin sensitivity and glucose metabolism has not yet been examined.

Herein we provide first evidence of the therapeutic potential of PDX for lipid-induced inflammation and insulin resistance. Importantly, we also reveal an unanticipated mechanism of action whereby PDX enhances both hepatic and peripheral glucose metabolism in vivo by increasing the prototypic myokine IL-6.

Results

PDX reduces lipid-induced inflammation in macrophages

We had previously reported that increased protectin biosynthesis was associated with reduced macrophage infiltration and inflammation in adipose tissue of high fat fed mice (White et al., 2010). We therefore decided to first investigate whether protectins could directly affect macrophage function in a model that is relevant to obesity-related inflammation. We thus tested the ability of PDX to block palmitate-induced inflammation in J77A4.1 macrophages *in vitro*. Macrophage exposure to palmitate (400μM) for 16h induced secretion of the inflammatory chemokines CCL2/MCP-1 and CCL5/RANTES (Figure 1, A and B) as well as the cytokines TNFα, IL-2, IL-6 and IL-10 (Figure 1, C-F). Inducible nitric oxide synthase (iNOS) activity, measured by nitrite production in the medium, and protein expression as well as c-Jun N-terminal Kinase (JNK) activation, as measured by phosphorylation on threonine183/tyrosine185, were also stimulated by palmitate exposure (Figure 1, G-I). Importantly, co-administration of nanomolar doses (10 and 100 nM) of PDX significantly reduced each of these inflammatory responses (Figure 1, A-I).

Since it is unknown whether other novel omega-3 derived resolution mediators also posses the potential to limit lipid-induced macrophage activation we decided to test the effectiveness of the resolvins, RvE1 (Figure S1, A-G) and RvD1 (Figure S2, A-G), to block palmitate-induced chemokine, cytokine and iNOS activity in parallel experiments. Interestingly, each mediator displayed distinctive profiles of action. Indeed, RvE1 potently inhibited secretion of the cytokines TNFα, IL-2, IL-6 and IL-

10 while exerting more modest effects on CCL2/MCP1 and iNOS activity but had no significant effect on the chemokine CCL5/RANTES. In contrast, RvD1 effectively blunted TNFα, IL-2, and iNOS but had no significant effect on either CCL2/MCP-1, CCL5/RANTES, or IL-6.

PDX prevents lipid-induced insulin resistance in mice

To test whether the anti-inflammatory activity of PDX *in vitro* translates into protection against lipid-induced inflammation and insulin resistance *in vivo* we next employed a 6h lipid infusion paired to a 4mU/kg hyperinsulinemic-euglycemic (HIE) clamp to induce systemic insulin resistance in lean 14 week old C57BL/6J mice and determined whole-body insulin sensitivity as well as hepatic and peripheral insulin action (see design in Figure S3). This technique was recently established in our laboratory as an acute model of systemic insulin resistance that is characterized by impaired insulin action in both skeletal muscle and liver (Charbonneau and Marette, 2010).

PDX treated lipid-infused mice were administered PDX (1μg i.v.) immediately prior to and 2.5h into the 6h lipid infusion. As expected, plasma free fatty acids (FFA's) were raised by more than 10 fold in both the lipid and lipid+PDX groups compared to saline-infused controls (Table 1). Interestingly, although pre-infusion glycemia was not different among groups (Table 1), administration of PDX lead to a significant lowering of pre-clamp glycemia compared to both the saline and lipid treated animals (Figure 2A, $P<0.01$), suggesting that PDX might directly modulate glucose metabolism. Furthermore, whereas lipid infusion caused a rise in pre-clamp insulinemia in the lipid group ($P<0.05$) this was not the case for the lipid+PDX treated mice (Table 1). In accordance with these data, the glucose infusion rate (GIR) required to maintain euglycemia during the clamp, a measure of whole body insulin sensitivity, was reduced by ~70% in lipid-infused mice compared to saline-infused controls; however, this insulin desensitizing effect was completely blocked by PDX administration ($P<0.001$; Figure 2, B and C).

The clamp studies revealed that the protective action of PDX results from improved insulin sensitivity in both muscle and liver since insulin action on peripheral glucose

uptake (Figure 2D) and suppression of hepatic glucose production (Figure 2E) were both significantly improved by PDX administration. It is noteworthy that PDX administration only partially restored peripheral insulin sensitivity but actually improved hepatic insulin action significantly beyond that of saline-infused mice ($P<0.05$). In line with the improved insulin sensitivity in these tissues, we found that Akt phosphorylation was reduced in both liver and skeletal muscle of lipid-infused mice (Figure 2, F and I) but maintained with PDX treatment.

PDX suppresses activation of established mediators of insulin resistance

We hypothesized that PDX likely exerts its protective action on lipid-induced insulin resistance by inhibiting the activation of established inflammatory mediators of insulin resistance, such as iNOS and JNK (Hirosumi et al., 2002; Perreault and Marette, 2001). Compared to saline, lipid infusion led to a robust induction of iNOS protein expression in both liver ($P<0.001$) and muscle ($P<0.05$) and this was significantly repressed in each of these important metabolic tissues by PDX administration (Figure 2, G and J). JNK activation, as determined by phosphorylation on Thr183/Tyr185, was also induced by lipid infusion in liver but not in muscle (data not shown) and this too was abrogated by PDX administration ($P<0.05$; Figure 2H). These data support our findings in macrophages exposed to palmitate, and suggest that PDX prevents lipid-induced insulin resistance at least in part by preventing the activation of inflammatory mediators of insulin resistance.

PDX inhibits lipid-induced inflammation in vivo

Since PDX effectively reduced the activation of two well established inflammatory mediators of insulin resistance we were interested to determine whether this was due to upstream inhibition of lipid-induced chemokine and cytokine secretion. We thus examined a panel of inflammatory chemokines and cytokines in circulation (Figure 3). In line with our observations in palmitate treated macrophages, lipid infusion *in vivo* led to robust induction of the pro-inflammatory chemokines CCL2/MCP-1 and CCL5/RANTES and this was significantly suppressed by PDX administration (Figure 3, A and B). Remarkably, this was also the case for the pro-inflammatory Th1 cytokines, TNFα, IFNγ, IL-1β, and IL-2 as well as the Th-17

cytokine, IL-17 (Figure 3, C-G). However, unexpectedly, in contrast to its effect on all other chemokines and cytokines, PDX actually promoted an ~7 fold increase in IL-6 beyond that induced by lipid infusion alone ($P<0.001$; Figure 3H). These findings provide strong evidence that PDX is a potent inhibitor of lipid-induced inflammation; however, the observation that PDX robustly induces circulating IL-6 suggests that the mechanism of action of PDX is likely more complex than first anticipated.

PDX promotes IL-6 expression in skeletal muscle

The role of IL-6 in insulin resistance and glucose metabolism is the subject of fierce debate in the literature (Mooney, 2007; Pedersen and Febbraio, 2007). While IL-6 is frequently associated with the pathogenesis of obesity-related inflammation and metabolic disorders (Bastard et al., 2002) there are multiple reports which suggest that IL-6 may actually promote glucose and lipid metabolism (Carey et al., 2006; Petersen et al., 2005) and blunt inflammation (Wunderlich et al., 2010). Furthermore, IL-6 was identified as the prototypic 'myokine' (muscle derived cytokine) and has been proposed as a beneficial 'exercise factor' that regulates systemic metabolism during and post-exercise by mediating crosstalk with liver and adipose tissue (Febbraio and Pedersen, 2005). In light of these data, we felt it was important to first establish the potential site of IL-6 release in our study. We therefore examined IL-6 protein expression in both skeletal muscle and liver. Here we observed that the expression profile of IL-6 protein in skeletal muscle was very similar to that in plasma but this was not the case for liver where no significant differences were observed (Figure 4, A and B). Since PDX was seen to inhibit IL-6 release from palmitate treated macrophages in the earlier in vitro studies (Figure 1E) these data suggest that PDX likely raises circulating IL-6 by promoting its release from skeletal myocytes rather than myeloid cells within skeletal muscle tissue.

A very recent report demonstrated that an IL-6-dependent pathway underlies the insulin sensitizing actions of adiponectin in liver (Awazawa et al., 2011). We therefore felt it was important to also determine whether PDX influenced circulating

adiponectin levels in our study. However, here we found that adiponectin likely does not account for the PDX mediated rise in circulating and skeletal muscle IL-6 since both lipid-infused groups displayed similar significantly lower levels of total adiponectin in plasma ($P<0.05$; Figure 4C).

PDX activates AMPK in skeletal muscle

IL-6 is thought to increase glucose metabolism in skeletal muscle by activating AMP-activated protein kinase (AMPK) (Carey et al., 2006; Kelly et al., 2009; Kelly et al., 2004). We therefore examined the effect of PDX administration on skeletal muscle AMPK and its downstream target Acetyl-CoA carboxylase (ACC). We observed that PDX administration promotes a robust phosphorylation of AMPK on Thr172 compared to both saline and lipid-infused groups ($P<0.05$; Figure 4D) and this was associated with significantly greater phosphorylation of ACC on ser79 ($P<0.05$ vs saline, $P<0.01$ vs lipid; Figure 4E). These data suggest that muscle derived IL-6 possibly contributes to the improved skeletal muscle glucose metabolism in PDX treated mice by stimulating AMPK in an autocrine manner.

PDX promotes activation of STAT-3 in liver

In addition to its autocrine action in skeletal muscle, muscle derived IL-6 is believed to regulate hepatic glucose production in an endocrine manner via STAT-3 mediated transcriptional suppression of PGC1α, PEPCK and G6Pase (Inoue et al., 2006; Inoue et al., 2004). Accordingly, we found that PDX administration induced phosphorylation of hepatic STAT-3 on ser727 ($P<0.05$; Figure 4F) and this was associated with greater transcriptional suppression of mRNAs encoding for the transcription factor PGC1α ($P<0.05$; Figure 4G), as well the important gluconeogenic enzyme, PEPCK ($P<0.01$; Figure 4H), compared to saline-infused mice. This effect of PDX likely explains the greater suppression of hepatic glucose production observed with PDX treatment during the HIE clamp (Figure 2D). There was also a tendency for reduced expression of the gluconeogenic enzyme G6Pase in PDX treated animals but this did not reach significance ($P=0.08$; Figure 4I). Importantly, in accordance with the findings of the HIE clamp, mice infused with

lipid alone displayed significantly elevated expression of PGC1α ($P<0.01$; Figure 4G) and PEPCK ($P<0.05$; Figure 4H) compared to the saline-infused controls.

IL-6 is required for the beneficial effects of PDX on glucose metabolism

To test our hypothesis that IL-6 underlies the beneficial effects of PDX on glucose metabolism we performed a second set of lipid infusion HIE-clamp studies in 10 week old IL-6 null (KO) mice alongside wild type (WT) C57BL/6J control mice. An additional saline-infused group treated with PDX was added to the study to determine whether PDX might also improve glucose metabolism in insulin sensitive mice. In order to ensure that we could detect any potential differences between the insulin sensitive saline-infused animals these experiments were performed as 2.5mU/kg rather than 4mU/kg HIE clamps. The physiological parameters for this lipid-infusion HIE clamp study are shown in Table 2.

In accordance with the findings of the original clamp study (Figure 2A), here we observed that PDX administration induced a significant fall in pre-clamp glycemia in WT mice. Importantly, this reduction was present in both saline-infused and lipid-infused WT mice compared to their vehicle treated counterparts ($P<0.05$; Figure 5, A and F) but was completely absent in the PDX treated KO mice. These data suggest that the glucose lowering actions of PDX are IL-6 dependent. PDX treatment also significantly enhanced the GIR required to maintain euglycemia in saline-infused WT mice ($P<0.05$) but this was not the case in KO animals (Figure 5, B and C). The clamp data revealed that the improved insulin sensitivity witnessed in WT PDX treated saline-infused mice was the result of superior hepatic insulin action since peripheral insulin action was not different among groups but PDX treatment significantly improved the suppression of hepatic glucose production ($P<0.05$; Figure 5, D and E). Interestingly, hepatic insulin action was significantly reduced in saline-infused KO mice compared to their WT counterparts ($P<0.05$) suggesting that IL-6 is required for full insulin mediated suppression of hepatic glucose production in insulin sensitive animals.

As demonstrated for the original clamp study in Figure 2, vehicle treated lipid-infused mice required less glucose than saline-infused mice to maintain

euglycemia during the HIE clamp and PDX administration significantly increased the GIR in WT mice ($P<0.05$; Figure 5, G and H). Importantly, the beneficial effect of PDX administration on GIR in lipid-infused animals was completely lost in KO mice (Figure 5, G and H). These studies revealed that in contrast to the saline-infused mice, PDX improves both peripheral ($P<0.05$) and hepatic ($P<0.001$) insulin action in lipid-infused mice (Figure 5, I and J). Interestingly, the beneficial effect of PDX on peripheral insulin action in lipid-infused mice was completely absent in KO mice whereas the effect on hepatic insulin action was only partially lost. These data suggest that whereas IL-6 accounts for the entire beneficial effects of PDX in normal unchallenged animals, other anti-inflammatory mechanisms triggered by PDX also likely contribute to its beneficial effects in an inflammatory setting such as during lipid challenge.

AMPK activation by IL-6 is not required for PDX action on muscle glucose metabolism

Since our initial study revealed that the PDX mediated rise in skeletal muscle IL-6 was associated with greater activation of AMPK we felt it was important to determine whether this effect of PDX was IL-6 dependent. In line with our original study (Figure 4, A and D), here we found that administration of PDX causes a robust increase in skeletal muscle IL-6 in both saline and lipid infused WT mice ($P<0.01$, Figure 6, A and G) that is associated with a parallel rise in phosphorylation of AMPK on Thr172 ($P<0.05$, Figure 6, B and H). As expected PDX failed to raise IL-6 in skeletal muscle of saline and lipid infused KO mice (Figure 6, A and G); however, this was not associated with reduced levels of AMPK phosphorylation compared to WT PDX treated mice (Figure 6, B and H). To the contrary, we found that genetic deletion of IL-6 leads to equally elevated levels of AMPK phosphorylation in saline infused mice compared to their vehicle treated WT counterparts regardless of the treatment received ($P<0.05$, Figure 6B). More importantly, we observed that the stimulatory effect of PDX on AMPK phosphorylation is completely maintained in lipid infused animals, which in contrast to their saline infused counterparts, display similar basal levels of AMPK

phosphorylation to vehicle treated WT mice ($P<0.05$, Figure 6H). Together these data suggest that IL-6 is not required for PDX mediated activation of AMPK.

PDX regulates the STAT-3-gluconeogenesis axis in an IL-6 dependent manner

We next evaluated the role of IL-6 in PDX mediated activation of hepatic STAT-3 and the transcriptional suppression of hepatic gluconeogenesis. In support of our previous findings (Figure 4F), here we observed that PDX promotes robust phosphorylation of hepatic STAT-3 on ser727 in both saline and lipid infused WT mice ($P<0.01$, Figure 6, C and I). Importantly, in contrast to AMPK, this effect of PDX was found to be completely absent in KO mice. The activation of STAT-3 by PDX was not associated with further suppression of PGC1α in insulin sensitive saline infused mice (Figure 6D). However, PDX administration in saline infused WT mice clearly had an additive effect on the transcriptional repression of PEPCK downstream of PGC1α ($P<0.01$, Figure 6E). In line with STAT-3, this effect of PDX was found to be completely absent in saline infused KO mice which displayed significantly elevated levels of PEPCK expression compared to vehicle treated WT mice ($P<0.05$). PDX administration also tended to improve the suppression of G6Pase in saline infused WT mice ($P=0.0614$) but this was not the case for their KO counterparts where G6Pase expression was also increased compared to vehicle treated WT mice ($P<0.01$, Figure 6F). These data suggest that IL-6-STAT-3 mediated suppression of PEPCK and G6Pase likely accounts for the glucose lowering (Figure 5 A) and insulin sensitizing (Figure 5, B-E) actions of PDX in saline infused mice. Furthermore, the elevated expression of these enzymes in KO mice likely explains the slight impairment in hepatic insulin action detected during the clamp (Figure 5E).

In contrast to the insulin sensitive saline infused mice, PDX administration significantly improved the suppression of PGC1α ($P<0.01$) alongside PEPCK ($P<0.05$) and G6Pase ($p<0.001$) in lipid infused WT mice (Figure 6J-L). Importantly, these effects of PDX were entirely absent in lipid infused KO mice. As observed for saline infused mice, systemic absence of IL-6 lead to an increase in

PEPCK expression compared to vehicle treated WT mice ($P<0.01$, Figure 6K); however, this was not the case for G6Pase whose expression was significantly reduced compared to vehicle treated WT mice ($P<0.01$, Figure 6L). This inverse regulation of G6Pase mRNA expression in lipid infused KO mice likely represents a compensatory mechanism to restrain hepatic glucose output in conditions of insulin resistance. Together these data support our working hypothesis that the IL-6-STAT-3 pathway underlies the insulin sensitizing and glucoregulatory actions of PDX in liver through transcriptional repression of PGC1α, PEPCK and G6Pase.

Discussion

In the present study we have identified the docosanoid resolution mediator, PDX, as a novel glucoregulatory agent with exciting potential for combating insulin resistance and T2DM owing to a combination of potent anti-inflammatory, glucose lowering and insulin sensitizing actions. Surprisingly this anti-inflammatory mediator appears to stimulate glucose metabolism *in vivo* by promoting the release of the prototypic myokine IL-6. To the best of our knowledge this is the first report of an agent other than contraction/exercise that directly promotes skeletal muscle IL-6 expression.

In line with the currently described mechanisms of action of IL-6 in the literature, our findings support a model wherein PDX-dependent IL-6 release promotes the suppression of hepatic glucose production in an endocrine fashion via STAT-3 mediated transcriptional repression of PGC1α, PEPCK, and G6Pase (Inoue et al., 2006; Inoue et al., 2004). We also found that PDX stimulates AMPK in skeletal muscle however this action does not appear to require muscle IL-6 release.

Since the favorable effect of PDX on skeletal muscle glucose metabolism was entirely absent in IL-6 null mice it is presently unclear what contribution AMPK makes to the beneficial actions of PDX reported herein. Interestingly, we found that PDX did not further improve peripheral glucose disposal in saline-infused mice whereas there was a substantial improvement in their lipid challenged counterparts. These data suggest that PDX induced IL-6 likely improves muscle glucose metabolism by protecting against the lipid insult rather than by directly

promoting glucose uptake in this tissue. In contrast, PDX administration clearly potentiated insulin mediated suppression of hepatic glucose production in both lipid and saline infused mice suggesting that PDX induced IL-6 directly modulates glucose production in liver. Our studies in IL-6 null mice support this notion and point toward a mechanism previously described by Inoue et al (Inoue et al., 2004) whereby STAT-3 restricts PEPCK and G6Pase expression independently of insulin mediated suppression of PGC1α. Importantly, in lipid challenged mice it appears that PDX improves the insulin mediated suppression of PGC1α while also activating STAT-3 mediated suppression of PEPCK and G6Pase.

To the best of our knowledge this is the first report where IL-6 KO mice were studied using the HIE clamp in conditions of lipid excess. Our findings are in complete disagreement with a role for IL-6 in the development of lipid-induced insulin resistance since we found that lack of IL-6 does not prevent insulin resistance in lipid-infused mice. To the contrary, we found that insulin sensitive saline-infused IL-6 KO mice display a slight defect in hepatic insulin action that is associated with altered regulation of hepatic PEPCK and G6Pase but not PGC1α. However, this slight effect on hepatic insulin action did not cause a significant change of whole body glucose metabolism. Our data thus join a growing body of work (Awazawa et al., 2011; Carey et al., 2006; Pedersen and Febbraio, 2007; Wunderlich et al., 2010) that argues for a positive role of IL-6 in the regulation of glucose metabolism.

Interestingly, in addition to potentiating insulin action, PDX administration also induced a characteristic lowering of basal glycemia that was IL-6 dependent and preceded insulin administration in both saline and lipid-infused mice. This suggests that PDX and IL-6 might also represent promising therapeutic targets as insulin independent glucose lowering agents. This finding is in agreement with work showing that exposure of mouse soleus to IL-6 and soluble IL-6 receptor increases glucose transport *ex vivo* (Gray et al., 2009) and also a recent study which showed that the hypoglycemic response to endotoxemia is absent in IL-6 KO mice (Tweedell et al., 2011). Importantly, our data suggest that this glucose lowering

effect of PDX is dependent on IL-6 mediated activation of the hepatic STAT-3 pathway which independently suppresses the expression of PEPCK and G6Pase (Inoue et al., 2004).

In addition to providing the first evidence of the insulin sensitizing and glucoregulatory actions of PDX this is also the first report demonstrating the powerful ability of PDX to suppress lipid-induced inflammation. Importantly, we observed that PDX inhibits lipid-induced secretion of pro-inflammatory chemokines and cytokines as well as activation of two well established inflammatory mediators of insulin resistance, iNOS and JNK, in macrophages in culture as well as skeletal muscle and liver *in vivo*. This is the first report in any setting documenting that PDX can inhibit iNOS and JNK. Although it was beyond the scope of this study it will be interesting to determine in future studies whether PDX interferes with toll like receptor activation or whether another mechanism is involved in its anti-inflammatory activity in the setting of lipid excess. It is plausible that activation of AMPK might underlie part of the anti-inflammatory activity of PDX (Centeno-Baez et al., 2011; Pilon et al., 2004).

In conclusion, we have identified the docosanoid resolution mediator, PDX, as a novel agent that carries potent therapeutic potential for lipid-induced inflammation and insulin resistance. What is more we have unraveled an unanticipated mechanism of action whereby PDX enhances both hepatic and peripheral glucose metabolism *in vivo* by increasing the prototypic myokine IL-6. These data add to the growing body of evidence supporting a beneficial role for IL-6 in glucose metabolism and also suggest that protectins likely underlie part of the beneficial actions of omega-3 fatty acids for obesity-related inflammation and insulin resistance.

Experimental Procedures

Macrophages

J774A.1 murine macrophages were maintained in DMEM (10% FBS) until 80% confluence. A 2mM palmitate solution or methanol vehicle in alpha-MEM (12% BSA) was added to fresh DMEM (10% FBS) to give a final concentration of 400µM palmitate. Concomitantly, PDX, RvE1, RvD1 (10 or 100nM, Cayman chemical), or vehicle was added to the media. After 16 hours, the media was collected and cells were lysed and scraped in 200µl of ice cold lysis buffer (50mM HEPES pH7.5, 150mM NaCl, 1mM EGTA, 20mM b-glycerophosphate, 1%NP40, 10mM NaF, 2mM Na3VO4, 1x protease inhibitor cocktail (Sigma)).

Paired lipid-infusion HIE clamp studies

14-week old male C57BL/6J mice from Jackson Labs were used for the first paired lipid infusion-HIE clamp study. These mice were placed on a standard laboratory chow diet with free access to food and water and kept in a 12h light 12h dark cycle at the Laval University hospital research centre animal facility. Mice were randomly assigned to saline, lipid or lipid + PDX groups. Five days prior to the experiment, mice were anesthetized and PE-10 catheters (Harvard Apparatus, QC, Canada) were inserted into the left common carotid artery and the right jugular vein for blood sampling and infusions respectively. Mice were fasted for 5h leading up to the protocol. Immediately prior to the start of the lipid infusion, PDX (1µg) or an equal volume of vehicle was administered via the jugular catheter to each group. Mice were then infused for 6h with saline ($5ml.kg^{-1}.h^{-1}$) or lipid (20% intralipid emulsion (Baxter, ON, Canada) $5ml.kg^{-1}.h^{-1}$ with $20IU.ml^{-1}$ heparin (LEO pharma, ON, Canada)). 2.5h into the infusion PDX (1µg) or vehicle was again administered to the appropriate groups and the HIE clamp was initiated as previously described (Charbonneau and Marette, 2010; Xu et al., 2009). The HIE clamp protocol consisted of a 90min tracer equilibration period followed by a 120min experimental period. Pre-clamp glycemia was taken immediately prior to the tracer equilibration period. A 5-µCi bolus of [3^{-3H}]glucose was given at the start of the tracer equilibration period followed by a 0.05-µCi/min infusion for 90min. Blood samples

were drawn for the assessment of glycemia, insulin and glucose turnover levels. Following the 90min tracer equilibration period the HIE clamp began with a primed-continuous infusion of human insulin (16mU/kg bolus followed by 4 $mU.kg^{-1}.min^{-1}$, Humulin R; Eli Lilly, Indianapolis, IN). The [3^{-3H}] glucose infusion was increased to 0.2µCi/min for the remainder of the experiment. Euglycemia (6.0-7.0mM) was maintained during clamps by infusing 20% dextrose as necessary. Blood samples were taken continuously to determine glucose specific activity as well as insulin concentrations. Mice received saline-washed erythrocytes from donor mice throughout the experimental period (5–6$µl.min^{-1}$) to prevent a fall of ≥5% hematocrit. HGP and Rd were determined using Mari's non-steady-state equations for a two-compartmental model (Mari, 1992).

For the second paired lipid infusion HIE clamp study, 10 week old male B6.129S2-$Il6^{tmkopf}$/J (IL-6 KO) and control C57BL/6J (WT) mice from Jackson Labs were used. Mice from each genetic background were randomly assigned to saline-vehicle, saline-PDX, lipid-vehicle and lipid-PDX groups. The lipid-infusion clamp study was performed as described above, except the clamp was performed with a 2.5$mU.kg^{-1}.min^{-1}$ insulin infusion as per (Mulvihill et al., 2011). All animal procedures were approved and carried out in accordance with directions of the Laval University and Canadian Councils for Animal Care.

Western blotting

Snap frozen gastrocnemius muscle and liver from mice were pulverized in liquid nitrogen then lysed overnight at 4°C in the lysis buffer described for the macrophage experiments. Immunoblotting of macrophage, liver and muscle lysates was then performed as previously described (White et al., 2010). Briefly, 50µg of protein was loaded onto a 7.5% acrylamide gel, subjected to SDS-PAGE then transferred onto nitrocellulose membranes. Membranes were then blocked and probed with the appropriate antibodies. Antibodies for p-Akt ser473, p-JNK thr183/tyr185, JNK, p-AMPK thr172, AMPK, p-ACC ser79, ACC, p-STAT-3 ser727 and STAT-3 were obtained from Cell Signaling Technology (MA, USA). Antibodies

for total Akt and iNOS were from Santa Cruz Biotechnology (CA, USA) and BD Transduction Laboratories™ (Canada) respectively.

Real-time RT-PCR

RNA was extracted from homogenized liver tissue using an RNeasy® fibrous tissue mini kit from QIAGEN. RNA was then reverse transcribed to cDNA using the high-capacity cDNA reverse transcription kit from applied biosystems. Real-time RT-PCR for Ppargc1, Pck1, G6Pc, and GAPDH was then performed using Taqman assay on demand probes and primers from Applied Biosystems in a CFX96 real-time system from BIO-RAD. The relative expression of genes of interest was then determined by normalization to the housekeeping gene GAPDH using the comparative CT method for relative gene expression (Schmittgen and Livak, 2008).

Analytical methods

Chemokines and cytokines were quantified in macrophage media, or mouse plasma using the MILLIPLEX™ MAP mouse cytokine/chemokine kit (Millipore Corporation, MA, USA). Nitrite accumulation in media was determined by Greiss assay as previously described (Pilon et al., 2004). Plasma insulin levels were assessed by RIA (Linco, MI, USA). FFA were measured using an enzymatic colorimetric assay (Wako Chemicals, VA, USA). Skeletal muscle and liver IL-6 were quantified using the mouse IL-6 ELISA kit from R&D systems. Total plasma adiponectin was determined using the ELISA from ALPCO.

Statistical Analysis

A one-way ANOVA was used for all data. Bonferonni was the post-hoc test employed. Results were considered significant when $P<0.05$.

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Figure Legends

Figure 1. PDX blunts lipid induced inflammation in macrophages

J774A.1 macrophages were exposed to palmitate (400μM) or vehicle for 16h in the presence or absence of 10 or 100nM of PDX. (A-F) PDX administration reduced macrophage chemokine and cytokine secretion in media (G) PDX also blunted nitrite accumulation in macrophage media (H-I) Immunoblots for iNOS, pJNKthr183/tyr185 and total JNK in macrophage lysates show that PDX blocks lipid-induced activation of these intracellular inflammatory mediators. Quantification of densitometry analyses are shown below the representative gels. All data are mean ±SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs vehicle; † $P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ vs palmitate. See also Figures S2 and S3 for effects of RvE1 and RvD1.

Figure 2. PDX prevents lipid-induced insulin resistance in mice

Pre-clamp glycemia is shown in panel A. (B) Glycemia and glucose infusion rate (GIR) during the HIE clamp. (C) Mean GIR for last 60min of HIE clamp is reduced by lipid-infusion but restored by PDX administration (D) Peripheral insulin action expressed as fold increase in Rd during the clamp is improved in PDX treated mice (E) PDX markedly improved hepatic insulin action expressed as percent suppression of hepatic glucose production (HGP) during the clamp (F-J) Immunoblots for pAKTser473, total AKT, iNOS, pJNKthr183/tyr185, and total JNK in gastrocnemius muscle and liver show that PDX maintains insulin signal transduction to Akt by blunting activation of JNK and iNOS. Quantification of densitometry analyses are shown below the representative gels. Lanes were run on the same gel but were non contiguous. All data are mean ±SEM, ND not

detected, * P<0.05, **P<0.01, ***P<0.001 vs Saline; † P<0.05, †† P<0.01, †††P<0.001 vs Lipid. See also Figure S3 for study design.

Figure 3. PDX inhibits lipid-induced inflammation in mice

PDX administration prevented lipid-induced elevations in plasma chemokines (A-B) as well as Th1 (C-F) and Th17 (G) cytokines during the paired lipid infusion HIE clamp. PDX also provoked an increase in IL-6 (H). All data are mean ±SEM, ND not detected, * P<0.05, ***P<0.001 vs Saline; † P<0.05, ††† P<0.001vs Lipid.

Figure 4. PDX stimulates skeletal muscle IL-6 expression

(A-B) Skeletal muscle and liver IL-6 protein expression. (C) Total plasma adiponectin. (D-E) Immunoblots for pAMPK thr172, total AMPK, pACC ser79, and total ACC in skeletal muscle. (F) Immunoblots for pSTAT-3 ser727 and total STAT-3 in liver. Quantification of densitometry analyses for immunoblots are shown below the representative gels. In each case lanes were run on the same gel but were non contiguous. (G-I) Relative mRNA expression for Ppargc1, Pck1 and G6Pc. All genes were normalized to GAPDH using the delta delta CT method. All data are mean ±SEM, * P<0.05, **P<0.01 vs Saline; † P<0.05, †† P<0.01vs Lipid.

Figure 5. IL-6 is required for the insulin sensitizing actions of PDX

Pre-clamp glycemia for PDX and vehicle (VEH) treated saline-infused animals are shown in panel A. (B) Glycemia and GIR during the HIE-clamp. (C) Mean GIR for last 60min of HIE clamp is improved by PDX administration in C57BL/6J (WT) but not IL-6 null (KO) mice. (D-E) Peripheral and hepatic insulin action in saline-infused

animals during the HIE clamp. (F) Pre-clamp glycemia for PDX and VEH treated lipid-infused animals. (G) Glycemia and GIR during the HIE-clamp. (H) Mean GIR for last 60min of HIE clamp is improved by PDX in WT but not KO mice. (I-J) Peripheral and hepatic insulin action in lipid-infused animals during the HIE clamp. All data are mean ±SEM, * P<0.05, ***P<0.001 vs WT VEH; † P<0.05, †† P<0.01vs WT PDX; § P<0.05 vs KO VEH.

Figure 6. Role of IL-6 in PDX mediated activation of AMPK and STAT-3

Panel A shows IL-6 protein expression in skeletal muscle of PDX and vehicle (VEH) treated saline-infused animals. (B-C) Immunoblots for pAMPK thr172/ total AMPK in muscle and pSTAT-3 ser727/ total STAT-3 in liver. (D-F) Relative mRNA expression for Ppargc1, Pck1 and G6Pc. (G) IL-6 protein expression in skeletal muscle of PDX and vehicle (VEH) treated lipid-infused animals. (H-I) Immunoblots for pAMPK thr172/ total AMPK in muscle and pSTAT-3 ser727/ total STAT-3 in liver. (J-L) Relative mRNA expression for Ppargc1, Pck1 and G6Pc. For all immunoblots quantification of densitometry analyses are shown below the representative gels. In each case lanes were run on the same gel but were non contiguous. For all real time RT-PCR data genes of interest were normalized to GAPDH using the delta delta CT method. All data are mean ±SEM, * P<0.05, **P<0.01, ***P<0.001 vs WT VEH; § P<0.05 vs KO VEH.

Table 1

	Saline	Lipid	Lipid + PDX
FFA's (mEq.L^{-1})			
Pre-infusion	0.204±0.001	0.206±0.004	0.226±0.018
Post-clamp	0.186±0.003	2.365±0.098 ***	2.793±0.371***
Glycemia (mmol.L^{-1})			
Pre-infusion	8.4±0.13	8.2±0.15	8.6±0.13
Clamp (final 60min)	6.5±0.08	6.4±0.08	6.5±0.08
Insulin (pmol.mL^{-1})			
Pre-clamp	21.9±3.0	36.5±4.5 *	23.1±4.2
Post-clamp	105.9±9.3	106.5±11.5	103.7±9.4

Data are mean ± SEM, * P<0.05 vs Saline, *** P<0.001 vs Saline

Table 2

	Saline				Lipid			
	WT VEH	**WT PDX**	**KO VEH**	**KO PDX**	**WT VEH**	**WT PDX**	**KO VEH**	**KO PDX**
FFA's (mEq.L^{-1})								
Pre-infusion	0.23±0.016	0.24±0.025	0.24±0.019	0.21±0.016	0.22±0.022	0.25±0.019	0.22±0.008	0.25±0.023
Post-clamp	0.10±0.02	0.10±0.04	0.10±0.03	0.11±0.03	1.03±0.18§§	1.12±0.20§§	1.04±0.11§§	1.00±0.20§§
Glycemia (mmol.L^{-1})								
Pre-infusion	9.4±0.11	8.4±0.96	10.3±0.92	10.8±0.72	9.2±0.66	8.6±0.80	9.1±0.71	10.1±0.48
Clamp (final 60min)	6.2±0.19	6.2±0.09	6.72±0.22	6.62±0.10	6.5±0.11	6.4±0.10	6.7±0.12	6.5±0.17
Insulin (pmol.mL^{-1})								
Pre-clamp	26.1±6.1	19.8±8.3	26.4±6.7	26.0±4.2	25.1±6.4	16.9±4.0	22.5±6.6	20.2±4.9
Post-clamp	150.4±41	149.2±19	148.3±14	150.9±47	152.1±23	150.6±20	153.7±34	149.3±29

Data are mean ± SEM. §§ P<0.01 vs saline control counterpart.

Figure 1

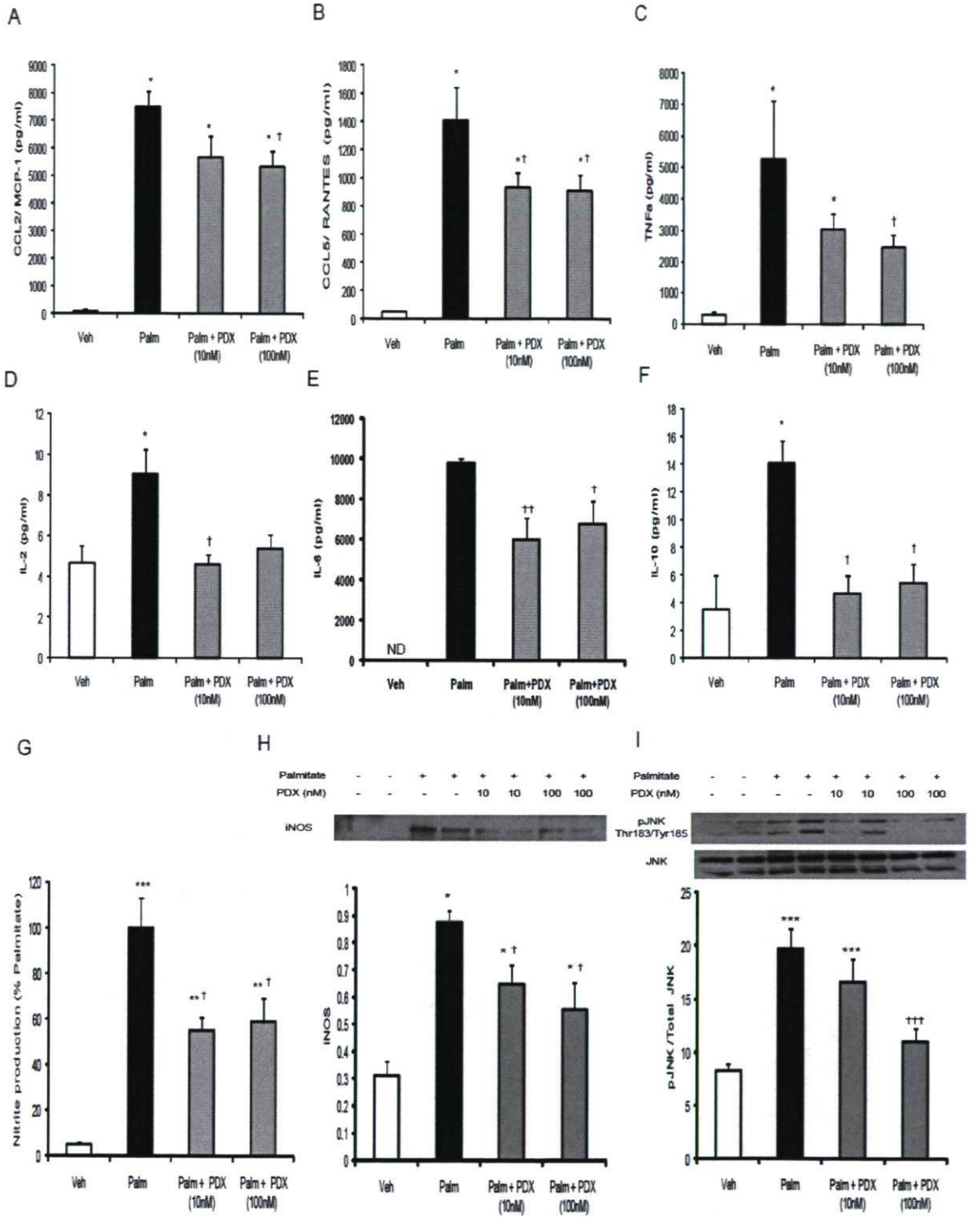

A
B
C
CCL2/ MCP-1 (pg/ml)
CCL5/ RANTES (pg/ml)
TNFa (pg/ml)
Veh
Palm
Palm + PDX (10nM)
Palm + PDX (100nM)
D
E
F
IL-2 (pg/ml)
IL-6 (pg/ml)
IL-10 (pg/ml)
ND
Palm+PDX (10nM)
Palm+PDX (100nM)
G
H
I
Palmitate
PDX (nM)
iNOS
pJNK Thr183/Tyr185
JNK
Nitrite production (% Palmitate)
pJNK /Total JNK

Figure 2

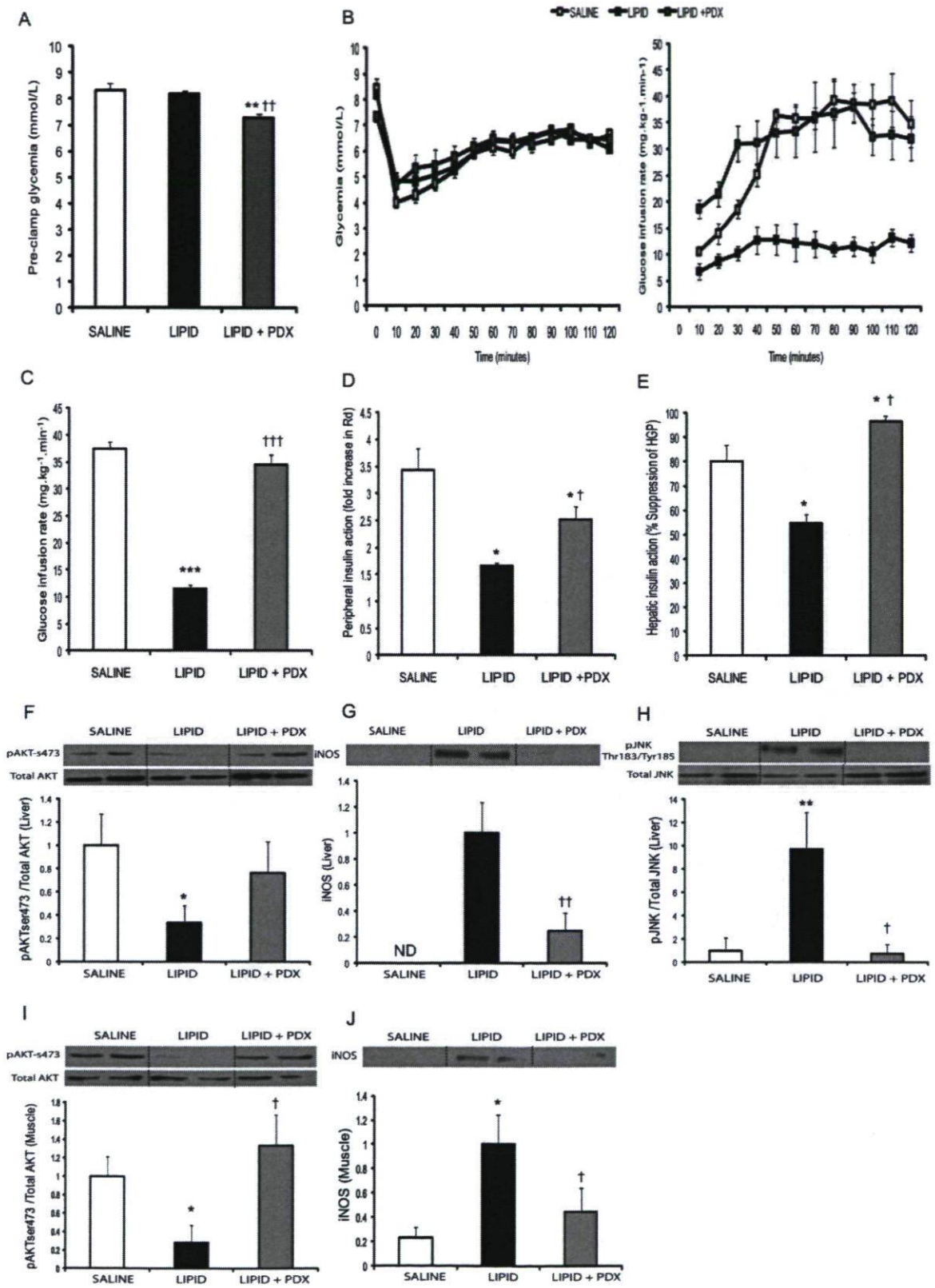

A
Pre-clamp glycemia (mmol/L)
SALINE
LIPID
LIPID + PDX
B
SALINE
LIPID
LIPID +PDX
Glycemia (mmol/L)
Time (minutes)
Glucose infusion rate (mg.kg-1.min-1)
Time (minutes)
C
Glucose infusion rate (mg.kg-1.min-1)
D
Peripheral insulin action (fold increase in Rd)
E
Hepatic insulin action (% Suppression of HGP)
F
pAKT-s473
Total AKT
pAKTser473 /Total AKT (Liver)
G
iNOS
iNOS (Liver)
ND
H
pJNK Thr183/Tyr185
Total JNK
pJNK /Total JNK (Liver)
I
pAKT-s473
Total AKT
pAKTser473 /Total AKT (Muscle)
J
iNOS
iNOS (Muscle)

Figure 3

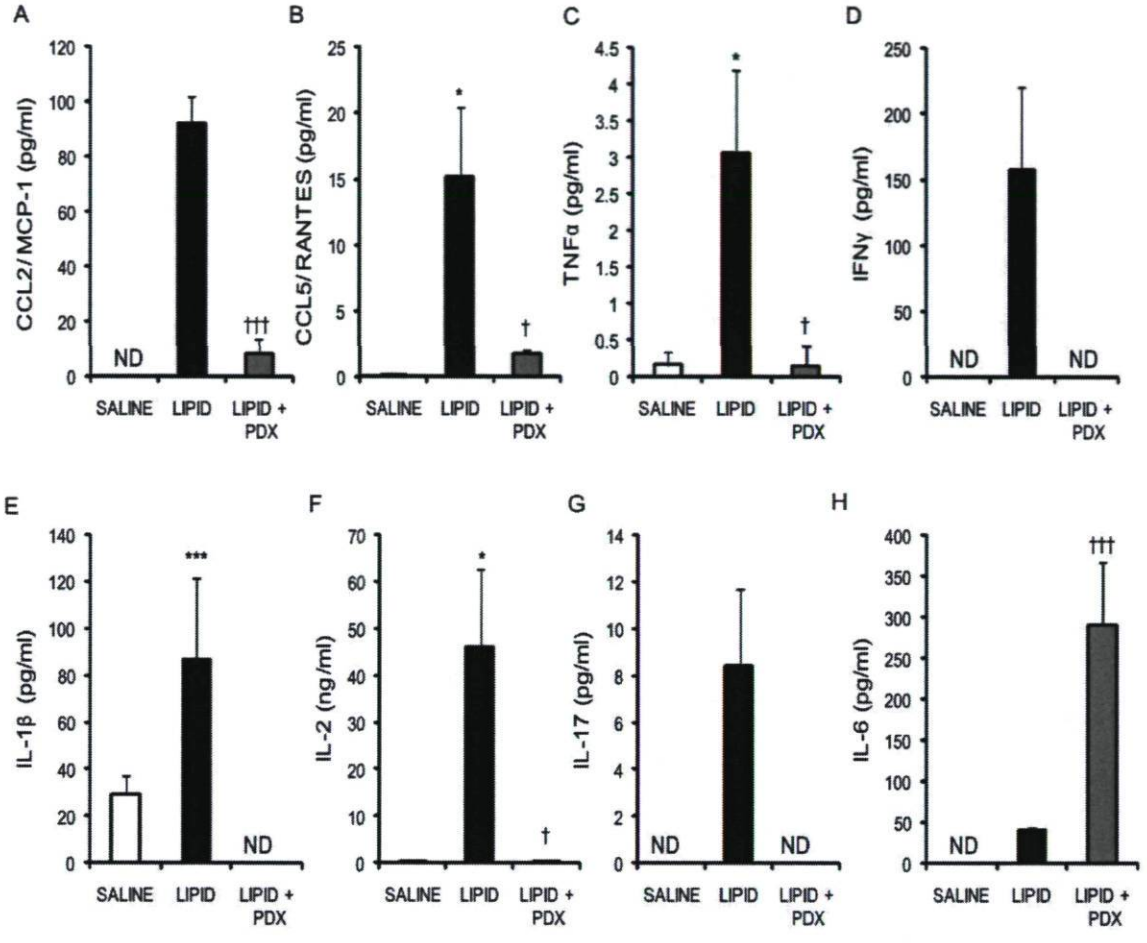

A
CCL2/MCP-1 (pg/ml)
120
100
80
60
40
20
0
ND
†††
SALINE
LIPID
LIPID + PDX
B
CCL5/RANTES (pg/ml)
25
20
15
10
5
0
*
†
SALINE
LIPID
LIPID + PDX
C
TNFα (pg/ml)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
†
SALINE
LIPID
LIPID + PDX
D
IFNγ (pg/ml)
250
200
150
100
50
0
ND
ND
SALINE
LIPID
LIPID + PDX
E
IL-1β (pg/ml)
140
120
100
80
60
40
20
0
***
ND
SALINE
LIPID
LIPID + PDX
F
IL-2 (ng/ml)
70
60
50
40
30
20
10
0
*
†
SALINE
LIPID
LIPID + PDX
G
IL-17 (pg/ml)
14
12
10
8
6
4
2
0
ND
ND
SALINE
LIPID
LIPID + PDX
H
IL-6 (pg/ml)
400
350
300
250
200
150
100
50
0
ND
†††
SALINE
LIPID
LIPID + PDX

Figure 4

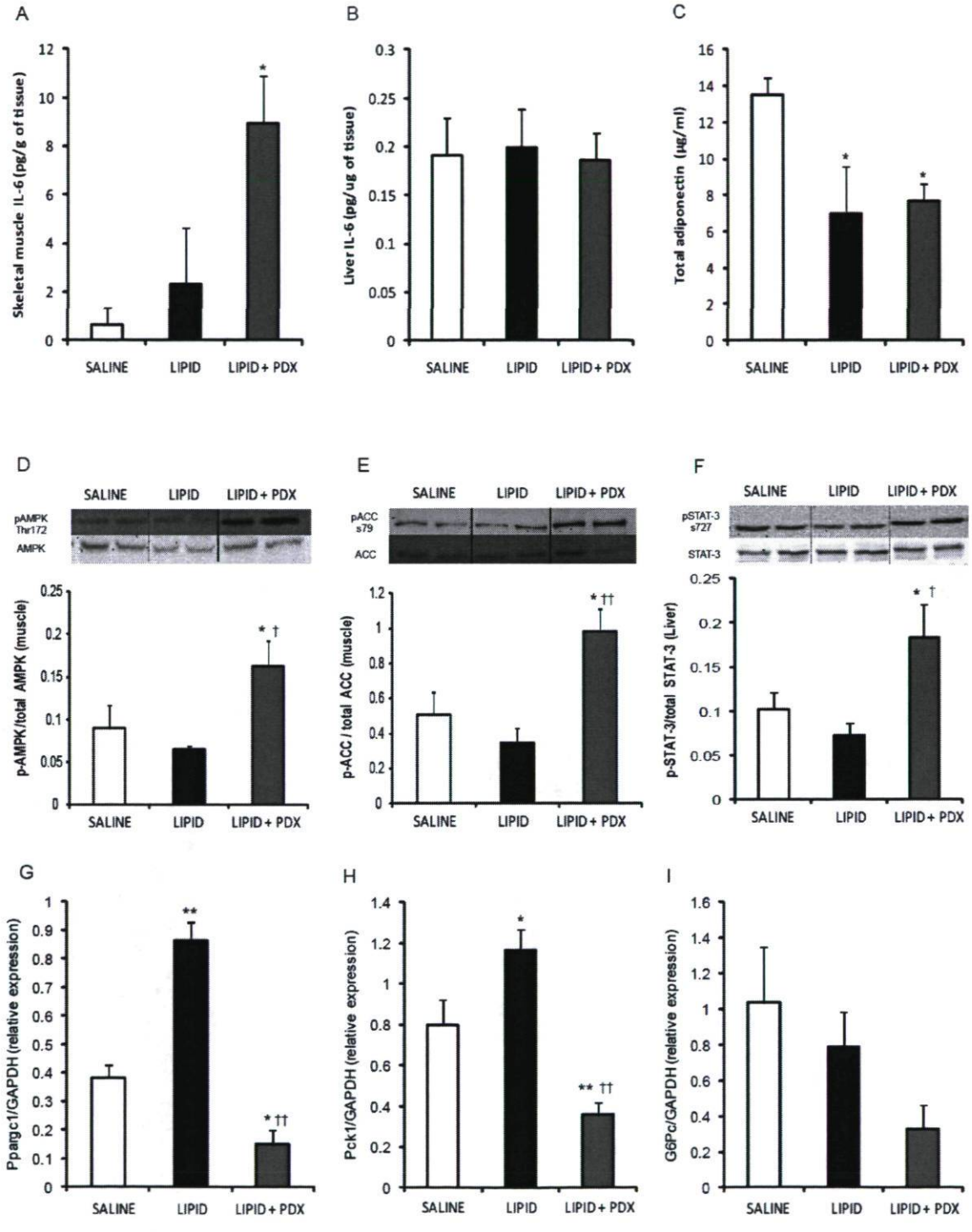

A
Skeletal muscle IL-6 (pg/g of tissue)
SALINE
LIPID
LIPID + PDX
B
Liver IL-6 (pg/ug of tissue)
C
Total adiponectin (µg/ml)
D
pAMPK Thr172
AMPK
p-AMPK/total AMPK (muscle)
E
pACC s79
ACC
p-ACC / total ACC (muscle)
F
pSTAT-3 s727
STAT-3
p-STAT-3/total STAT-3 (Liver)
G
Ppargc1/GAPDH (relative expression)
H
Pck1/GAPDH (relative expression)
I
G6Pc/GAPDH (relative expression)

Figure 5

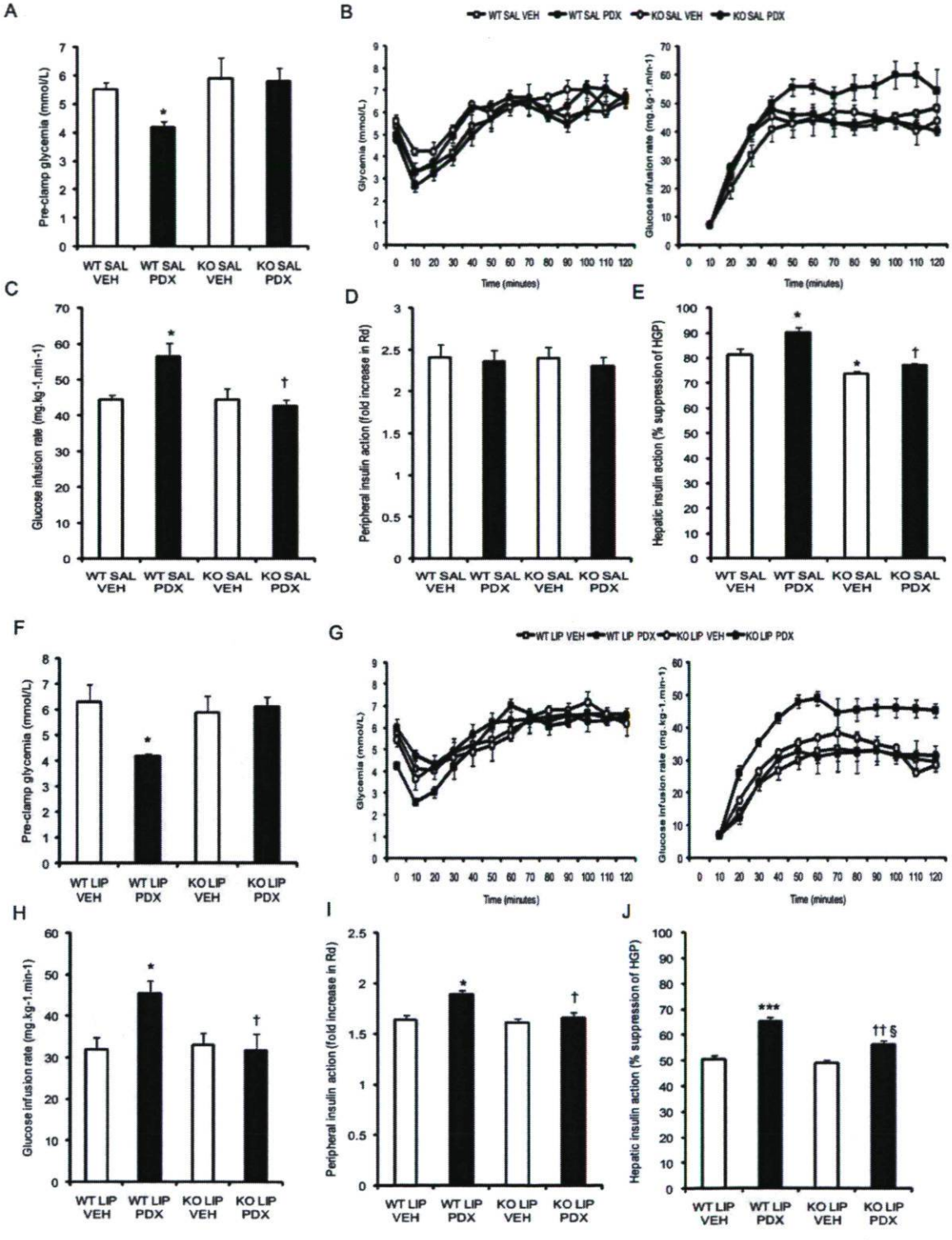
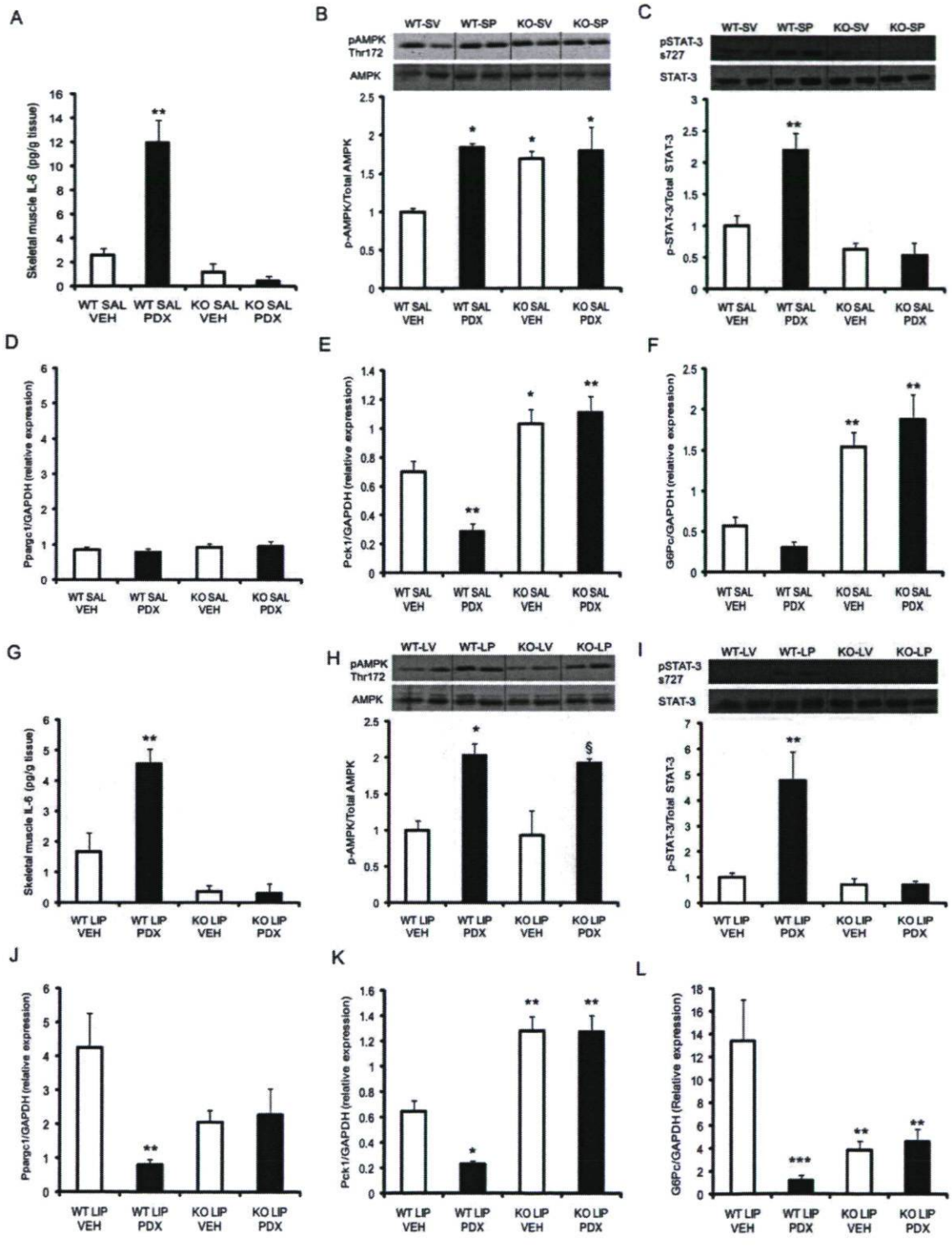

A
Pre-clamp glycemia (mmol/L)
WT SAL VEH
WT SAL PDX
KO SAL VEH
KO SAL PDX
B
WT SAL VEH
WT SAL PDX
KO SAL VEH
KO SAL PDX
Glycemia (mmol/L)
Glucose infusion rate (mg.kg-1.min-1)
Time (minutes)
C
Glucose infusion rate (mg.kg-1.min-1)
D
Peripheral insulin action (fold increase in Rd)
E
Hepatic insulin action (% suppression of HGP)
F
Pre-clamp glycemia (mmol/L)
WT LIP VEH
WT LIP PDX
KO LIP VEH
KO LIP PDX
G
WT LIP VEH
WT LIP PDX
KO LIP VEH
KO LIP PDX
Glycemia (mmol/L)
Glucose infusion rate (mg.kg-1.min-1)
Time (minutes)
H
Glucose infusion rate (mg.kg-1.min-1)
I
Peripheral insulin action (fold increase in Rd)
J
Hepatic insulin action (% suppression of HGP)

Figure 6


A
Skeletal muscle IL-6 (pg/g tissue)
WT SAL VEH
WT SAL PDX
KO SAL VEH
KO SAL PDX
B
WT-SV
WT-SP
KO-SV
KO-SP
pAMPK Thr172
AMPK
p-AMPK/Total AMPK
C
pSTAT-3 s727
STAT-3
p-STAT-3/Total STAT-3
D
Ppargc1/GAPDH (relative expression)
E
Pck1/GAPDH (relative expression)
F
G6Pc/GAPDH (relative expression)
G
Skeletal muscle IL-6 (pg/g tissue)
WT LIP VEH
WT LIP PDX
KO LIP VEH
KO LIP PDX
H
WT-LV
WT-LP
KO-LV
KO-LP
p-AMPK/Total AMPK
I
p-STAT-3/Total STAT-3
J
Ppargc1/GAPDH (relative expression)
K
Pck1/GAPDH (relative expression)
L
G6Pc/GAPDH (Relative expression)

Supplemental Figure Legends

Figure S1- RvE1 reduces fatty acid-induced inflammation in macrophages
J774A.1 macrophages were exposed to palmitate (400μM) or vehicle for 16h in the presence or absence of 10nM or 100nM of RvE1. (A-E) Chemokines and Cytokines detected in macrophage media (F) Nitrite detected in macrophage media. All data are mean ± SEM. * P<0.05, *** P<0.001 vs vehicle; † P<0.05, ††† P<0.001 vs palmitate.

Figure S2- RvD1 reduces fatty acid-induced inflammation in macrophages
J774A.1 macrophages were exposed to palmitate (400μM) or vehicle for 16h in the presence or absence of 10nM or 100nM of RvD1. (A-E) Chemokines and Cytokines detected in macrophage media (F) Nitrite detected in macrophage media. All data are mean ± SEM. * P<0.05, *** P<0.001 vs vehicle; † P<0.05 vs palmitate.

Figure S3- Paired lipid infusion HIE clamp study design
5h fasted mice were infused with lipid or saline for 6h. Prior to the initiation of the infusion a blood sample was taken for the determination of pre-infusion glycemia and FFA's. At t=150 minutes the stabilization period of the HIE clamp procedure was begun by steady infusion of 3^{-3H} glucose and erythrocytes. Immediately, prior to the commencement of the stabilization period a blood sample was taken for the determination of pre-clamp glycemia and insulinemia. The HIE clamp was begun at t=240 with infusion of insulin and glucose. Groups were administered 1ug of PDX or equal volumes of vehicle immediately prior to the infusion and at t=150. Clamp glycemia represents the mean of the measures taken during the last 60minutes of the procedure. Post-clamp insulin was determined from the blood sample taken at sacrifice.

Figure S1

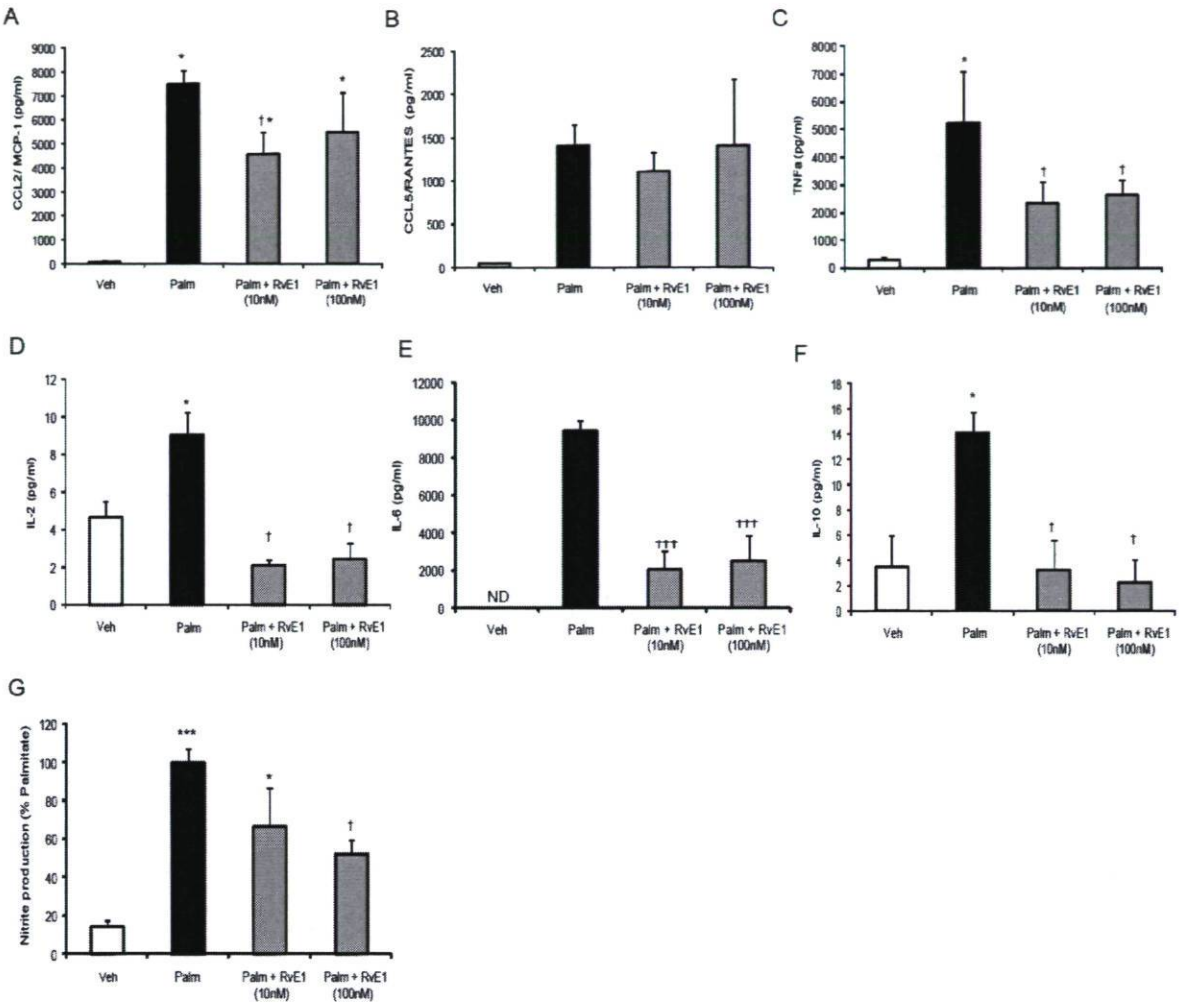

A
CCL2/ MCP-1 (pg/ml)
B
CCL5/RANTES (pg/ml)
C
TNFa (pg/ml)
D
IL-2 (pg/ml)
E
IL-6 (pg/ml)
ND
F
IL-10 (pg/ml)
G
Nitrite production (% Palmitate)
Veh
Palm
Palm + RvE1 (10nM)
Palm + RvE1 (100nM)

Figure S2

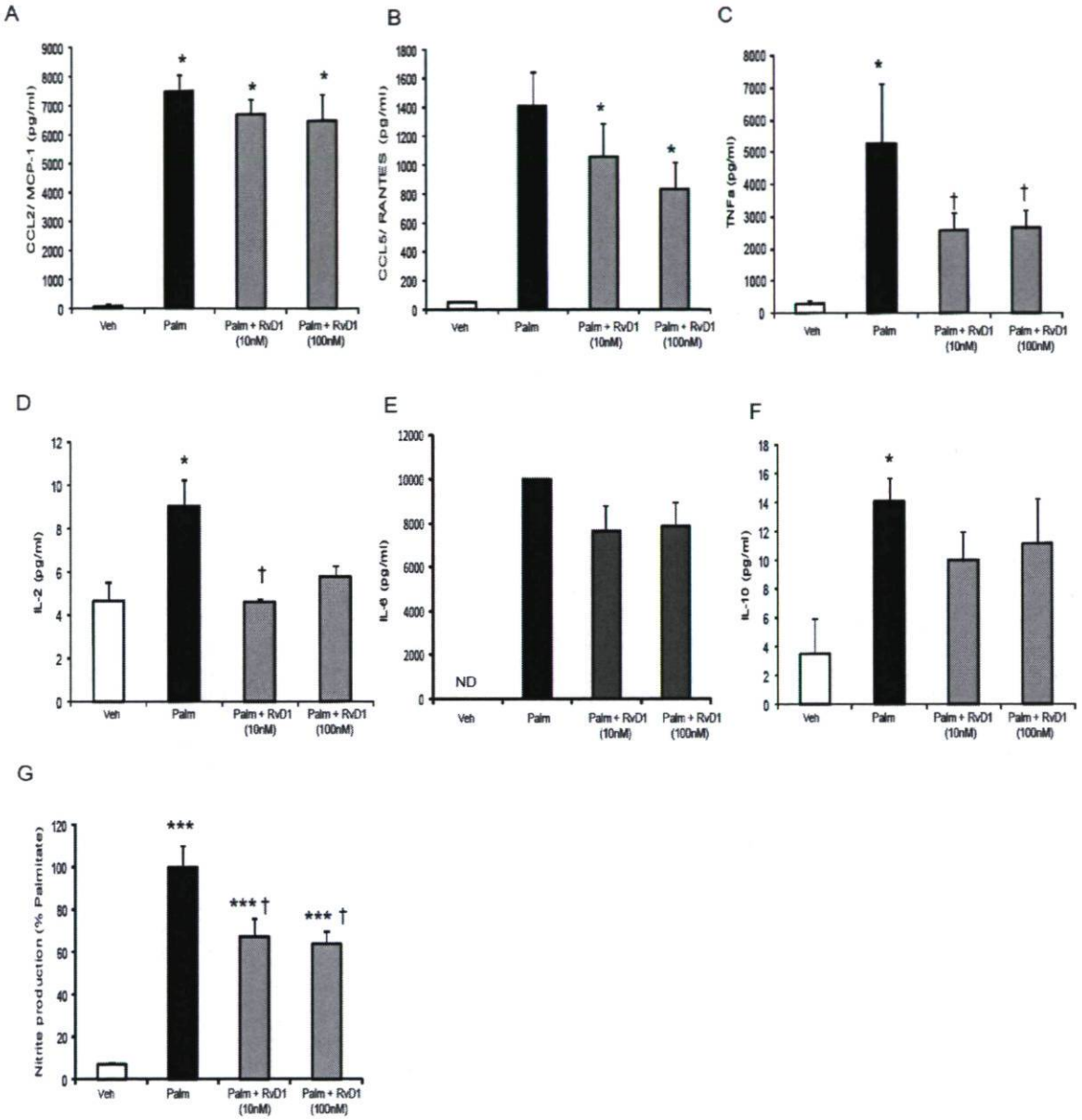
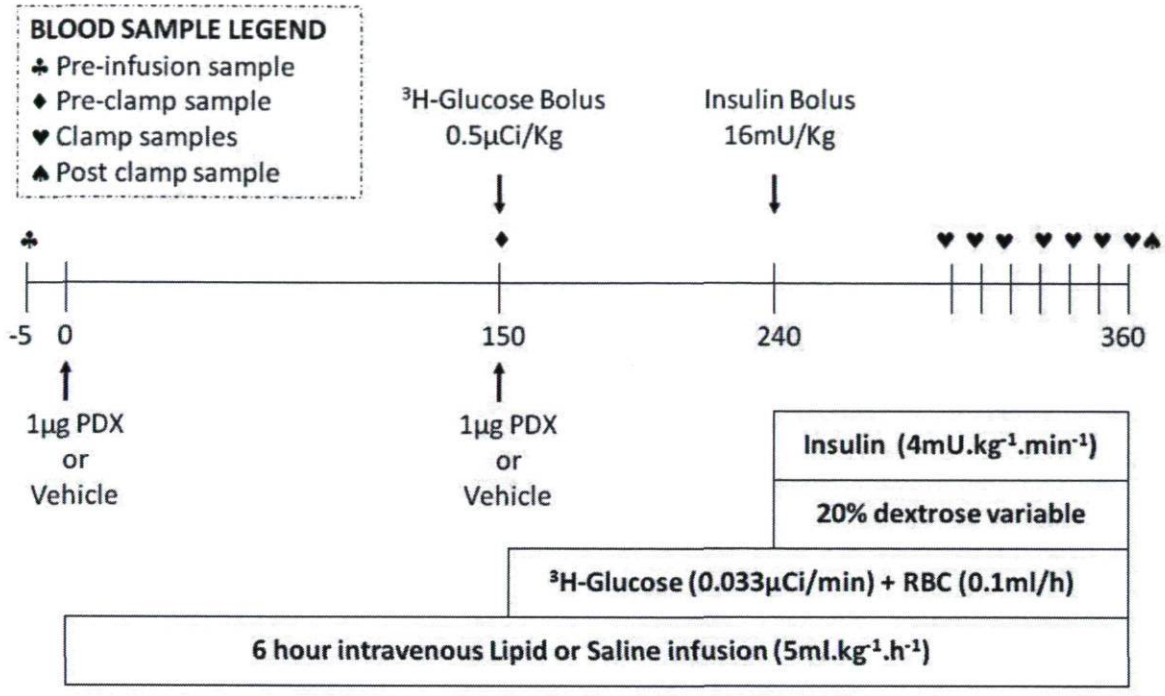

A
CCL2/ MCP-1 (pg/ml)
B
CCL5/ RANTES (pg/ml)
C
TNFa (pg/ml)
D
IL-2 (pg/ml)
E
IL-6 (pg/ml)
ND
F
IL-10 (pg/ml)
G
Nitrite production (% Palmitate)
Veh
Palm
Palm + RvD1 (10nM)
Palm + RvD1 (100nM)

Figure S3


BLOOD SAMPLE LEGEND
♣ Pre-infusion sample
♦ Pre-clamp sample
♥ Clamp samples
♠ Post clamp sample
³H-Glucose Bolus
0.5µCi/Kg
Insulin Bolus
16mU/Kg
-5
0
150
240
360
1µg PDX
or
Vehicle
1µg PDX
or
Vehicle
Insulin (4mU.kg⁻¹.min⁻¹)
20% dextrose variable
³H-Glucose (0.033µCi/min) + RBC (0.1ml/h)
6 hour intravenous Lipid or Saline infusion (5ml.kg⁻¹.h⁻¹)

CHAPTER III

Transgenic restoration of ω-3 PUFA alters the gene expression profile and morphology of adipose tissue in obese high fat fed mice.

Résumé

Contexte: L'administration diététique des acides gras polyinsaturés (AGPI) ω-3 est souvent associée à des changements dans la morphologie et/ou fonction du tissu adipeux chez la souris obèse. Cependant, il est difficile de savoir si ces observations sont une conséquence indirecte d'une réduction du gain de poids ou si cela résulte d'actions directes des AGPI ω-3. Nous avons donc étudié des souris transgéniques *fat-1* qui convertissent des AGPI ω-6 endogènes en AGPI ω-3. Ces souris présentent un gain de poids et une accrétion de la graisse identiques à leurs homologues sauvages.

Méthodologie / Principaux résultats: Nous avons effectué des analyses par biopuces Affymetrix dans le tissu adipeux viscéral des souris *fat-1* et leurs homologues sauvages nourries d'une diète riche en gras. Par la suite, nous avons effectué une analyse de voies avec le programme GenMAPP/MAPPFinder 2,0. Ceci a fait ressortir l'adipogenèse, la biosynthèse du cholestérol, la signalisation de l'insuline, la synthèse/ régulation des prostaglandines et des petits ligands RCPGs comme des domaines clés où les gènes différemment exprimés ont été clairement identifiés. En conformité avec le profil d'expression pro-adipogénique, l'analyse histologique a révélé une diminution de la grosseur des adipocytes, reflétée par un décalage vers la gauche dans la distribution de la taille des adipocytes chez les animaux transgéniques. Par ailleurs, une analyse par PCR quantificatif a confirmé que l'ARNm de PPARγ a été régulé à la hausse chez la souris *fat-1*. La restauration transgénique des AGPI ω-3 a également amélioré l'expression des récepteurs endocannabinoïdes et diminué l'expression de l'enzyme FAAH, responsable du catabolisme des endocannabinoïdes.

Conclusions / Signification: Ces données révèlent que des AGPI ω-3 coordonnent des programmes d'expression génique d'une manière indépendante de la réduction de la prise de poids ou de l'accumulation de graisse et met en évidence une influence importante des AGPI ω-3 sur l'adipogenèse et sur les voies des endocannabinoïdes dans le tissu adipeux.

Transgenic restoration of ω-3 PUFA alters the gene expression profile and morphology of adipose tissue in obese high fat fed mice.

Phillip J. White[1] and André Marette[1]

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* Correspondence: andré.marette@criucpq.ulaval.ca

Running title: Altered gene expression profile in adipose of obese *fat-1* mice

Abstract

Background: Dietary administration of ω-3 polyunsaturated fatty acids (PUFA) is often associated with altered adipose tissue (AT) morphology/function in obese mice. However, it is unclear whether these observations are an indirect consequence of reduced weight gain or result from direct actions of ω-3 PUFA. Here we studied the AT of *fat-1* transgenic mice that convert endogenous ω-6 to ω-3 PUFA. These mice display equivalent weight gain and fat accretion to their wild-type (WT) counterparts.

Methodology/Principal Findings: We performed Affymetrix microarray in epididymal AT of high fat-fed *fat-1* mice and their WT counterparts. GenMAPP/MAPPFinder 2.0 pathway analysis highlighted adipogenesis, cholesterol biosynthesis, insulin signaling, prostaglandin synthesis/regulation and small ligand GPCRs as key areas where differentially expressed genes were significantly overrepresented. In accordance with the pro-adipogenic expression profile, histological analysis revealed a left shift in adipocyte size distribution in transgenic animals. Furthermore, real-time RT-PCR confirmed that mRNA for PPARγ was upregulated in *fat-1* mice. Transgenic restoration of ω-3 PUFA also enhanced endocannabinoid receptor expression while downregulating expression of the endocannabinoid catabolic enzyme Faah.

Conclusions/Significance: These data reveal that ω-3 PUFA coordinately regulate AT gene expression programs in a manner that is independent of restriction of weight gain or fat accrual and highlight an important influence of ω-3 PUFA on adipogenesis and endocannabinoid pathways in AT.

Introduction

Adipose tissue plays an important role in the maintenance of metabolic homeostasis due to its dual role as a repository for surplus nutrients and a prominent endocrine tissue. It is well appreciated that obesity-related alterations in adipose tissue function particularly in the visceral depots has dramatic consequences for systemic glucose and lipid metabolism [1]. For instance, the onset of inflammation in visceral adipose tissue is thought to be fundamental to the development of obesity-related metabolic complications [2]. Interestingly, adipocyte size in visceral adipose tissue is positively correlated with insulin resistance [3,4], glucose intolerance [5], inflammation [6] and circulating triglycerides and cholesterol [7,8] suggesting that proper adipose function is intimately linked to cell size.

Administration of marine oils containing the ω-3 polyunsaturated fatty acids (ω-3 PUFA) eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA 22:6 n-3) has been reported to improve both glucose [9,10,11] and lipid metabolism [12,13,14,15] in obesity. Interestingly, these beneficial effects of ω-3 PUFA have been associated with reduced adipose inflammation [16,17,18,19], increased expression and secretion of the insulin sensitizing adipokine adiponectin [20,21,22], raised adipose mitochondrial activity [23], and inhibition of high fat diet-induced remodeling of adipose tissue [24]. Furthermore, ω-3 PUFA have been identified as potential endogenous ligands for the peroxisome proliferator activated receptor (PPAR) family of nuclear receptors that play a vital role in adipocyte differentiation and lipid metabolism [20,25,26,27]. Together these reports appear to suggest that direct actions of ω-3 PUFA in adipose tissue are likely responsible for at least part of the beneficial effects of these essential fatty acids.

However the interpretation of many of the aforementioned studies is complicated by the fact that a common effect of ω-3 PUFA supplementation in rodent diets is reduced weight gain and fat accretion [28,29]. Although this is likely attributable to altered palatability of the experimental diet there is growing reference in the literature to the anti-obesity effects of ω-3 PUFA [30,31,32,33]. Thus it is not clear

whether the reported actions of ω-3 PUFA in adipose tissue arise as a secondary result of reduced weight gain and limited adipose expansion or are indeed direct actions of ω-3 PUFA.

To alleviate this issue we have employed the *fat-1* transgenic mouse which has been genetically engineered to ubiquitously express the *fat-1* ω-3 fatty acid desaturase from *C. elegans*. This enzyme, not found in mammals, efficiently converts endogenous ω-6 to ω-3 PUFA such that in *fat-1* transgenic mice fed a diet rich in ω-6 and deficient in ω-3 PUFA the tissue ω-6: ω-3 ratio is approximately 1:1 compared to 50:1 in wild type animals [34]. Importantly, we recently demonstrated that when exposed to a high fat diet, *fat-1* transgenic mice are protected from obesity-linked insulin resistance, glucose intolerance and adipose inflammation despite equivalent weight gain and visceral adiposity as their wild type counterparts [19]. The *fat-1* transgenic mouse therefore represents the ideal model to study the effects of ω-3 PUFA in adipose tissue in an environment that is not confronted by dietary issues.

To expand upon our original findings and better characterize the actions of ω-3 PUFA specifically in adipose tissue herein we performed affymetrix microarray followed by GenMAPP/MAPPFinder 2.0 pathway analysis in epididymal adipose tissue from high fat fed *fat-1* transgenic mice and their wild type counterparts. We also examined adipose morphology and corroborated key findings from the microarray using real time RT-PCR.

Results

Body composition of mice used for microarray study

For the our microarray study, 6 week old *fat-1* transgenic mice and their wild type littermates were fed a high fat (HF) diet for 8 weeks with 55% of Kcal from fat as reported in our previous study [19]. Importantly, once again we found that *fat-1* transgenic mice exposed to a HF diet (F1HF) display equivalent body mass, epididymal fat accretion and liver weight as their HF-fed wild-type counterparts (WTHF; Figure 1A-C).

Effect of ω-3 PUFA on adipose tissue gene expression profile

To identify genes regulated by ω-3 PUFA in adipose tissue, RNA extracted from epididymal adipose tissue of high fat fed *fat-1* transgenic mice and their wild type counterparts was hybridized onto an affymetrix mouse 430 2.0 GeneChip®. This high density oligonucleotide array contains 45 000 probes for the analysis of gene expression levels from over 34 000 well characterized mouse genes. After normalization of the dataset using the robust multi-array average (RMA) algorithm [35], an EB Wright and Simon analysis [36] was used to determine the fold change between the two groups and to generate P values. Gene probesets where the fold change was ≥ 1.5 and the P value was < 0.05 were considered significantly different. 4435 probesets met these criteria. Of these, 2185 probesets were found to be upregulated and 2250 were found to be downregulated (see Figure 2).

In light of the vast number of differentially expressed genes detected in the microarray we elected to perform pathway analysis using the GenMAPP/MAPPFinder 2.0 software package to provide statistically oriented focus to our study [37]. The combination of GenMAPP with MAPPFinder analysis allows for the identification of established biological pathways where differentially expressed genes are significantly overrepresented. The input parameters used to identify differentially expressed genes were maintained as fold change ≥ 1.5 and P value < 0.05. Significantly represented pathways were identified by a Z-score ≥ 2 and a Westfall-Young adjusted P-value < 0.05. Using these criteria adipogenesis, cholesterol biosynthesis, insulin signaling, prostaglandin synthesis/regulation and small ligand GPCRs were highlighted as key pathways where differentially expressed genes were significantly overrepresented (Table 1).

All genes from the adipogenesis pathway found to be differentially regulated by ω-3 PUFA are listed in Table 2. Notably, the important transcriptional regulator of adipocyte differentiation, PPARγ (1.6394 fold) as well as its heterodimer partner RXRγ (1.6307 fold) were found to be upregulated in *fat-1* transgenic mice. In contrast, GATA3 an inhibitor of pre-adipocyte transition to fully differentiated adipocyte [38] was robustly downregulated (-2.647 fold). In line with other reports

[20,21,22], we also found that the adipocyte secretory product adiponectin was upregulated (2.2154 fold) by transgenic restoration of ω-3 PUFA. These data suggest that ω-3 PUFA promote adipogenesis in epididymal adipose tissue.

Transgenic restoration of ω-3 PUFA was also found to affect the expression of 53.3% of genes in the cholesterol biosynthesis pathway (Table 3). Importantly, the gene encoding HMG Co-A Reductase (Hmgcr), the rate limiting enzyme in the cholesterol biosynthesis pathway, was found to be downregulated (-1.881 fold). The reduced expression of this rate limiting enzyme was accompanied by an apparent compensatory increase in the expression of six downstream enzymes, including mevalonate kinase (1.5642 fold) which is found immediately downstream of Hmgcr in the cholesterol biosynthesis pathway. Additional manual data mining for other differentially expressed genes related to cholesterol metabolism also revealed a robust reduction in the expression of endothelial lipase (Lipg), with two out of three probesets for this gene displaying greater than 10 fold reduction in expression (probesets *1450188_s_at* and *1421262_at* displayed fold changes of -10.13006 and -13.40117 respectively $P<0.0001$ in both cases). These data suggest that ω-3 PUFA potentially exert some of their well appreciated beneficial effects on circulating cholesterol via direct actions in adipose tissue.

In line with our previous report of improved insulin sensitivity in high fat fed *fat-1* mice [19], differentially expressed genes were also found to be significantly overrepresented in the insulin signaling pathway (Table 4). Most importantly, the negative modulators of insulin receptor activation, Enpp1 (Ectonucleotide pyrophosphatase phosphodiesterase 1) [39] and Ptprf (Protein tyrosine phosphatase, receptor type, F otherwise known as LAR) [40], were robustly downregulated in *fat-1* transgenic mice (-23.17 and -2.467 fold respectively) while the expression of insulin receptor substrate 3 (Irs3) was upregulated (1.8748 fold). These changes were associated with increased expression of glycogen synthase 2 (2.4313 fold) and the immediate early response gene, Fos (1.74 fold). There was also some regulation of genes involved in vesicular trafficking and the Igf1

receptor/MAP kinase-axis of the insulin signaling pathway. All differentially expressed genes are listed in Table 4.

In accordance with the widely appreciated influence of ω-3 PUFA on eicosanoid formation, the prostaglandin synthesis/regulation pathway was also highlighted as an important area modulated by ω-3 PUFA in adipose tissue (Table 5). Of note, the expression of the prostaglandin synthases, Ptgs1 (-1.885 fold) and Ptgds (-24.47 fold), was robustly downregulated in *fat-1* mice while the expression of three prostaglandin receptors, Ptger2 (2.024 fold), Ptgfr (2.17 fold) and Ptgir (1.516 fold) was found to be increased. The expression of thromboxane synthase, Tbxas1 (1.8084 fold), was also found to be increased in transgenic mice. These data suggest that the regulation of prostanoid synthesis and signaling by ω-3 PUFA likely involves a genomic component that accompanies the well described [41,42,43] competitive inhibition of arachidonic acid (20:4 n-6) metabolism in this pathway.

Lastly, the small ligand class of GPCRs was found to be significantly overrepresented in our microarray study (Table 6). In addition to the regulation of the prostanoid receptors described above, transgenic restoration of ω-3 PUFA also resulted in increased expression of the shingosine-1 phosphate receptors, Edg1 (1.7463) and Edg3 (1.5424) as well as the endocannabinoid receptors Cnr1 (1.6074 fold change) and Cnr2 (1.7284 fold change). Manual data mining for other genes related to endocannabinoid signaling revealed that the expression of both probesets for the endocannabinoid catabolic enzyme, fatty acid amide hydrolase (Faah), was significantly reduced in *fat-1* mice (probesets *1421969_a_at* and *1434091_at* displayed fold changes of -2.5588 and -1.8228 respectively, $P<0.001$ in both cases). The elevated expression of endocannabinoid receptors accompanied by reduced expression of Faah is suggestive of reduced endocannabinoid tone in adipose tissue of *fat-1* mice.

Transgenic restoration of ω-3 PUFA alters the adipocyte size profile

Since the pathway analysis highlighted adipogenesis as a key area regulated by transgenic restoration of ω-3 PUFA, we felt it was pertinent to determine whether this gene expression profile had any impact on adipocyte size. We thus quantified cell size in hematoxylin and eosin stained sections of epididymal adipose tissue taken from wild type and *fat-1* transgenic mice in our initial study [19]. Figures 3A and 3B show representative histological sections and adipocyte size distribution profiles for high fat fed *fat-1* transgenic mice and their wild type counterparts. Importantly, despite equivalent fat mass the adipocyte size distribution profile of *fat-1* transgenic mice displayed a prominent left shift that was characterized by significantly more mid-sized adipocytes (500-2499μM^2; $P<0.001$) and less large (2500-4999μM^2; $P<0.01$) and very large adipocytes (5000-9999μM^2; $P<0.05$). Furthermore, real time RT-PCR for PPARγ corroborated the findings of the microarray and revealed a significant increase in the expression of this key adipogenic transcription factor in the epididymal adipose tissue of *fat-1* mice (Figure 3C).

ω-3 PUFA influence the endocannabinoid pathway in adipose tissue

Another striking finding of the microarray study was that ω-3 PUFA influence the expression of multiple components of the endocannabinoid pathway. Since there is very little information in the literature regarding the influence of ω-3 PUFA on endocannabinoid signaling/metabolism in adipose tissue we felt it was important to validate these findings. We therefore performed real time RT-PCR in epididymal adipose tissue of high fat fed *fat-1* mice and their wild type counterparts for Cnr1, Cnr2 and Faah. In line with the findings of the microarray study, we found that transgenic restoration of ω-3 PUFA significantly increased the expression of endocannabinoid receptor Cnr1 (Figure 4A; $P<0.05$) while reducing the expression of the catabolic enzyme Faah (Figure 4C; $P<0.05$). However, although ω-3 PUFA also appeared to increase expression of Cnr2 this effect did not reach significance (Figure 4B; $P=0.109$). These data confirm the findings of the microarray study.

Discussion

In this study we examined the influence of ω-3 PUFA on adipose tissue gene expression and morphology in obese high fat fed *fat-1* transgenic mice and their wild type counterparts. By employing transgenic expression of the *fat-1* ω-3 fatty acid desaturase as a means to elevate ω-3 PUFA *in vivo* we were able to effectively study the role of ω-3 PUFA in adipose tissue in an environment that is not confounded by dietary issues and in which weight gain and adiposity develop normally on a high fat diet (Figure 1 and [19]). Herein, we clearly demonstrate that ω-3 PUFA coordinately regulate adipose tissue gene expression programs in a manner that is independent of restriction of weight gain or fat accrual. Pathway analysis of microarray data highlighted adipogenesis, cholesterol biosynthesis, insulin signaling, prostaglandin synthesis/regulation, and small ligand GPCRs as five key areas regulated by ω-3 PUFA in adipose tissue. Importantly, we were also able to independently validate some key gene expression changes using real time RT-PCR and show that the pro-adipogenic gene expression profile was accompanied by significant changes in adipocyte size distribution.

Our microarray data suggests that the left shift in the adipocyte size distribution profile of *fat-1* mice is the result of an altered gene expression pattern characterized by robust reduction of the inhibitor of pre-adipocyte transition, GATA3 [38], combined with increased expression of pro-adipogenic transcription factors such as PPARγ. It is plausible that this pattern of gene expression would promote hyperplasia over hypertrophy in the expanding adipose tissue and thereby account for the greater number of midsized and lesser number of large adipocytes in adipose tissue of the same mass. Indeed, synthetic PPARγ agonists such as the Thiazolidinediones are well known to stimulate adipocyte hyperplasia [44,45,46,47]. Importantly, our findings support those of others showing increased expression of PPARγ following exposure to the long chain ω-3 PUFA, DHA and EPA [22,48]. Since excessive adipocyte hypertrophy is thought to underlie the pathological changes that take place in adipose tissue through mechanisms such as endoplasmic reticulum stress [49], hypoxia [50,51,52] and necrosis-like cell

death [53,54] this important adipogenic influence of ω-3 PUFA is likely responsible for many of the protective properties of these essential fatty acids in obesity.

We also employed real time RT-PCR to validate microarray data concerning genetic regulation of the endocannabinoid pathway by ω-3 PUFA in *fat-1* mice. It is noteworthy that the two most studied members of the endocannabinoid family, 2-arachidonylglycerol (2-AG) and anandamide/*N*-arachidonoylethanolamine (AEA), are metabolites of the ω-6 PUFA, arachidonic acid (20:4; n-6) [55]. Since ω-3 PUFA are known to interfere with the formation of ω-6 derived metabolites [41,42,56,57], our observation of elevated expression of endocannabinoid receptors in association with reduced expression of the catabolic enzyme, Faah, likely represents reduced endocannabinoid presence in adipose tissue of *fat-1* mice. This is in line with the work of Batetta et al [58] and Piscitelli et al [59] which shows that administration of krill oil containing the long chain ω-3 PUFA, EPA and DHA, lowers levels of AEA and 2-AG, in visceral adipose tissue of obese zucker rats and high fat fed mice respectively. To the best of our knowledge this is the first report of the effect of ω-3 PUFA on the expression of endocannabinoid receptors and catabolic enzymes in adipose tissue. Since, elevated endocannabinoid tone has been found to contribute to the peripheral metabolic derangements taking place in obesity [60] this effect of ω-3 PUFA warrants further investigation.

Another interesting outcome of our microarray study was the impact of transgenic restoration of ω-3 PUFA on enzymes related to cholesterol biosynthesis in adipose tissue. The pathway analysis revealed that ω-3 PUFA lower the expression of the rate limiting enzyme of the cholesterol biosynthesis pathway, HMG Co-A reductase, in adipose tissue resulting in a corresponding increase in the expression of six downstream enzymes. Although we are the first to report this effect of ω-3 PUFA in adipose tissue our findings are in line with studies showing that administration of either EPA or DHA reduces the expression of HMG Co-A reductase in liver [30]. Interestingly, a recent study on the effects of the HMG Co-A reductase inhibitor, Pitavastatin, in adipose tissue revealed that inhibition of this enzyme alters the adipocyte size distribution profile significantly decreasing the

frequency of large adipocytes without altering fat pad mass [61]. Given that this observation is very similar to our finding with transgenic restoration of ω-3 PUFA (Figure 3), these data suggest that the ω-3 dependent inhibition of HMG Co-A reductase expression likely contributes to the altered adipose morphology observed in *fat-1* mice.

In addition to the effect on HMG Co-A reductase we also found that transgenic restoration of ω-3 PUFA robustly inhibits the expression of endothelial lipase in adipose tissue. Elevated expression of endothelial lipase is thought to be responsible for reduced levels of circulating high density lipoprotein (HDL) cholesterol in obesity [62,63]. Therefore it is likely that this effect of ω-3 PUFA in adipose tissue accounts for at least part of the increase in HDL cholesterol often associated with fish oil administration [9,13]. It is noteworthy that the HMG Co-A reductase inhibitor Pitavastatin has also been reported to inhibit the expression of endothelial lipase [64]. Thus the activities of ω-3 PUFA in adipose tissue appear to resemble those of the Statin class of lipid lowering drugs.

Since we had previously reported that high fat fed *fat-1* transgenic mice display improved insulin sensitivity [19] it was not surprising to see that the insulin signaling pathway was also highlighted in our pathway analysis. Importantly, the robust reduction of the expression of two well known inhibitors of insulin signal transduction adds further mechanistic insight into the manner by which ω-3 PUFA likely improve insulin sensitivity in adipose tissue. Enpp1 is increased in adipose tissue of insulin resistant humans [65] and is known to interact with the insulin receptor and interfere with autophosphorylation of the beta subunit [39,66]. Similarly, Ptprf is increased in adipose tissue of obese humans [40] and is found to directly dephosphorylate the insulin receptor by virtue of its intrinsic phosphatase activity [67]. Interestingly, both Enpp1 [68] and Ptprf [69] were recently reported to be robustly downregulated during the early phase of adipogenesis while overexpression of either was found to prevent adipocyte differentiation and maturation. Hence, beyond insulin sensitization the enhanced suppression of these enzymes likely also contributes to the pro-adipogenic phenotype present in *fat-1*

mice. Future studies are required to determine whether ω-3 PUFA directly regulate the expression of Enpp1 and Ptprf through interaction with transcription factors or whether the regulation of these enzymes occurs secondary to the improved adipogenic profile or previously reported anti-inflammatory actions of ω-3 PUFA in adipose tissue [19].

Finally, in light of our earlier work [19] which showed increased activity of ω-3 resolution mediator biosynthetic pathways in adipose tissue of *fat-1* mice we felt it was important to determine whether these bioactive ω-3 metabolites could contribute to the adipogenic profile revealed herein. We therefore exposed 3T3-L1 adipocytes to effective anti-inflammatory doses (100nM) of Resolvin E1 (RvE1), Resolvin D1 (RvD1) or Protectin DX (PDX) throughout their differentiation. However, in contrast to the PPARγ agonist Rosiglitazone, we found that administration of RvE1, RvD1 or PDX has no impact on lipid accrual in differentiating 3T3-L1 adipocytes (data not shown). These data suggest that resolvins and protectins likely do not posses any direct adipogenic activity. Nevertheless, these findings do not rule out the possibility that ω-3 derived resolution mediators could contribute indirectly to the improved adipogenic profile in high fat fed *fat-1* mice. Indeed, we have previously demonstrated that the inducible form of nitric oxide synthase (iNOS), a mediator of adipose inflammation, negatively regulates PPARγ activity and adipogenesis in a nitric oxide dependent manner [70]. Thus these anti-inflammatory mediators might act indirectly to sustain rather than promote adipogenesis in obesity by counteracting an endogenous inflammatory brake that normally restricts this process. Alternatively, other novel ω-3 derived resolution mediators that were not tested in this study, such as the newly described Maresins [71], or combined actions of multiple resolution mediators might be responsible for the adipogenic profile observed in *fat-1* mice. Thus additional studies are warranted to examine these different possibilities.

In conclusion, the microarray-pathway analysis approach employed in *fat-1* transgenic mice in this study led to the identification of five focal points of ω-3 PUFA action in adipose tissue. These data should serve as starting points for

future studies on the mechanism of action of ω-3 PUFA in fat. Interestingly, genes affected in the cholesterol and insulin signaling pathways were also found to have secondary roles in the regulation of adipogenesis. These data provide a likely explanation for the altered adipocyte size distribution apparent in *fat-1* mice and imply that the genomic influence of ω-3 PUFA in adipose tissue is highly coordinated towards this outcome. Importantly, the influence of ω-3 PUFA on adipose tissue morphology and gene expression programs identified herein occurred independently of any restriction of weight gain or fat accrual.

Materials and Methods

Animals

Mice used in the microarray study (n=3 per group) were from a different cohort of mice than those described in [19]; however, tissues used to validate the findings of the microarray study by real time RT-PCR and histological analyses were from the mice described in [19]. The dietary treatment used for both cohorts of mice was identical. Briefly, six-week-old male hemizygous *fat-1*(+/-) mice and their wild type littermates bred at the Laval University hospital research center were fed high fat diets (diet-9302, 55% Kcal from fat, Harlan Teklad) and sacrificed after 8 weeks. At sacrifice epididymal adipose tissues excised for the microarray study were rapidly homogenized in QIAZOL (QIAGEN) and snap frozen in liquid nitrogen. Histology was performed in sections of epididymal adipose that were placed in 4% paraformaldehyde at sacrifice whereas the samples used for the real time RT-PCR came from tissues that were rapidly excised and snap-frozen in liquid nitrogen. Animal procedures were approved and carried out in accordance with the Laval University and Canadian Councils for Animal Care.

RNA extraction, quantitative real time RT–PCR and microarray hybridization

RNA was extracted for real time RT-PCR and microarray studies using the RNeasy lipid tissue mini kit (QIAGEN) by following the manufacturer's instructions. For real time RT-PCR, RNA was then reverse transcribed to cDNA using the high-capacity cDNA reverse transcription kit from Applied Biosystems. Real-time PCR for Pparg,

Cnr1, Cnr2, Faah and GAPDH was then performed using Taqman assay on demand probes and primers from Applied Biosystems in a CFX96 real-time system from BIO-RAD. The relative expression of genes of interest was then determined by normalization to the housekeeping gene GAPDH using the comparative C_T method for relative gene expression [72].

For microarray studies the RNA quality testing, quantification and hybridization to the Mouse 430 2.0 Affymetrix gene chip was performed at the McGill University and Génome Québec Innovation Center in Montréal, Québec.

Microarray data analysis

Array images were preprocessed into CEL files at the McGill University and Génome Québec Innovation Center in Montréal, Québec. Microarray data was then normalized using the FlexArray 1.6.1 software package. After normalization of the dataset using the robust multi-array average (RMA) algorithm [35] an EB Wright and Simon analysis [36] was applied to the data to determine the fold change between the two groups and to generate P values. This dataset was then imported into GenMAPP/MAPPFinder 2.0 for subsequent pathway analysis. The input parameters used to identify differentially expressed genes were a fold change ≥ 1.5 and a P value < 0.05. Significantly represented pathways were identified by a Z-score ≥ 2 and a Westfall-Young adjusted P-value < 0.05.

Adipose histology

Adipose tissue sections were embedded and mounted and hematoxylin eosin staining was performed at the University Laval microscopy facility. Images were acquired using a BX60 microscope (Olympus, NY) and an RT slider 2.3.0 camera (Diagnostic Instrument, Sterling Heights, MI). Adipocyte area analysis was performed using Image J software from NIH.

Statistical Analysis

Adipocyte size distribution data was analyzed by two-way ANOVA with Bonferroni as the post-hoc test used. Real time RT-PCR data were analyzed using Students t-test. Data were considered statistically significant when $P<0.05$.

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Figure Legends

Figure 1. Body composition of mice used in the microarray study

Body weight, epididymal fat pad mass and liver weight for the high fat fed WT (WTHF) and *fat-1* (F1HF) mice used in the microarray study are shown in panels A-C respectively. Body and tissue weights were measured at sacrifice after 8 weights of high fat feeding. Data are mean ±SEM.

Figure 2. Volcano plot of differentially regulated genes

An EB Wright and Simon analysis was applied to the microarray comparison between the adipose tissue taken from high fat fed *fat-1* mice and their wild type counterparts (n=3 mice per group). The negative log10-transformed p-values are plotted against the log2 fold change. Dark dots represent the 4335 differentially expressed probesets that show fold changes > 1.5 fold and P values <0.05. Lighter dots represent all other probesets.

Figure 3. Transgenic restoration of ω-3 PUFA alters adipocyte size distribution

Representative images of adipose tissue sections from high fat fed wild type (WTHF) mice and their *fat-1* (F1HF) counterparts are shown for two different magnifications in panel A. Adipocyte size distribution profiles are displayed in Panel B (n=7 mice per group). Panel C shows relative mRNA expression for PPARγ normalized to GAPDH using the delta delta CT method. All data are mean ±SEM, * P<0.05, **P<0.01, ***P<0.001.

Figure 4. ω-3 PUFA influence the endocannabinoid pathway in adipose tissue

Panels A-C show relative mRNA expression for CnR1, CnR2 and Faah in adipose tissue of high fat fed wild type (WTHF) mice and their *fat-1* (F1HF) counterparts. Genes were normalized to GAPDH using the delta delta CT method. All data are mean ±SEM, * P<0.05.

Table 1. MAPPs where differentially expressed genes were significantly overrepresented.

MAPP name	Number changed/ number measured	Number measured/ number on MAPP	Z-score	Adjusted P-value
Adipogenesis	33/130	130/132	4.386	0.017
Cholesterol biosynthesis	8/15	15/15	4.715	0.007
Insulin signaling	37/157	157/159	4.142	0.023
Prostaglandin synthesis regulation	11/30	30/31	3.936	0.046
Small ligand GPCRs	8/18	18/19	4.034	0.033

Table 2. Differentially expressed genes in the adipogenesis pathway

Gene function	Gene	Fold change
Inhibitor of pre-adipocyte to adipocyte transition	Gata3	-2.647
Transcription factors/Modulators	PPARy	1.6394
	RXRy	1.6307
	Nr1h3	1.6713
	Cebp/B	1.54
Adipocyte secretory products	Adpn	2.2154
	Pbef1	1.5896
	Spock1	-2.459
Growth factors/Hormones	Igf1	1.7646
Insulin action genes	Irs3	1.8748
Possible lipodystrophy genes	Lpin1	1.5265
Miscellaneous	Serpine1	2.1043
	Twist1	1.8054
	Stat1	1.9198
	Stat5b	1.8384
	Socs3	1.5875
	Trib3	1.7895
	Egr2	-4.46

Table 3. Differentially expressed genes in the cholesterol biosynthesis pathway

Gene function	Gene	Fold change
Convert HMG-CoA to Mevalonate	Hmgcr	-1.881
Convert Mevalonate to Mevalonate-5-P	Mvk	1.5642
Convert Mevalonate-5-P to Mevalonate-5-PP	Pmvk	1.693
Convert Mevalonate-5-PP to Delta-Isopentenyl	Mvd	1.5113
Convert Squalene-2,3-Epoxide to Lanosterol	Lss	2.0161
Convert Lanosterol to Lathosterol	Cyp51	1.5849
Convert Lathosterol to 7-dehydro Cholesterol	Sc5d	1.8018

Table 4. Differentially expressed genes in the insulin signaling pathway

Gene Function	Gene	Fold change
Modulators of insulin action	Enpp1	-23.17
	Ptprf	-2.467
	Socs3	1.5875
	Trib3	1.7895
Insulin receptor substrate	Irs3	1.8748
PDK/Akt Signaling	Sgk	1.5452
Immediate early gene	Fos	1.74
Metabolic regulation	Gys2	2.4313
Vesicular trafficking	Flot1	1.5016
	MGI:1345171	2.4266
	Ehd1	1.8525
	Rapgef1	1.6815
	Cap1	-2.41
	Cblc	-1.757
Receptor	Igf1r	-3.257
Ras-MAPK Signaling	Rrad	2.0601
MEK/MAP Kinases	Mapk13	-3.426
	Map3k1	-1.581
	Map3k8	1.5339
	Map4k1	1.7873

Table 5. Differentially expressed genes involved in prostaglandin synthesis/regulation

Gene Function	Gene	Fold change
Prostaglandin receptor	Ptger2	2.024
	Ptgfr	2.17
	Ptgir	1.516
Prostaglandin synthases	Ptgs1	-1.885
	Ptgds	-24.47
	Tbxas1	1.8084

Table 6. Differentially expressed small ligand GPCRs

Gene Function	Gene	Fold change
Cannabinoid receptors	Cnr1	1.6074
	Cnr2	1.7284
Prostanoid receptors	Ptger2	2.024
	Ptgfr	2.17
	Ptgir	1.516
S1P receptors	Edg1	1.7463
	Edg3	1.5424

Figure 1

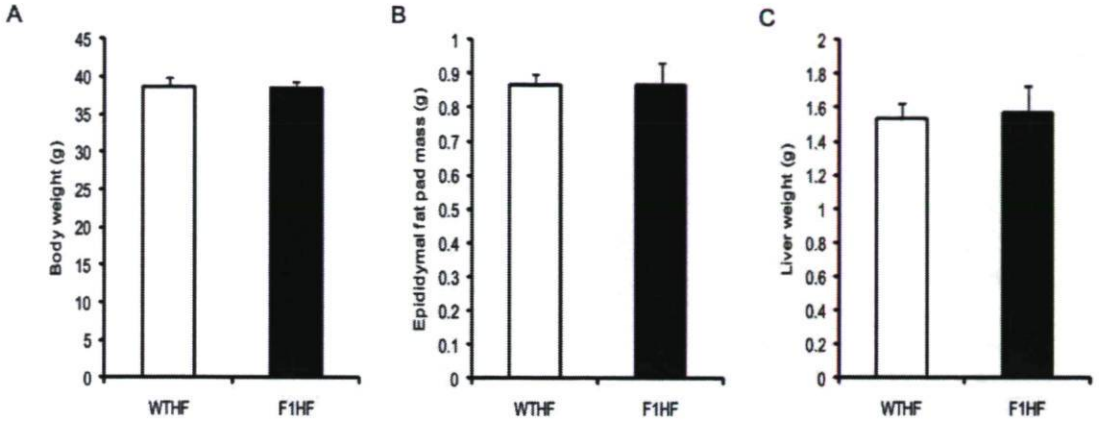

A
Body weight (g)
45
40
35
30
25
20
15
10
5
0
WTHF
F1HF
B
Epididymal fat pad mass (g)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WTHF
F1HF
C
Liver weight (g)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WTHF
F1HF

Figure 2

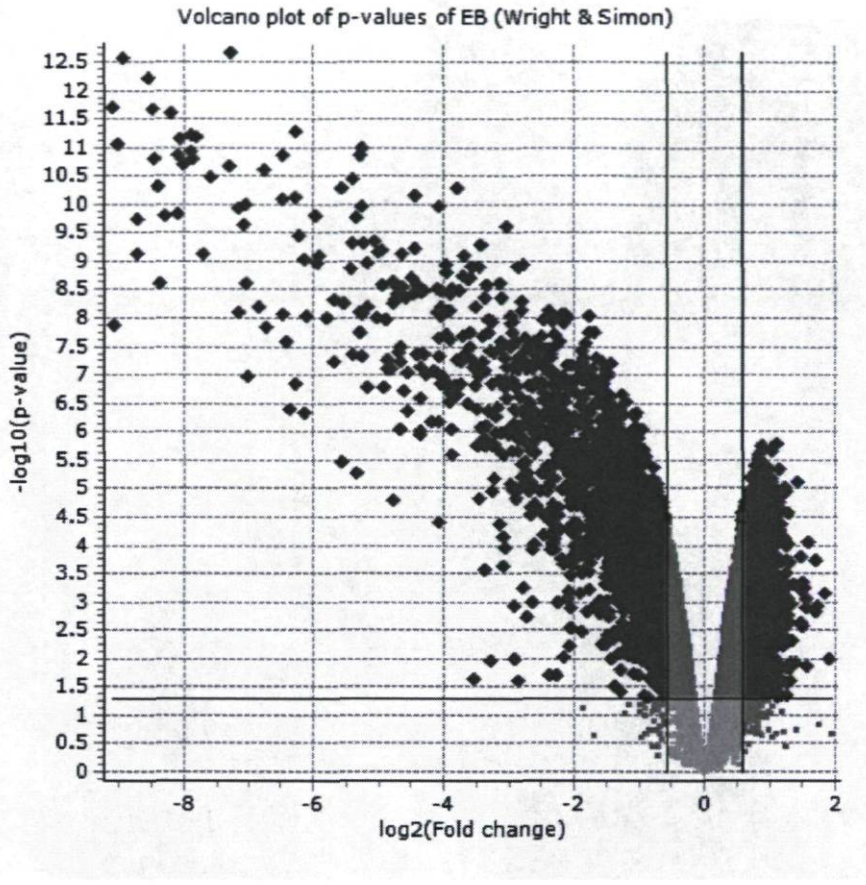

Volcano plot of p-values of EB (Wright & Simon)
-log10(p-value)
log2(Fold change)
12.5
12
11.5
11
10.5
10
9.5
9
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-8
-6
-4
-2
0
2

Figure 3

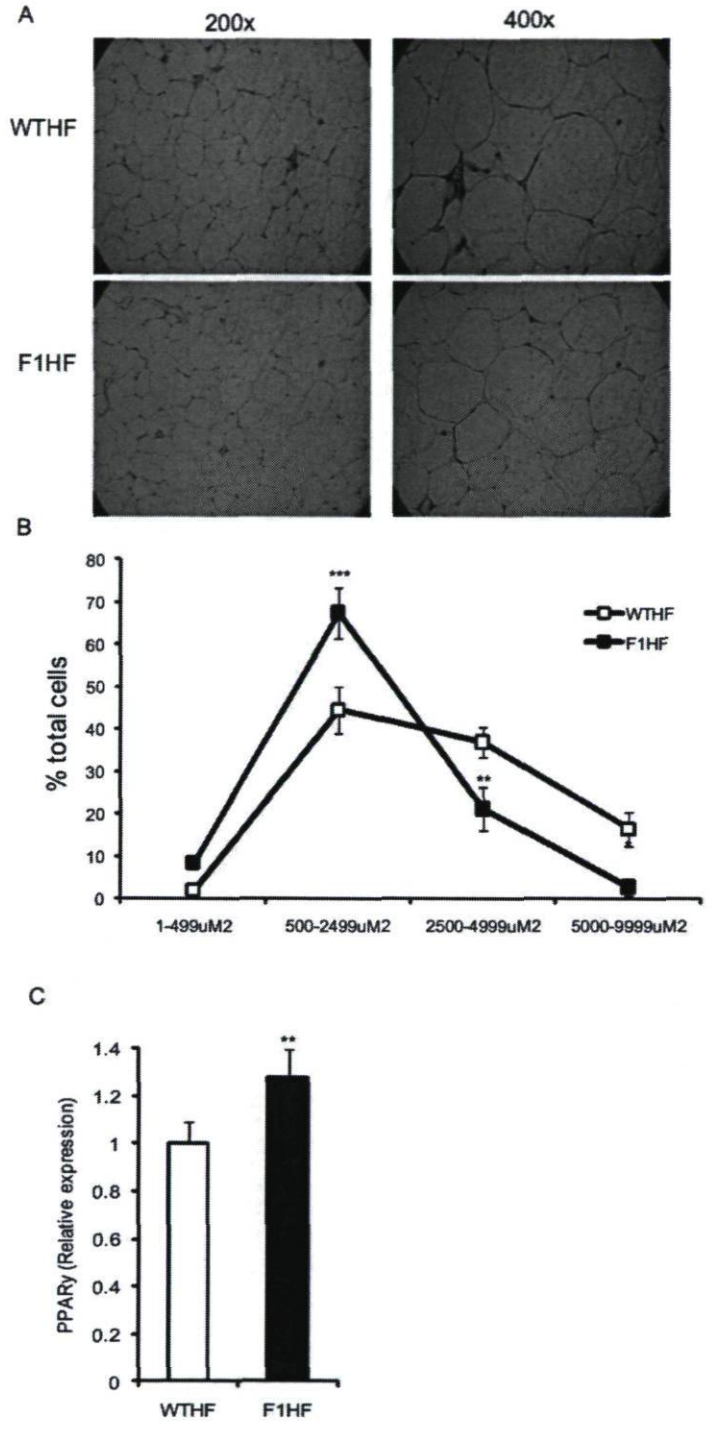
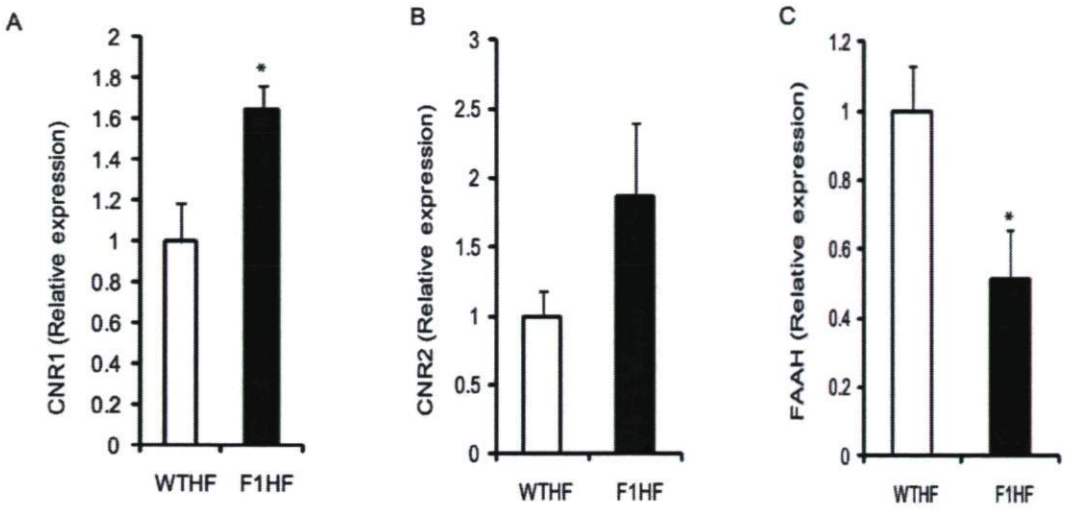

A
200x
400x
WTHF
F1HF
B
% total cells
80
70
60
50
40
30
20
10
0
***
**
WTHF
F1HF
1-499uM2
500-2499uM2
2500-4999uM2
5000-9999uM2
C
PPARγ (Relative expression)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
WTHF
F1HF

Figure 4


A
CNR1 (Relative expression)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
WTHF
F1HF
B
CNR2 (Relative expression)
3
2.5
2
1.5
1
0.5
0
WTHF
F1HF
C
FAAH (Relative expression)
1.2
1
0.8
0.6
0.4
0.2
0
*
WTHF
F1HF

Conclusion

Studying the metabolic effects and mechanisms of action of ω-3 PUFA in the context of high fat feeding has proven to be a major challenge for the field due to the fact that incorporation of ω-3 PUFA into rodent diets often prevents weight gain (401; 404-407). Herein we overcame this challenge by employing the *fat-1* transgenic mouse which possesses the unique ability to generate ω-3 PUFA from endogenous ω-6 PUFA by virtue of its ubiquitously expressed transgene encoding the *fat-1* ω-3 desaturase from *C. elegans* (403). Since this innovative model permits the elevation of ω-3 PUFA compared to wild type littermates without necessitating dietary intervention, we were able to study the influence of ω-3 PUFA in an environment that is not confronted by the same dietary issues that have overshadowed many previously published studies in this area. Importantly, we were able to show that transgenic based elevation of ω-3 PUFA does not restrict high fat diet induced weight gain or fat accretion.

This is an important outcome since it establishes the *fat-1* transgenic mouse as a model in which the influence of ω-3 PUFA can be studied in the context of high fat feeding without altered weight gain. However this finding does not necessarily negate the growing reference in the literature to the anti-obesity effects of dietary ω-3 PUFA. Indeed, such an effect may be reliant on neuronal or hormonal signals activated by ω-3 PUFA in the digestive tract (408-410) which are not present in this transgenic model. Nevertheless, the findings presented in *Chapter I* and *Chapter III* reveal that the effects of ω-3 PUFA on insulin sensitivity, glucose tolerance, resolution mediator biosynthesis, inflammation, adipose gene expression and adipocyte cell size distribution are not dependent upon an anti-obesity effect.

A major perceived limitation of this transgenic model is that ω-3 PUFA are generated at the expense of their ω-6 counterparts. However, this is also true for many dietary studies in which the fat source of ω-3 PUFA in the diet replaces a portion of the ω-6 PUFA (325; 339; 358-359; 393; 400; 404; 407; 411-412). In any case one should consider the effects reported for raising ω-3 PUFA to also be

representative of a reciprocal reduction in ω-6 PUFA. Thus it is probably more appropriate to discuss results in the context of the ω-6: ω-3 PUFA ratio rather than an increase or decrease in these individual classes of essential fatty acids.

An interesting characteristic of the *fat-1* model is its flexibility. Whereas exposure of *fat-1* mice to diets containing very high levels of ω-6 PUFA and very low levels of ω-3 PUFA will lead to a massive shift in the ω-6: ω-3 ratio compared to wild type counterparts (403) exposure to a diet containing balanced levels of these essential fatty acids will result in very little difference between the two groups of mice as reported for standard chow fed mice in *Chapter I*. This means the *fat-1* phenotype can be amplified or diminished depending on the diet chosen by the experimenter. For the studies described in *Chapters I* and *III,* the ω-6: ω-3 ratio of the high fat diet was rather modest (~18:1), and these essential PUFA made up only a small percentage of total fat intake (~14%) in contrast to saturated (~28%), monounsaturated (~30%) and trans fats (~28%) which were much more abundant. Future studies should exploit the flexibility of this transgenic model to examine the effect that more extreme modifications in the experimental diet would have on the *fat-1* phenotype. For example, a diet in which the major fat source was safflower oil (very rich in ω-6 PUFA) would not only increase the difference observed between *fat-1* mice and their wild type counterparts in terms of the ω-6: ω-3 ratio it would also increase the absolute amount of ω-3 produced by the *fat-1* desaturase due to the presence of higher levels of substrate for this enzyme. Such modifications in the experimental diet might facilitate the identification of less abundant ω-3 derived lipid mediators such as RvE1 and might also change the degree to which ω-3 PUFA improve the metabolic state of high fat fed mice. It is also possible that the anti-obesity effect of ω-3 PUFA will become apparent under more extreme circumstances. Thus further studies are warranted to fully characterize *fat-1* transgenic mice in the context of high fat feeding.

In *Chapters I* and *III* we effectively demonstrated the therapeutic potential of ω-3 PUFA for obesity-related insulin resistance and glucose intolerance in *fat -1* mice and revealed that these beneficial properties were associated with improved

resolution mediator synthesis, reduced inflammation (*Chapter I*) and an altered adipose gene expression and cell size distribution profile (*Chapter III*). Ensuing studies should be directed towards understanding the contribution that different cells and tissues make to the beneficial systemic effects of ω-3 PUFA. Whereas it was seemingly impossible to evaluate the importance of cell/tissue specific actions of ω-3 PUFA *in vivo* using dietary manipulations to raise ω-3 PUFA, the *fat-1* transgene is a tool that could be applied to clarify this area. Indeed, tissue and cell specific expression of this enzyme resulting from combining the transgene with a tissue specific promoter sequence (413) could provide a means to evaluate the influence of specific elevations in ω-3 PUFA content in just about any tissue or cell type.

In addition to the possibility of generating new cell/tissue specific lines of *fat-1* transgenic mice, chimeric mice in which bone marrow from *fat-1* mice is transplanted into previously irradiated wild type mice and vice versa (414) would provide an alternative option that would allow for the study of the role of ω-3 PUFA in the hematopoietic compartment vs. parenchymal cells without the requirement for the generation of a new mouse line. These experiments alone would provide very new and important insight into the role that the hematopoietic compartment plays in resolution mediator synthesis and the anti-inflammatory effects of ω-3 PUFA. Although we demonstrated that ω-3 derived resolution mediators can be biosynthesized in adipose, liver and skeletal muscle it is not yet known whether these mediators are produced by adipocytes, hepatocytes and myocytes or solely by the hematopoietic compartment in these tissues. It would therefore be enlightening to study whether *fat-1* transgenesis specifically in the hematopoietic or parenchymal cells would be sufficient to maintain the anti-inflammatory and metabolic effects of ω-3 PUFA observed in *Chapter I*. Furthermore, these studies could also provide important insight into the role of the hematopoietic compartment in the actions of ω-3 PUFA on the adipose gene expression and cell size distribution profiles described in *Chapter III*.

The most valuable finding to come from the study of the *fat-1* mice was the identification of greater biosynthesis of ω-3 derived resolution mediators, namely protectins, in adipose, liver and skeletal muscle of high fat fed transgenic mice since this finding led directly to the study of the therapeutic potential of PDX for lipid-induced insulin resistance presented in *Chapter II.* This study revealed that in addition to a predicted potent anti-inflammatory activity characterized by inhibition of pro-inflammatory cytokine secretion and JNK and iNOS activation, PDX also carries unanticipated therapeutic potential for metabolic disorders due to IL-6 dependent insulin sensitizing and glucoregulatory properties. These findings present PDX as a unique candidate for the treatment of obesity-related metabolic disorders due to its dual anti-inflammatory and glucoregulatory activity and thereby open the door to an exciting new area of research that carries great potential for a therapeutic outcome.

While the work presented in *Chapter II* clearly established that PDX exerts its novel glucoregulatory and insulin sensitizing actions in an IL-6 dependent manner an abundance of questions remain regarding the precise mechanism of action of PDX. Firstly, in light of the similar plasma and skeletal muscle IL-6 profiles it appears that PDX exerts its metabolic effects by stimulating skeletal muscle IL-6 release. However, it is unclear how PDX promotes IL-6 expression and release in skeletal muscle and whether IL-6 is released from the myocytes or the hematopoietic cells present in this tissue. With regard to this latter point, the studies presented in Figure 1 of *Chapter II* in which macrophages were treated with palmitate in the presence or absence of PDX provide anecdotal evidence to suggest that the IL-6 raising effect of PDX is likely muscle cell derived since PDX actually repressed IL-6 secretion in macrophages. To substantiate this finding, *in vitro* studies are presently ongoing to demonstrate that cultured muscle cells are capable of expressing and/or secreting IL-6 in response to PDX exposure. The establishment of this *in vitro* model will also facilitate the future study of the molecular mechanisms by which PDX promotes IL-6 expression and release. Complementary, *in vivo* studies employing IL-6 null chimeric mice in which IL-6 is present only in the hematopoietic cells and vice versa could also be used for this

objective. Should the effect of PDX on muscle IL-6 release be maintained in wild type mice lacking IL-6 in the hematopoietic compartment this would support the anecdotal evidence contained in Figure 1 of *Chapter II*.

Another major point of interrogation concerns the identity of the PDX receptor. Despite the absence of any information on this front, it is likely that PDX employs at least one G-protein coupled receptor (GPCR) to exert its beneficial effects given that this is the case for RvE1 (377; 379), RvD1 (385) and many other lipid mediators such as the prostanoids (415), leukotrienes (416), lipoxins (417) and endocannabinoids (418). Considering the enormous quantity of GPCRs that have been identified in the human and mouse genome (419) the elucidation of the PDX receptor/s appears to be a daunting task that may be the biological equivalent of finding a needle in a haystack. However commercial GPCR reporter libraries presently exist which permit high throughput screening of ligand-GPCR interactions. These libraries could originally be employed to identify candidate GPCRs for PDX. Alternatively, one could employ gene chips to examine the effect of PDX administration on the skeletal muscle GPCR mRNA expression pattern. Those GPCRs whose expression was modified by PDX administration would represent potential targets of PDX. The binding specificity of PDX to the candidate GPCRs identified by these methods could then be evaluated using radiolabeled PDX in the presence or absence of other bioactive lipids with similar structures e.g. RvD1. Once a small list of candidate receptors has been developed for which PDX binding is found to be specific, molecular tools such as siRNA and adenoviral constructs could be employed to knock down and overexpress the candidate receptors respectively. Final validation could then be performed by producing knockout mice and studying the effect of PDX in the absence of the candidate receptor *in vivo*. The possibility that PDX exerts its action via a receptor mediated mechanism provides an interesting avenue for the therapeutic extrapolation of our findings since highly specific synthetic agonists could potentially be developed to promote skeletal muscle IL-6 release. Thus in light of the potential therapeutic applications and the fact that identification of the receptor/s would greatly facilitate

the study of the fine regulation of PDX signaling, forthcoming studies should definitely be focused towards this objective.

Beyond the identification of the receptor/s for PDX, additional studies are also warranted to delineate the major proximal components of the PDX signaling pathway that lead to IL-6 release and also its anti-inflammatory activity. Given that exercise induced muscle contraction is also known to promote IL-6 release (307-308), findings from this field should be considered when planning experiments to examine the molecular pathway employed by PDX. On this note, calcium ionophores have been shown to promote skeletal muscle IL-6 expression and release suggesting that the pathway to IL-6 secretion in muscle may require a calcium signal (420). Although it is purely speculation, this mechanism of action would fit well with PDX signaling through a Gq/G_{11} coupled GPCR, since activation of the Gq/G_{11} pathway is known to promote calcium release from the endoplasmic reticulum in a phospholipase C (PLC)/inositol 1,4,5-triphosphate (IP3) dependent manner (421-422). Thus experiments employing co-administration of PDX with cell permeable calcium chelating agents and examining PLC activity and intracellular calcium mobilization should be performed to evaluate this possibility. Since the effect of exercise on resolution mediator synthesis in skeletal muscle has not yet been studied it would also be interesting to determine whether protectin synthesis is stimulated by skeletal muscle contraction during exercise and whether this might actually account for the contraction induced IL-6 release.

In contrast to the proximal components of the PDX pathway which remain largely unknown, in *Chapter II* we were able to identify hepatic STAT-3 and skeletal muscle AMPK as two distal components that likely contribute to the beneficial metabolic effects of PDX. Interestingly, whereas the stimulatory effect of PDX on hepatic STAT-3 was entirely lost in IL-6 null mice this was not the case for muscle AMPK. This finding suggests that PDX might directly activate AMPK in muscle and that this signal might have a role to play in IL-6 release. Direct activation of AMPK by PDX would also provide a mechanism to explain the inhibitory effect observed for PDX on iNOS expression and JNK activity (88). Future studies should therefore

be performed in our colony of muscle specific AMPK-KD mice that express a kinase dead mutant of the α2-AMPK (423) to more clearly delineate the role of this kinase in the metabolic and anti-inflammatory effects of PDX.

In addition to our findings concerning STAT-3 and AMPK we also identified two important inflammatory signaling mediators that are regulated by PDX. We found that PDX administration inhibited both iNOS and JNK activity in cultured macrophages as well as muscle and liver of lipid infused mice. Importantly, this was also the case in the liver and muscle of high fat fed *fat-1* transgenic mice which displayed higher endogenous production of protectins in these tissues. Future studies are now warranted to determine at which nodes PDX intersects with these inflammatory pathways. Since obesity-related inflammation involves dynamic interactions between multiple cellular players some studies should be directed towards defining the influence of PDX administration on neutrophil (92; 169), macrophage (90-91; 160; 353), T-cell (93; 95-97), B-cell (174), eosinophil (172), and mast cell (173) activity in the expanding adipose tissue. The influence of PDX administration on primary changes believed to promote inflammation in obesity such as adipocyte cell death (156), endoplasmic reticulum stress (180), TLR activation (187; 189; 191-192; 200) and hypoxia (176-178) should also be evaluated. These studies will provide important mechanistic insight into the anti-inflammatory actions of PDX for the obesity setting. It will be interesting to see whether PDX acts by simply interfering with the activation of inflammatory cells, pathways, and processes or if PDX also promotes the activation, expansion and/or recruitment of native cellular regulators of adipose inflammation such as the eosinophils (172), M2 macrophages (160) and T-regulatory T-cells (96-97). The study of the molecular mechanisms underlying the anti-inflammatory actions of PDX could lead to the identification of novel counter regulatory pathways with interesting potential for future drug development.

Aside from the aforementioned questions concerning the mechanism of action of PDX there are also many options that remain to be evaluated regarding the therapeutic application of our findings. For the experiments described in *Chapter II*

we employed an acute mode of PDX administration in an acute model of insulin resistance. Under these circumstances the administration of PDX was extremely effective as a means of preventing insulin resistance and inflammation in lipid-infused mice. Importantly, PDX also displayed hypoglycemic activity within the first 2.5 hours of treatment and substantially improved insulin sensitivity in saline-infused mice. The challenge ahead is to develop a therapeutic approach that will effectively restore insulin sensitivity and glucose control in the obesity setting.

Thus far we have trialed an insulin resistance reversal protocol in which mice that had been rendered obese and insulin resistant by 8 weeks of high fat feeding were treated with PDX or vehicle daily for two weeks (1µg per day I.P.). Unfortunately, in these preliminary experiments we found that two weeks of PDX administration was not sufficient to see any beneficial effect on glucose tolerance or insulin sensitivity in high fat fed animals (Figure 1D-F). However, in contrast to their high fat fed counterparts, daily PDX administration significantly improved each of these parameters in chow fed mice (Figure 1G-I). It is noteworthy that the improved glucose tolerance in these mice was not associated with a stimulatory effect on circulating insulin, to the contrary, PDX treatment appeared to reduce the requirement for insulin during the GTT (Figure 1H). These preliminary findings from chow fed mice support our original findings in saline infused animals and suggest that the glucoregulatory actions of PDX are insulin independent. Furthermore, in accordance with the work of Holmes et al (424) our data argue against the possibility that repeated stimulation of skeletal muscle IL-6 release could lead to a negative outcome in terms of glucose regulation and insulin sensitivity.

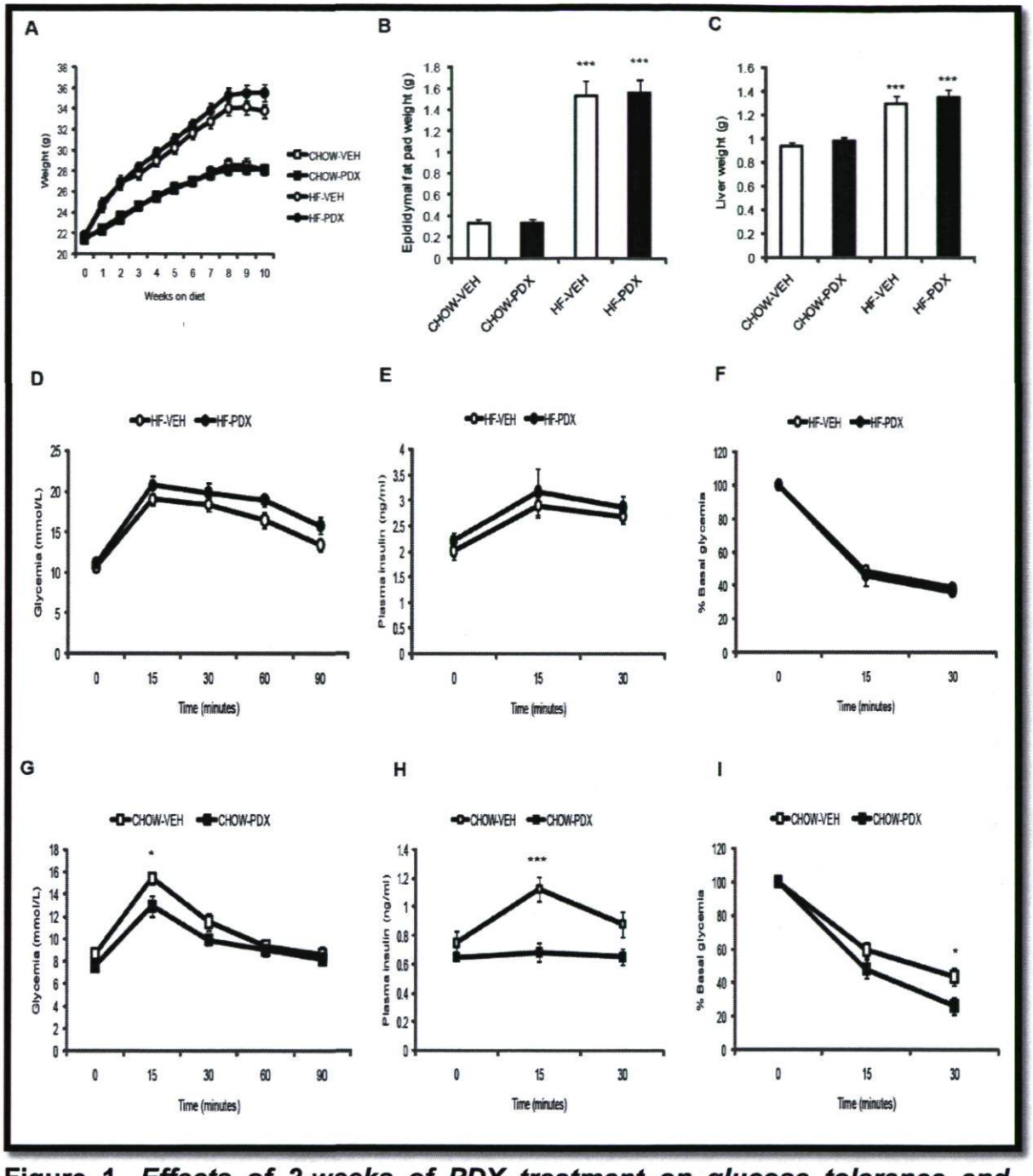


Figure 1. ***Effects of 2-weeks of PDX treatment on glucose tolerance and insulin sensitivity in high fat fed mice and their chow fed counterparts.***

*Panels A, B, and C. weight gain, epididymal fat pad and liver weights for C57BL6/J mice treated with PDX or vehicle (VEH) for two weeks after being fed either standard chow or high fat (HF) diets for 8 weeks. Data are mean±SEM, *** P<0.001 vs chow fed counterparts. Panels D and E. glucose and insulin excursions for HF-fed mice during the 1g/kg glucose tolerance test. Panel F. glucose excursions for HF-fed mice during the 1.5U/kg insulin tolerance test. Panels G and H. glucose and insulin excursions for chow-fed mice during the 1g/kg glucose tolerance test. Panel I. Glucose excursion for chow-fed mice during the 1.5U/kg insulin tolerance test. Data are mean±SEM. *P<0.05 vs VEH treated counterparts.*

There are multiple plausible explanations for the lack of beneficial effect of PDX treatment in high fat fed mice in this study. Most likely is that the quantity, frequency and/or duration of PDX administration was insufficient for this chronic disease model. Alternatively, it is also plausible that the mode of administration may have been inappropriate for this mediator in obese animals since the greater amount of fat in the peritoneal cavity (Figure 1C) may have reduced or slowed PDX absorption into the circulation following intraperitoneal injections. This possibility is supported in part by the finding that PDX had clear effects in lean chow fed mice but not their obese high fat fed counterparts.

In light of these questions concerning the mode of administration, future studies should be designed to evaluate the efficiency of different treatment modes (e.g. oral, intravenous, intraperitoneal) at raising PDX in circulation of obese mice. Furthermore, since the pharmacokinetics of this mediator have not yet been studied it would be valuable to evaluate the half life of PDX *in vivo* and to study its break down products. Such information would be useful for determining the required frequency of administration and might also lead to the identification of new bioactive metabolites. Since high fat feeding is known to provoke an acute inflammatory response in adipose tissue during the post-prandial period (170; 425) a treatment modality that raises PDX specifically at feeding times might be most appropriate. Application of nanotechnology to encapsulate PDX into microspheres (426-427) that could then be included in the food source and absorbed in the GI tract would be an excellent development for the timely administration of this mediator.

An alternative to the direct administration approach is the use of pharmaco-nutritional combination strategies to boost endogenous resolution mediator production. This strategy is possible because the non steroidal anti-inflammatory drug (NSAID) aspirin (ASA) has been found to trigger the synthesis of novel epimeric forms of ω-3 and ω-6 derived resolution mediators (428). This occurs because ASA mediated acetylation of COX-2 does not simply inhibit COX activity as once thought but rather alters its regioselectivity for lipid substrates allowing it to

act like a LOX and convert EPA (20:5 n-3), DHA (22:6 n-3), and AA (20:4 n-6) into epimeric forms of the first intermediates of their respective pathways (428) (Figure 2). Thus far ASA triggered (AT) formation of E-series Resolvins (366), D-series Resolvins (429-430) and Protectins (430) as well as ω-6 derived Lipoxins (431-432) has been described. Although much less studied, it is interesting to note here that the thiazolidinedione (TZD) pioglitazone, an insulin sensitizing agent, and the statin atorvastatin, a commonly used cholesterol lowering agent, can also promote epimeric resolution mediator synthesis (433). These agents appear to act by altering COX-2 activity in a manner similar to ASA and also by stimulating protein kinase A (PKA) mediated phosphorylation of 5-LOX, the other key enzyme in these pathways (434). The phosphorylation of 5-LOX promotes its association with COX-2 enhancing the formation of sequential oxidation products (434). Importantly, the so-called AT-resolution mediators appear to have equivalent or greater activity than their ASA independent epimers (366; 430-431); and they may also be more resistant to enzymatic degradation (429). These findings suggest that combination therapy in which ω-3 PUFA are administered in partner with ASA, statins or TZDs may be a novel and effective strategy of promoting resolution mediator synthesis in metabolic tissues. These data also suggest that resolution mediators likely contribute to the beneficial effects of ASA, statins and TZDs. It is noteworthy that if successful, this therapeutic approach carries the potential to be rapidly translated into clinical trials since fish oil, ASA, statins and TZDs are all commonly employed by obese insulin resistant individuals.

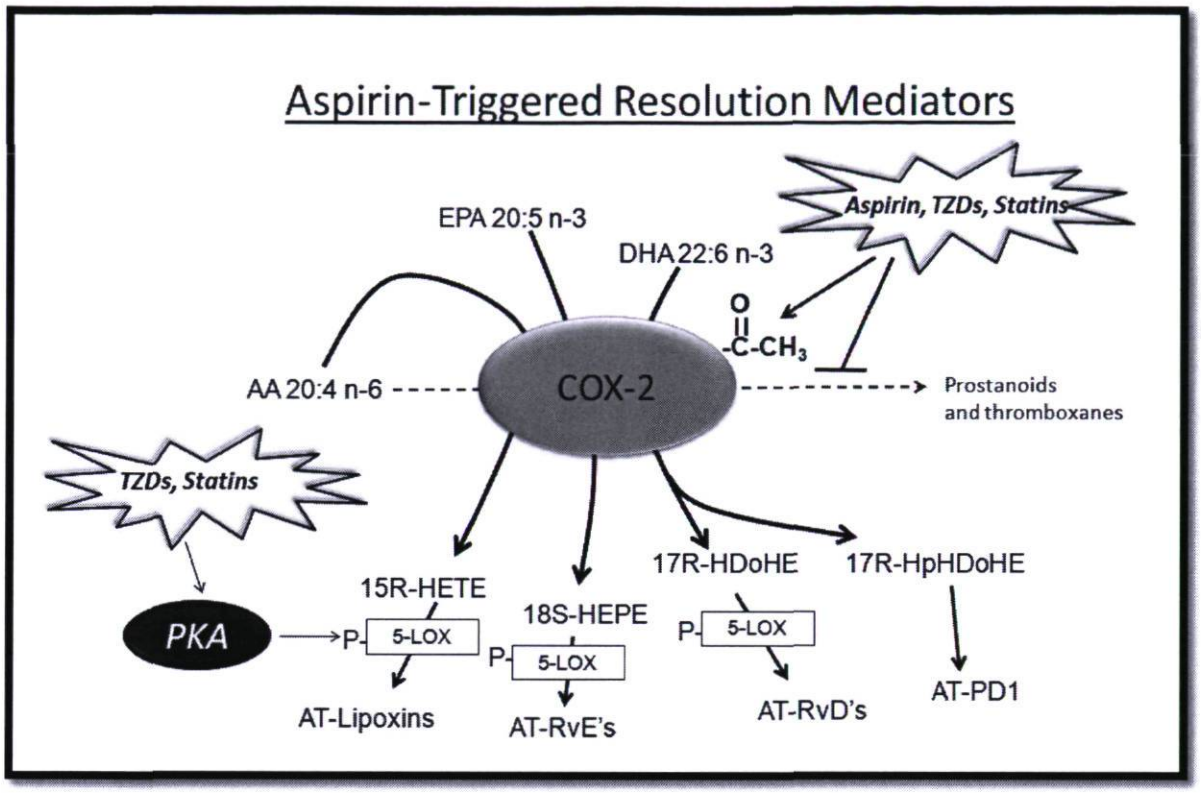


Figure 2. ***Aspirin Triggered (AT) resolution mediator synthesis***

In addition to being easily transferable to the clinical setting the combined pharmaco-nutrition intervention approach described above also has the benefit of promoting the formation of other resolution mediators such as RvE1. Importantly, recent studies suggest that RvE1 is also a promising target for the prevention of insulin resistance and T2DM. Although RvE1 has yet to be detected in metabolic tissues, Gonzalez-Periz et al (386) showed that administration of RvE1 for 1 week can improve hepatic steatosis in genetically obese ob/ob mice by reducing both lipid storage and macrophage accumulation in liver. These data are in line with our findings in Supplementary Figure 1 of *Chapter II* showing that RvE1 blunts lipid-induced macrophage activation *in vitro*. Although they did not study glucose metabolism in these mice the authors also suggested that RvE1 might improve insulin sensitivity in ob/ob mice since adipose mRNA expression of adiponectin, PPARγ, IRS-1 and GLUT4 was also increased.

Beyond this work in obese mice, studies in other disease models on the mechanism of action of RvE1 provide important insight into the promising

therapeutic potential of RvE1 for insulin resistance and T2DM. Indeed, RvE1 has been identified as an endogenous agonist of the GPCR ChemR23 (379) that was recently shown to play an important role in adipogenesis through interactions with its peptide ligand Chemerin (435-436). These data suggest that RvE1 by virtue of its receptor may be an important regulator of adipose biology. Importantly, ChemR23 is also known to be expressed in liver and pancreas suggesting that RvE1 might also regulate glucose homeostasis through actions in these tissues (377). Interestingly, RvE1 was recently found to exert analgesic effects by inhibiting the activation of transient receptor potential vanilloid type-1 (TRPV1) in sensory neurons via a ChemR23 dependent mechanism (437). This is of interest for metabolism because ablation of TRPV1 activity via capsaicin treatment was recently shown to improve glucose tolerance (438). Moreover TRPV1 activity has been reported to promote pancreatic inflammation and type 1 diabetes (438). As a whole these data highlight the exciting potential of another ω-3 derived resolution mediator for the treatment of insulin resistance and T2DM through novel mechanisms that remain to be unraveled. Subsequent studies should therefore be directed towards evaluating the therapeutic potential of RvE1 and other novel classes of ω-3 derived resolution mediators such as RvD1 and the maresins (372) in addition to PDX.

In conclusion, we effectively employed a novel experimental model, the *fat-1* transgenic mouse, to improve the current understanding of the metabolic actions of ω-3 PUFA in the obesity setting. Our findings show that transgenic manipulation of the ω-6: ω-3 ratio in order to raise ω-3 PUFA prevents obesity-linked inflammation, insulin resistance and glucose intolerance in a manner that is independent of restriction of weight gain or fat accrual. Furthermore, we revealed that these beneficial effects are associated with improved resolution mediator synthesis in metabolic tissues and altered gene expression and adipocyte size distribution profiles in visceral adipose tissue. Importantly, closer study of ω-3 derived resolution mediators led to the identification of PDX as an exciting new therapeutic candidate for obesity-related metabolic disorders due to its dual glucoregulatory and anti-inflammatory activities. Collectively, the findings described in this thesis

provide a strong foundation for the future investigation of the mechanisms of action of ω-3 PUFA and the development of their bioactive derivatives as therapeutic candidates for obesity-related pathologies.

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