**Modulation of Adipokines by n-3 Polyunsaturated Fatty Acids and Ensuing
Changes in Skeletal Muscle Metabolic Response and Inflammation**

by

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ABSTRACT

MODULATION OF ADIPOKINES BY N-3 POLYUNSATURATED FATTY ACIDS AND ENSUING CHANGES IN SKELETAL MUSCLE METABOLIC RESPONSE AND INFLAMMATION

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Adipose tissue represents an important endocrine organ that secretes a multitude of adipokines known to mediate inflammation, lipid metabolism, and insulin sensitivity in peripheral tissues such as skeletal muscle. Specifically, adiponectin stimulates skeletal muscle fatty acid oxidation and is associated with improvements in insulin response. Long-chain n-3 polyunsaturated fatty acids (PUFA) are well known for their anti-inflammatory and insulin-sensitizing properties, and their dietary consumption is associated with a more favourable circulating adipokine profile, including increased adiponectin. However, whether n-3 PUFA can directly stimulate adiponectin secretion from human adipocytes, as well as the underlying mechanisms involved, is unknown. In contrast to n-3 PUFA, diets high in saturated fatty acids (SFA) are thought to decrease adiponectin and increase pro-inflammatory adipokines, as well as blunt skeletal muscle response to both adiponectin and insulin, possibly via activation of inflammatory pathways. The role of n-3 PUFA in mediating the communication between adipose tissue and skeletal muscle, as well as preventing SFA-induced impairments in skeletal muscle function, has yet to be examined.

In this thesis, it was found that long-chain n-3 PUFA increase adiponectin secretion from human adipocytes via a peroxisome proliferator-activated receptor gamma-dependent

mechanism. The effects of n-3 PUFA on adiponectin secretion were additive when combined with the thiazolidinedione, rosiglitazone. Secondly, incorporation of n-3 PUFA into a high SFA diet prevented impairments in adiponectin response and both prevented and restored impairments in insulin response in rodent skeletal muscle. Interestingly, these findings were paralleled by prevention of SFA-induced increases in toll-like receptor 4 expression by n-3 PUFA, suggesting inflammatory changes may be involved. Finally, dietary n-3 PUFA and SFA modulated the secretion of adipose tissue-derived factors from visceral rodent adipose tissue and subsequent exposure of isolated skeletal muscle to such factors induced acute changes in inflammatory gene expression without affecting insulin sensitivity.

Together, the findings in this thesis suggest that n-3 PUFA modulate adipokine secretion from adipose tissue and that adipose-derived factors mediate skeletal muscle inflammation and response to adiponectin and insulin. Ultimately, this work highlights the importance of considering n-3 PUFA as a therapeutic strategy in the prevention and treatment of obesity and related pathologies.

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List of Abbreviations

ACC	acetyl CoA carboxylase
ACM	adipose conditioned medium
Ad	adiponectin
AdipoR1/R2	adiponectin receptor 1/2
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
APPL1/2	adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif 1/2
BADGE	bisphenol-A-diglycidyl ether
BMI	body mass index
BSA	bovine serum albumin
CoA	coenzyme A
CPT1	carnitine palmitoyltransferase 1
CVD	cardiovascular disease
DAG	diacylglycerol
DHA	docosahexaenoic acid
DMEM	Dulbecco's modified eagle medium
EDL	extensor digitorum longus
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid

ER	endoplasmic reticulum
FAO	fatty acid oxidation
FAT/CD36	fatty acid translocase/cluster of differentiation 36
FBS	fetal bovine serum
FFA	free fatty acid
G6P	glucose 6 phosphate
gAd	globular adiponectin
GLUT4	glucose transporter 4
HMW	high molecular weight
I κ B α	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α
IKK	I κ B kinase
IL-6/15	interleukin-6/15
IRS-1	insulin receptor substrate-1
JNK	c-Jun N-terminal kinases
M199	medium 199
MCP-1	monocyte chemoattractant protein-1
mRNA	messenger RNA
NF- κ B	nuclear factor- κ B
p	phosphorylation
PBS	phosphate buffer saline
PEPCK	phosphoenolpyruvate carboxykinase
PPAR α/γ	peroxisome proliferator-activated receptora/ γ

PPRE	PPAR-responsive elements
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
SE	standard error
SEM	standard error of the mean
SFA	saturated fatty acids
SOCS3	suppressor of cytokine signalling-3
T2D	type 2 diabetes
TAG	triacylglyceride
TLR4	toll-like receptor 4
TNF- α	tumour necrosis factor- α
TZDs	thiazolidinediones

Chapter 1: Review of Literature

This review of literature is adapted from the following:

1. **Tishinsky JM**, Robinson LE, and Dyck DJ (2011) Insulin-sensitizing properties of adiponectin. *Biochimie* 2012 Jan 31. [Epub ahead of print].
2. **Tishinsky JM**, Dyck DJ, and Robinson LE (2012) Lifestyle factors increasing adiponectin synthesis and secretion. *Vitamins and Hormones* Vol 90, 2012.

Section 1.1: Introduction: obesity and adipose tissue biology

Obesity is currently considered to be an epidemic. In 2005, the Canadian Community Health Survey estimated that 17% of adults aged 25 to 64 years were obese (body mass index (BMI) ≥ 30 kg/m²) and 53% were overweight or obese (BMI ≥ 25 kg/m²) (149). Although genetics are undoubtedly a contributing factor (124), the alarming increase in obesity rates in today's society is largely credited to lifestyle factors, including increased caloric intake and physical inactivity. Given that obesity is strongly linked to the development of numerous pathologies (54), most notably type 2 diabetes and cardiovascular disease (CVD), there is a growing interest in understanding the underlying link between excess adipose tissue and metabolic disease.

In addition to its role as a storage depot for lipid, adipose tissue represents an active endocrine organ that secretes hundreds of adipose-derived cytokines (or adipokines) into the circulation (72). These adipokines include inflammatory mediators such as tumour necrosis factor (TNF)- α , interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, and resistin as well as the anti-inflammatory adipokine, adiponectin. These adipose-derived factors act as important regulators of lipid metabolism and insulin sensitivity in peripheral tissues, such as

skeletal muscle and the liver (48). In obesity, adipose tissue expands and is characterized by adipocyte hypertrophy, increased macrophage infiltration, impaired blood flow, hypoxia, increased free fatty acid release, and altered adipokine secretion (**Figure 1.1**). While adiponectin secretion is downregulated in obesity, release of pro-inflammatory cytokines is increased (164), contributing to a state of chronic whole body low-grade inflammation.

Figure 1.1: Lean vs. Obese Adipose Tissue. In obesity, adipose tissue exhibits adipocyte hypertrophy, increased macrophage infiltration and an altered secretory profile. Adipocyte = white, macrophage = black. Thick arrows = increased release of adipose-derived factors; thin arrows = decreased release of adipose-derived factors.

Section 1.2: Adiponectin

1.2.1 Adiponectin Overview

a) Adiponectin isoforms and its receptors:

Adiponectin plays a pivotal role in protecting against the development and progression of obesity, type 2 diabetes, and CVD (12, 59, 96, 139). Adiponectin circulates in different oligomeric forms of trimeric, hexameric, or high molecular weight (HMW) (65), with the HMW isoform most closely correlated with whole body insulin sensitivity (56). While circulating adiponectin exists predominantly in full-length form, its proteolytic cleavage produces a globular form of adiponectin (gAd) that represents <1% of total adiponectin (46) and possesses biological activity, particularly in skeletal muscle (108). Unlike other adipokines, circulating adiponectin concentrations are inversely correlated with increasing adiposity (6) and insulin resistance (58).

Two transmembrane proteins, AdipoR1 and AdipoR2, have been identified as receptors for adiponectin (190). AdipoR1 is expressed ubiquitously among tissues and is most highly concentrated in skeletal muscle (190). AdipoR1 has a high affinity for gAd and a lower affinity for full-length adiponectin, suggesting that gAd acts primarily in skeletal muscle (190). In contrast, AdipoR2 is expressed predominantly in the liver and has an intermediate affinity for both gAd and full-length adiponectin, suggesting that full-length adiponectin exerts its functions predominantly in the liver (190). In addition, Hug et al. have identified a third adiponectin receptor, T-cadherin, that is exclusively expressed in endothelial and smooth muscle cells (61).

b) Adiponectin signalling in liver and muscle:

Adiponectin is known to exert insulin-sensitizing effects in both the liver and skeletal muscle, two key tissues involved in glucose homeostasis. Upon binding to its receptor, it is believed that adiponectin signals primarily through AMP-activated protein kinase (AMPK) to inhibit gluconeogenesis in the liver and to promote fatty acid oxidation in skeletal muscle (163, 191). An adaptor protein (APPL1) upstream of AMPK seems necessary for adiponectin signalling given that adiponectin-induced AMPK activation in myocytes is enhanced or suppressed when APPL1 is overexpressed or inhibited, respectively (100). In contrast, APPL2, an isoform of APPL1, acts as a negative regulator of adiponectin signalling (176). In addition to AMPK, adiponectin also activates peroxisome proliferator-activated receptor (PPAR)- α (190, 197), a nuclear hormone receptor that leads to upregulation of numerous lipid and glucose metabolism genes (52). As such, adiponectin treatment of hepatocytes and myocytes stimulates PPAR- α ligand activity and increases fatty acid oxidation in myocytes (190), while the targeted disruption of AdipoR2 in the liver of diabetic mice decreases expression of PPAR- α target genes involved in glucose metabolism (194).

c) Adiponectin effects in liver and muscle:

In the first study to provide evidence for an insulin-sensitizing effect of adiponectin in liver, the administration of a single dose of recombinant, full-length adiponectin led to a significant decrease in circulating glucose levels in wildtype mice and normalized hyperglycemia in three diabetic mouse models (11). To further elucidate the mechanism by which adiponectin improves whole body insulin-sensitivity, Combs et al. (24) used a pancreatic insulin clamp technique to determine if the adiponectin-mediated decrease in circulating glucose levels could

be attributed to a decrease in hepatic glucose production, an increase in skeletal muscle mediated glucose clearance, or both. This study found an adiponectin-mediated inhibition of hepatic glucose production without any changes in glucose clearance, glycolysis, or glycogenesis (24). While no change in peripheral glucose clearance was found in the aforementioned work, it is important to recognize that those studies utilized full-length adiponectin, known to signal primarily via AdipoR2, which is expressed predominantly at the liver (190). There is evidence to suggest that gAd signals via AdipoR1 to enhance glucose uptake in skeletal muscle (190), further contributing to overall improvements in whole body insulin sensitivity (see below).

Adiponectin is thought to improve hepatic insulin sensitivity primarily by suppressing high rates of endogenous glucose production, a known contributor to systemic glucose intolerance (98) (**Figure 1.2**). In support of this, numerous studies have demonstrated that adiponectin decreases expression of key gluconeogenic genes, including glucose 6-phosphatase and phosphoenolpyruvate carboxykinase (96, 100, 176, 197). Although the precise signalling mechanism by which adiponectin downregulates gluconeogenesis is presently unknown, AMPK is thought to be involved. Yamauchi et al. demonstrated that adiponectin activates AMPK in both isolated hepatocytes and in liver from mice *in vivo*, and this was associated with a reduction in circulating glucose levels (191). When co-administered with an adenovirus that encodes for a dominant negative form of AMPK, adiponectin failed to reduce glucose levels (191), suggesting that activation of hepatic AMPK is required for adiponectin-mediated improvements in glucose tolerance.

Figure 1.2: Proposed mechanism by which adiponectin suppresses hepatic glucose production. Upon binding to its receptor, full-length adiponectin decreases gluconeogenic enzyme expression through an AMPK-dependent mechanism, resulting in downregulated gluconeogenesis. AMP-activated protein kinase (AMPK), glucose 6-phosphate (G6P), phosphoenolpyruvate carboxykinase (PEPCK).

The first study to demonstrate that adiponectin enhances fatty acid oxidation in skeletal muscle was conducted by Lodish's group in 2001 (46). Administration of gAd decreased elevated plasma fatty acid levels in response to high fat feeding, and this was attributed to increased skeletal muscle fatty acid oxidation evidenced by an acute stimulation of oleate oxidation by gAd in cultured C2C12 myocytes (46). Mechanistically, gAd in skeletal muscle is thought to activate AMPK and downregulate acetyl CoA carboxylase (ACC) in skeletal muscle (108), thereby decreasing malonyl CoA and alleviating inhibition of carnitine palmitoyltransferase (CPT) 1, allowing for entry of fatty acids into the mitochondria for oxidation (**Figure 1.3**). However, the direct activation of AMPK by gAd in oxidative muscle

remains controversial (163) and, as such, it is possible that gAd may act directly to deactivate ACC (108). There is evidence to suggest that the accumulation of lipid species in skeletal muscle may play a causative role in impairing insulin signalling (121), although this too remains controversial. Therefore, it is possible that adiponectin may enhance skeletal muscle insulin sensitivity by increasing fatty acid oxidation and reducing intramuscular lipids. In support of this, gAd-stimulated fatty acid oxidation has been associated with an increase in glucose transport in both cultured myocytes (191) and isolated skeletal muscle (16). In summary, there is good evidence to suggest that adiponectin exerts insulin sensitizing effects in both liver and skeletal muscle by downregulating hepatic glucose production and enhancing fatty acid oxidation and glucose transport, respectively.

Figure 1.3: Proposed mechanism by which adiponectin stimulates skeletal muscle fatty acid oxidation. Upon binding to its receptor, globular adiponectin deactivates ACC, either directly or via stimulation of AMPK, to decrease malonyl CoA. This acts to relieve the inhibition on CPT1, allowing for fatty acid uptake into the mitochondria for oxidation. AMP-activated protein kinase (AMPK), acetyl CoA carboxylase (ACC), carnitine palmitoyltransferase (CPT)1.

d) Peripheral adiponectin resistance

Coupled with a decrease in circulating adiponectin, there is also compelling evidence that tissue responsiveness to adiponectin is impaired in obesity and related diseases. Insulin has been shown to downregulate adiponectin receptor expression in both myocytes and hepatocytes (166), suggesting that hyperinsulinemia may impair peripheral adiponectin signalling. In support of this, decreased skeletal muscle expression of AdipoR1 and AdipoR2 has been reported in mouse models of obesity and diabetes (166, 194), as well as in individuals with a strong family history of diabetes compared to those with no family history (22). There are also reports of decreased hepatic adiponectin receptor expression in patients with non-alcoholic fatty liver disease (145) and chronic hepatitis C virus-infected patients (26), two pathologies that are closely associated with insulin resistance. In contrast, no correlation between adiponectin receptor expression and insulin sensitivity was found in human myotubes (151), and no difference in AdipoR1 or AdipoR2 expression was found in skeletal muscle of lean and obese diabetic individuals (28), indicating that adiponectin resistance may occur downstream of the adiponectin receptor.

Several studies have aimed to directly assess tissue responsiveness to adiponectin in obesity and insulin resistance (16, 21, 92, 108, 166). In a study using cultured primary myotubes from skeletal muscle of lean, obese, and obese diabetic subjects, Chen et al. reported a blunted activation of AMPK signalling and fatty acid oxidation by adiponectin in both the obese and obese diabetic myotubes (21). These findings are supported by an impaired ability of adiponectin to activate AMPK in skeletal muscle of ob/ob mice (166), as well as a reduction in adiponectin-stimulated fatty acid oxidation in muscle from obese subjects compared to lean (16). Furthermore, adiponectin was unable to stimulate hepatic AMPK expression or lower circulating glucose levels in insulin receptor knockout mice compared to wildtype mice (92). Finally, in

addition to reports of adiponectin resistance in obese and diabetic models, it has been demonstrated that impairments in skeletal muscle response to adiponectin are inducible by high fat feeding in rodents (108). For example, as little as 3 days of high fat intake abolished adiponectin's stimulatory effect on skeletal muscle fatty acid oxidation in rats and, importantly, this preceded the development of diet-induced insulin resistance which occurred at 4 weeks (108). In summary, these studies illustrate that the state of hypoadiponectinemia observed in obesity is exacerbated by an impaired tissue response to circulating adiponectin, resulting in a loss of adiponectin action.

1.2 b Lifestyle factors that regulate adiponectin synthesis and secretion

Given the known insulin-sensitizing properties of adiponectin (193), and that circulating adiponectin concentrations are markedly reduced in obesity and type 2 diabetes (28, 151), strategies to augment adiponectin synthesis and secretion from adipose tissue are of significance. The following section will discuss the regulation of adiponectin by saturated fatty acids (SFA), long-chain n-3 polyunsaturated fatty acids (PUFA), and thiazolidinediones (TZDs).

a) Saturated fatty acids

Consumption of saturated fatty acids (SFA) has been demonstrated to modulate the secretion of adipose tissue-derived factors such as adiponectin (118), representing a possible mechanistic link between high dietary SFA intake and elevated risk of developing insulin resistance (172) and CVD (188).

Evidence from cell culture studies:

Studies examining SFA-induced modulation of cellular adiponectin content have yielded conflicting results. In 3T3-L1 adipocytes, treatment with 250 μ M palmitic acid for 48 hours led to a significant decrease in adiponectin gene expression (17). Similarly, exposure of cultured rat adipocytes to 300 μ M palmitic acid induced a 43% and 36% decrease in adiponectin mRNA and protein expression, respectively. (187). However, neither of these studies measured adiponectin concentration in the medium, making it impossible to determine if gene expression changes reflected SFA-induced changes in cellular or secreted adiponectin protein.

In contrast to the aforementioned studies, our laboratory demonstrated that treatment of 3T3-L1 murine adipocytes with 125 μ M palmitic acid for 24 hours had no effect on either adiponectin gene expression or secreted adiponectin protein measured in the culture medium (119). Our results are consistent with reports that short-term (2 hour) incubations of primary rat adipocytes with 0.06 - 0.5 mM concentrations of palmitic acid did not alter secreted adiponectin protein in the medium (158). Lastly, in a study that treated 3T3-L1 adipocytes with palmitic and stearic acid for 24 hours, both 10 and 100 μ M concentrations of these SFA increased secreted adiponectin protein (138). These discrepancies among studies may be somewhat attributable to differences in both fatty acid concentration and exposure time, although it is apparent that these contributing factors do not consistently account for differences in findings and more work is required to fully understand the role of SFA in adiponectin synthesis and secretion.

Evidence from rodent studies:

The majority of *in vivo* studies have reported a negative association between dietary SFA and adiponectin. For example, consumption of a high SFA diet led to a significant decrease in

adiponectin gene expression in both diabetic and wildtype mice (162), as well as in male Wistar rats (137). In a study by Bueno et al., mice fed a diet high in lard (17.5% of kcal) had significantly lower serum adiponectin concentrations both acutely (2 days) and chronically (8 weeks) (17). Furthermore, acute ingestion of the lard-enriched diet led to a decrease in adiponectin mRNA expression in retroperitoneal adipose tissue (17). Interestingly, no significant changes in adiponectin gene expression in the epididymal or subcutaneous fat pads were found, suggesting that SFA-induced modulation of adiponectin mRNA may be adipose tissue depot specific. Additionally, as little as 3 days of a high SFA diet (60% of kcal) induced a significant decrease in plasma adiponectin concentrations in Sprague-Dawley rats (110). As with the cell culture evidence discussed above, it is noteworthy that most rodent studies in this area have focused primarily on lard-based diets (containing palmitic acid as the most abundant SFA, as well as high levels of the monounsaturated oleic acid, 18:1n-9) and the ability of different types of SFA to modulate adiponectin requires further study.

Evidence from human studies:

Plasma concentrations of SFA have been found to be negatively associated with circulating adiponectin levels in both lean and obese (non-diabetic) humans (43). More specifically, percentages (of total fatty acids) of myristic and palmitic acid in the blood were correlated strongly with lower circulating adiponectin (43). These findings are supported by an intervention study in which consumption of a high SFA (19% of kcal) diet for 8 weeks in overweight subjects at risk for the metabolic syndrome led to decreased adiponectin gene expression in subcutaneous adipose tissue, as well as significantly lower plasma adiponectin levels (169).

Collectively, cell culture, rodent, and human studies suggest that SFA may decrease adiponectin, providing a potential mechanistic link between SFA consumption and elevated risk of metabolic disease.

b) Long-chain n-3 polyunsaturated fatty acids

Since SFA are a significant dietary component, particularly in western societies, delineating the role of other fatty acids thought to play a more protective role in metabolism is of value. Dietary consumption of the long-chain n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) is associated with a reduced risk of developing type 2 diabetes and CVD (12). The anti-inflammatory and insulin-sensitizing properties of long-chain n-3 PUFA are thought to be at least partly attributable to the regulation of adipose tissue-derived factors, including adiponectin (130).

Evidence from cell culture studies:

Studies using cultured adipocytes report conflicting findings regarding the regulation of adiponectin gene expression, cellular protein content, and secretion by long-chain n-3 PUFA. Work from our laboratory showed that treatment of 3T3-L1 murine adipocytes with 125 μ M of EPA and DHA for 24 hours induced an increase in both adiponectin gene expression and secreted protein concentration in the culture medium (119). Our findings are consistent with those of Banga and colleagues who observed a significant increase in adiponectin secretion from rat adipocytes treated overnight with 25 μ M EPA or DHA (9). Interestingly, this occurred in the absence of any changes in cellular gene or protein expression adiponectin (9), indicating that n-3 PUFA may exert a direct stimulatory effect on adiponectin translation. EPA has also been

shown to effectively reverse the decrease in adiponectin secretion from 3T3-L1 adipocytes that were induced by a co-culture with RAW264 macrophages (64). The restoration of adiponectin secretion occurred in parallel with a decrease in macrophage TNF- α mRNA expression (64), suggesting that n-3 PUFA may elicit changes in adiponectin secretion from adipose tissue by regulating the paracrine interactions between adipocytes and macrophages.

In contrast to the above studies, incubation of rat adipocytes with 100 or 200 μ M EPA for 96 hours resulted in a decrease in both adiponectin gene expression and secreted protein (95). Similarly, treatment of 3T3-L1 adipocytes with 250 μ M EPA or DHA for 48 hours decreased adiponectin gene expression, with lipid peroxidation reported as a possible mechanism for this effect of n-3 PUFA (17). Lastly, EPA had no effect on adiponectin gene expression in 3T3-L1 adipocytes after 12 hours (113), possibly due to the relatively short fatty acid exposure time. Due to the discrepancies in these studies, further research is necessary to elucidate the modulation of adiponectin by n-3 PUFA. It is also noteworthy that all of the cell culture work to date has utilized mouse and rat adipocyte models and, as such, there is a strong need to evaluate the effects of n-3 PUFA on adiponectin in human adipocytes.

Evidence from rodent studies:

Numerous rodent-based studies also provide evidence for a role of n-3 PUFA in modulating adiponectin secretion. For example, 15 days of dietary fish oil, rich in both EPA and DHA, increased plasma adiponectin levels in a dose-dependent manner in mice compared with those fed an isocaloric safflower oil diet, rich in linoleic acid (18:2 n-6) (113). Interestingly, fish oil induced adiponectin gene expression in epididymal adipose tissue, but elicited no change in adiponectin mRNA in subcutaneous fat, suggesting that the effects of n-3 PUFA may vary

depending on adipose tissue depot (113). Similar elevations in circulating adiponectin were observed in both genetically obese (ob/ob) mice and high-fat diet induced obese mice fed EPA (5% of kcal) (64).

Significant uptake of DHA in murine heart, liver, and adipose tissue occurs in as little as 4 days of supplementation; furthermore, plasma concentrations remain stable for a significant period i.e. 16 days after DHA supplementation has stopped (89). Although there is a general consensus that n-3 PUFA increase adiponectin in rodent models, there is also some evidence for a negative association between consumption of a fish oil-enriched diet and adiponectin (17). This negative association was only evident with acute (2 days) but not chronic (60 days) fish oil administration, and was accompanied by a reduction in adiponectin gene expression in retroperitoneal, but not epididymal or subcutaneous adipose tissue (17). Thus, it is possible that acute administration of a fish-oil enriched diet may produce a transient and depot-specific impairment in adiponectin secretion that is subsequently followed by a sustained elevation in adiponectin; however, this requires further study.

Additionally, n-3 PUFA have been demonstrated to counter the reduction in adiponectin secretion induced by high fat diets (44, 69, 162). For example, incorporation of EPA (16% of kcal) into a high fat diet resulted in plasma adiponectin concentrations that were similar to those in the low fat control group in wildtype mice (69). Similarly, inclusion of n-3 PUFA in a high fat diet effectively prevented a decrease in adiponectin gene expression and restored serum adiponectin levels in both wildtype (44) and db/db mice (162). Lastly, dietary fish oil has been demonstrated to restore impairments in adiponectin levels induced by long-term exposure to a high sucrose diet (136). In rats fed a sucrose-rich diet for 7 months, fish oil supplementation restored plasma adiponectin levels and this was also associated with a reversal of whole body

insulin resistance and dyslipidemia (136). These studies strongly suggest a valuable role for n-3 PUFA in normalizing diet-induced impairments in adiponectin, findings that may have important implications for common dietary habits in today's society.

Evidence from human studies:

Numerous clinical studies have found that n-3 PUFA consumption is positively correlated with circulating adiponectin concentrations in humans (3, 64, 76, 78, 116). EPA supplementation (1.8 g/day) for 3 months significantly increased plasma adiponectin levels in obese men and women with metabolic syndrome (64). Similarly, in overweight insulin resistant women undergoing a 24 week weight loss program, supplementation with 4.2 g/day of EPA and DHA led to an 18% increase in serum adiponectin, while weight loss alone had no effect (78). A positive correlation between plasma adiponectin levels and erythrocyte n-3 PUFA content has also been observed in dialysis patients (3). Furthermore, increases in serum adiponectin concentrations were observed in non-obese Japanese women consuming a minimum of 3 g/day of n-3 PUFA in the form of fish for 8 weeks, suggesting that n-3 PUFA can also elevate circulating adiponectin levels in lean, healthy populations (76). However, this study is limited by a relatively small sample size (17 subjects), as well as variability in the quantity and type of fish consumed. In contrast, in a study that supplemented hyperlipidemic patients with 1.8 g of EPA daily for 6 months, a significant increase in adiponectin levels was observed in only those subjects with type 2 diabetes and initial hypoadiponectinemia (116), implying that response to n-3 PUFA intake may be influenced by baseline adiponectin levels. Thus, additional studies are needed to clarify the role of n-3 PUFA in regulating adiponectin concentrations in healthy individuals.

Although the aforementioned studies provide evidence that dietary n-3 PUFA intake can augment circulating adiponectin in humans, there are also studies that observe no significant association (77, 82, 171). For example, moderately obese men and women who consumed 2.9 g/day of n-3 PUFA for 14 weeks had no change in either total plasma or HMW adiponectin (77). Likewise, 3.5 g/day of n-3 PUFA for 6 weeks failed to elicit any changes in circulating adiponectin levels in women with polycystic ovary syndrome (171). Finally, no significant changes in plasma adiponectin were found in non-obese healthy men consuming 2.4 g/day of EPA and DHA in the form of salmon for 4 weeks, although a trend ($P = 0.086$) for a positive role of n-3 PUFA was evident (82).

While most of the work to date has examined the ability of adiponectin to mitigate pre-existing hypoadiponectinemia present in obese and/or diabetic models, there does remain a need to further evaluate the role and physiological relevance of n-3 PUFA in modulating adiponectin secretion in lean, healthy populations. Furthermore, the ability of n-3 PUFA to target secretion of higher order multimers of adiponectin warrants examination. In summary, despite considerable variability among studies in terms of n-3 PUFA dosage, duration of dietary intake or supplementation, and composition of the background diet, there is evidence that n-3 PUFA increase adiponectin, thereby improving the secretory profile of adipose tissue and associated metabolic outcomes.

c) *Thiazolidinediones*

Thiazolidinediones (TZDs) are a class of antidiabetic drugs commonly prescribed to type 2 diabetic patients to improve insulin sensitivity (27). They were initially discovered in the early 1980s and include the clinically available drugs, rosiglitazone and pioglitazone, as well as

troglitazone, which was withdrawn from the market due to risk of hepatotoxicity (14). Although the precise mechanisms by which TZDs ameliorate insulin resistance remain somewhat unclear, considerable evidence suggests that an increase in adiponectin secretion is involved. In support of this, rosiglitazone-induced improvements in insulin sensitivity in obese mice are significantly attenuated when these mice concomitantly lack adiponectin (112). Additionally, research suggests that genetic variations in the adiponectin gene may actually be predictive of TZD responsiveness in type 2 diabetic patients (71), further implicating a role of adiponectin in TZD-mediated improvements in insulin sensitivity.

TZDs have been shown to increase adiponectin secretion from cultured murine 3T3-L1 adipocytes (25, 97), rat adipocytes (9), and isolated primary human adipocytes (107), as well as to increase circulating adiponectin in animal models (25, 97). Elevations in plasma adiponectin concentrations have also been achieved with both short- (14 days) (25) and long-term (12 weeks) (97, 126) TZD interventions in humans. In an initial study by Maeda et al., troglitazone led to a significant increase in plasma adiponectin concentrations in glucose intolerant individuals in the absence of any changes in body weight (97). In the same study, adiponectin expression was upregulated in adipose tissue from both lean and obese mice treated with troglitazone, pioglitazone or rosiglitazone, and troglitazone increased adiponectin mRNA expression in a dose- and time-dependent manner in 3T3-L1 adipocytes (97). Similarly, both obese and obese diabetic individuals exhibited a twofold increase in circulating adiponectin levels in response to 12 weeks of troglitazone administration (200). Interestingly, elevations in circulating adiponectin in response to TZD treatment have also been observed in healthy, nondiabetic individuals. Both Combs et al. (25) and Yu et al. (200) reported increased plasma adiponectin levels with TZD treatment in lean, healthy subjects with normal baseline adiponectin

concentrations, suggesting that TZD-mediated increases in adiponectin are not limited to obese or diabetic individuals typically characterized by initial hypoadiponectinemia.

In addition to modulating total adiponectin secretion, TZDs are thought to specifically increase higher order multimers of adiponectin (55, 120, 127). Scherer's group illustrated that rosiglitazone induced a dramatic increase in the ratio of HMW to total adiponectin in both diabetic mice and humans (120). Interestingly, improvements in insulin sensitivity with TZD treatment correlated with the ratio of HMW adiponectin to total, but not with total adiponectin itself (120), suggesting that the shift in the secretion of higher order multimers of adiponectin is the prevailing factor by which TZD-mediated increases in adiponectin enhance insulin sensitivity. Phillips et al. confirmed that TZD treatment increases circulating HMW adiponectin in type 2 diabetic individuals but found no effect of TZD treatment on cellular multimer distribution of adiponectin (127), indicating that TZDs may upregulate secretion of HMW adiponectin without necessarily increasing cellular content.

In short, these studies provide convincing evidence for a TZD-mediated increase in adiponectin, which may serve as an important mechanism by which TZD treatment enhances insulin sensitivity.

1.2.3 Mechanisms by which dietary fatty acids and TZDs regulate adiponectin:

Various mechanisms have been proposed for the role of SFA, long-chain n-3 PUFA, and TZDs in modulating adiponectin in rodents and humans. The following section will discuss the role of PPAR γ and AMPK activation, as well as changes in adipose tissue morphology and inflammation, as potential underlying mechanisms by which dietary fatty acids and TZDs influence adiponectin synthesis and secretion.

a) PPAR γ

PPAR γ represents a nuclear receptor protein involved in the regulation of adipogenesis and carbohydrate and lipid metabolism (90). PPAR γ is a ligand-activated transcription factor known to heterodimerize with retinoid x receptor and bind to PPAR-responsive elements (PPRE), thereby upregulating expression of its target genes (140). Iwaki et al. identified the existence of a PPRE in the promoter region of the adiponectin gene (65), and research strongly suggests that PPAR γ agonists serve to upregulate the expression of adiponectin (53, 113, 119). Interestingly, long-chain n-3 PUFA and their derivatives are activators of PPAR γ (189). Recently, both *in vitro* and *in vivo* studies have signified the necessity of PPAR γ in the n-3 PUFA-mediated upregulation of adiponectin (8, 113, 119). Work from our laboratory demonstrated that antagonism of PPAR γ significantly attenuates the n-3 PUFA-mediated induction of adiponectin secretion from mouse adipocytes (119), and these results are in agreement with findings in rat adipocytes (8).

Perhaps the strongest *in vivo* evidence for a role of PPAR γ in mediating n-3 PUFA-induced increases in adiponectin comes from a study in which the feeding of a 27% (of kcal) fish oil diet for 15 days led to a 2-3 fold increase in plasma adiponectin concentrations in mice (113). However, administration of the fish oil diet combined with the PPAR γ antagonist, BADGE completely abolished the n-3 PUFA-mediated rise in circulating adiponectin (113). It is also noteworthy that when PPAR α -null mice received the same fish oil diet, adiponectin levels increased to similar levels observed in the wildtype mice, suggesting that n-3 PUFA mediate adiponectin specifically via the gamma isoform of PPAR (113). Finally, in contrast to n-3 PUFA, evidence suggests that SFA may actually downregulate PPAR γ expression (169). In overweight subjects at risk for metabolic syndrome, consumption of a high SFA diet for 8 weeks

led to a significant decrease in PPAR γ expression in subcutaneous adipose tissue (169), which may serve as an underlying mechanism for the dietary SFA-induced impairment in adiponectin secretion, but this requires further study.

Similar to n-3 PUFA, PPAR γ is also the primary molecular target of TZDs (90). Data from an adiponectin knockout mouse model demonstrated that adiponectin is necessary for TZD-mediated improvements in insulin sensitivity (112), implicating a role for PPAR γ agonism in the upregulation of adiponectin. Indeed, other work has shown that pioglitazone directly stimulates expression of PPAR γ and this is required for TZD-mediated increases in adiponectin secretion from cultured adipocytes (53). Additionally, rosiglitazone treatment failed to increase adiponectin levels in patients with a dominant-negative PPAR γ mutation, further emphasizing that TZDs induce adiponectin transcription via a PPAR γ -dependent mechanism at the whole body level (25). Given that PPAR γ is well established as the primary receptor protein for TZDs (90), it is logical to largely accredit the enhancement of adiponectin secretion by TZD treatment to a PPAR γ -dependent mechanism.

In summary, substantial evidence exists for a mechanistic role of PPAR γ in the n-3 PUFA- and TZD-mediated upregulation of adiponectin.

b) AMPK

A more limited body of evidence suggests that lifestyle factors, including dietary n-3 PUFA, may also increase adiponectin via stimulation of AMPK. Pharmacological activation of AMPK by AICAR has been demonstrated to increase adiponectin gene expression in a dose-dependent manner in adipose tissue explants from normal and overweight individuals (91). Although the direct AMPK-mediated regulation of adiponectin secretion by n-3 PUFA has yet to

be examined, several groups have reported that n-3 PUFA can effectively stimulate AMPK (49, 94, 106). Incubation of both mature murine adipocytes and pre-adipocytes with 100-200 μ M EPA for 24 hours induced a significant increase in the phosphorylation of AMPK (94). Furthermore, dietary n-3 PUFA increased adiponectin mRNA expression in liver and skeletal muscle of high fat fed rats (106), and induced AMPK phosphorylation in liver, skeletal muscle, and adipose tissue of ob/ob mice (49).

In contrast to the abovementioned studies, 14 days of dietary fish oil (5% of kcal) did not affect AMPK phosphorylation and protein content in skeletal muscle, liver and heart in wildtype mice (34). In addition to using a healthy animal model, the n-3 PUFA feeding duration was relatively short (14 days vs. 5-6 weeks in preceding studies), which may provide an explanation for their conflicting results. Given that n-3 PUFA can successfully stimulate AMPK (49, 94, 106), and AMPK activation is known to increase adiponectin secretion (91), investigating the role of AMPK as a potential mechanism by which n-3 PUFA increase adiponectin is warranted. Additionally, while TZDs are classically accepted as PPAR γ agonists, a single dose of troglitazone has also been shown to phosphorylate AMPK in skeletal muscle, liver and adipose tissue in Sprague-Dawley rats (86), suggesting that TZDs may also regulate adiponectin via AMPK although this requires further study.

c) Adipose tissue morphology

In addition to increasing adiponectin expression and secretion directly, n-3 PUFA may also stimulate adiponectin by regulating adipogenesis and inflicting a change in adipocyte morphology. Since adipocyte size represents a known determinant of the adipokine secretory profile, with smaller adipocytes synthesizing and secreting more adiponectin than larger

adipocytes (192), such a change may provide another mechanism for lifestyle-induced modifications of adiponectin. Manickam and colleagues found that incubation of 3T3-L1 adipocytes with 100 μ M EPA exerted a suppressive effect on lipid droplet formation and this was associated with an elevation in adiponectin gene expression (99). Similarly, 25-200 μ M DHA inhibited adipocyte differentiation, induced apoptosis, and promoted lipolysis in post-confluent preadipocytes (74). Evidence from these *in vitro* studies is complemented by a study examining the n-3 PUFA-mediated regulation of adipocyte proliferation and differentiation in mice with diet-induced obesity (57). After consuming a high fat (35% of energy) corn oil-based diet for 8 weeks, the replacement of 15% of the dietary fat with an n-3 PUFA concentrate resulted in an increase in plasma adiponectin and a corresponding decrease in epididymal fat pad weight as well as adipocyte size (57).

Likewise, TZD administration has also been shown to regulate adipose tissue mass and morphology. More specifically, 15 days of troglitazone treatment resulted in a four-fold increase in the number of small adipocytes and induced apoptosis of larger adipocytes, without any change in total adipose tissue weight, in obese Zucker rats (117). Similarly, rosiglitazone treatment led to a significant decrease in adipocyte size in Sprague-Dawley rats fed either a low- or high-fat diet (66). Given that the secretion of adipokines, including adiponectin, from adipose tissue is highly dependent on both adiposity (6) and adipocyte size (193), it stands to reason that n-3 PUFA and TZD may upregulate adiponectin, in part, by reducing adipocyte hyperplasia and hypertrophy.

d) Macrophage infiltration and inflammation

Changes in the release of inflammatory adipokines from adipose tissue in obesity are largely accredited to an increase in macrophage infiltration (183). Macrophages have been shown to inhibit adiponectin secretion and promote the release of inflammatory cytokines, including TNF- α and IL-6 (156). Furthermore, there is considerable evidence to suggest that the release of pro-inflammatory adipokines from adipose tissue-derived macrophages can exert paracrine effects on adipocytes, thereby inhibiting the synthesis and secretion of adiponectin. Treatment of 3T3-L1 adipocytes with IL-6 decreased the expression and secretion of adiponectin by 50% and 75%, respectively (41). Similar impairments in adiponectin transcription are reported with TNF- α treatment in adipocytes (202). Recently, data has emerged to suggest that n-3 PUFA may act to prevent impairments in adiponectin secretion induced by inflammatory cytokines (60, 64, 123, 162). In a study by Itoh and colleagues using a co-culture system of 3T3-L1 adipocytes and RAW264 macrophages, 100-200 μ M EPA was shown to mitigate the co-culture-induced increase in TNF- α gene expression in macrophages (64). Furthermore, 5 weeks of EPA supplementation (1 g/kg/day) in high fat fed Wistar rats was shown to effectively prevent the induction of TNF- α expression in adipose tissue (123). From these two studies, it seems likely that n-3 PUFA may alleviate reductions in adiponectin by downregulating expression of pro-inflammatory adipocytes that interfere with its secretion.

Moreover, n-3 PUFA may also prevent the decrease in adiponectin that is induced by pro-inflammatory cytokines through inhibition of macrophage infiltration into the adipose tissue itself. Todoric et al. found that feeding a high SFA diet (30% of kcal from lard) for 6 weeks induced a marked increase in macrophage infiltration in obese diabetic mice and this was paralleled by an increase in inflammatory gene expression (162). However, replacement of 12%

of the lard with a high n-3 PUFA fish oil concentrate completely prevented the exacerbated state of adipose tissue macrophage infiltration and upregulated inflammatory gene expression that was triggered by the high SFA diet (162). Intriguingly, n-3 PUFA have also been shown to downregulate expression of matrix metalloproteinases required for macrophage infiltration into adipose tissue (60), supporting a role for n-3 PUFA in preventing inflammation by contributing to a remodelling of adipose tissue.

In addition to increases in macrophage infiltration in obesity, the activation of inflammatory pathways may also contribute to a downregulation of adiponectin secretion by pro-inflammatory cytokines. SFA are known ligands for toll-like receptor (TLR)4 and, upon binding, initiate an inflammatory cascade that culminates in the activation of nuclear factor (NF)- κ B and expression of inflammatory cytokines (101). Shi et al demonstrated that SFA treatment induced a TLR4 dependent activation of NF- κ B in macrophages and this resulted in increased IL-6 expression (144). Interestingly, research suggests that n-3 PUFA may aid in downregulating the SFA-mediated activation of TLR4 and NF- κ B, thereby preventing increases in pro-inflammatory adipokines that can inhibit adiponectin secretion. For example, 5 μ M EPA or DHA prevented lauric acid-induced activation of NF- κ B in RAW264 macrophages (88), while EPA effectively suppressed induction of NF- κ B activity by palmitic acid in RAW264 macrophages (64). Taken together, these studies suggest that n-3 PUFA may downregulate inflammatory signalling pathways induced by SFA, thereby alleviating the suppression of adiponectin by pro-inflammatory cytokines.

Figure 1.4: Modulation of adiponectin secretion from adipocytes. TZDs and n-3 PUFA activate PPAR γ , and possibly AMPK, to upregulate adiponectin secretion. SFA activate TLR4 and stimulate inflammatory pathways involving NF- κ B to downregulate adiponectin secretion. n-3 polyunsaturated fatty acids (PUFA), thiazolidinediones (TZD), AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor (PPAR) γ , saturated fatty acids (SFA), toll-like receptor (TLR)4, nuclear factor (NF)- κ B, tumour necrosis factor (TNF)- α , interleukin (IL)-6.

To summarize, there is good evidence to suggest that activation of PPAR γ and AMPK and changes in inflammatory signalling pathways are underlying mechanisms by which dietary fatty acids and TZDs regulate the synthesis and secretion of adiponectin. Adiponectin is also known to undergo considerable post-translational modifications prior to its release from adipocytes (180), and TZDs have been shown to regulate endoplasmic reticulum chaperone proteins involved in the release of adiponectin from the cell (181). Although it is not a focus of

this thesis, research investigating the role of n-3 PUFA in modulating the expression and function of these aforementioned chaperone proteins is also warranted.

Section 1.3: High saturated fat diets and skeletal muscle insulin response

While changes in adipokine secretion from adipose tissue in obesity are known to contribute to systemic inflammation and insulin resistance, the influence of adipose-derived factors on the function of peripheral tissues such as skeletal muscle also plays an important role in governing whole body metabolism.

1.3.1 Fatty acid overload and impairments in insulin response

Skeletal muscle is responsible for approximately 80% of insulin-stimulated glucose disposal (29), rendering it a vital tissue in the maintenance of whole body glucose homeostasis. Exposure of skeletal muscle to excess fatty acids is a known contributor to impairments in insulin responsiveness. Indeed, skeletal muscle insulin resistance has been induced by SFA exposure in cultured L6 myotubes (33) and isolated muscle strips (161), as well as in response to lipid infusion (35) and administration of a high SFA diet (108). Impairments in skeletal muscle insulin signalling in response to fatty acid surplus are attributed to several factors, most notably the accumulation of muscle lipid species (129, 199) and activation of intracellular inflammatory signalling pathways (144).

1.3.2 Accumulation of muscle lipids

The evidence supporting a connection between the accumulation of lipid species in skeletal muscle and impairments in insulin signalling is exceedingly convincing. Upon uptake

into skeletal muscle, fatty acids are either oxidized or partitioned into storage, primarily in the form of triacylglycerides (TAGs), as well as diacylglycerides (DAGs) and ceramides.

Accumulation of intramuscular TAGs is negatively correlated with insulin sensitivity and has traditionally served as a classic marker of insulin resistance (121). However, more recent studies suggest that TAGs are relatively inert in function and simply represent a surrogate marker for other more reactive lipid species (93). In fact, evidence suggests that TAG stores may even provide a protective effect with regards to insulin sensitivity by shunting fatty acids away from deleterious DAG and ceramide pools (93). Both DAGs and ceramides are also increased in obese muscle (105) and in contrast to TAGs, have been shown to directly interfere with insulin signalling (129, 199). DAGs are thought to signal via protein kinase C to interfere with phosphorylation of IRS-1 (199), whereas ceramides act to directly inhibit Akt (129). As such, the accumulation of intramuscular DAGs and ceramides represents an important link between fatty acid oversupply and the development of skeletal muscle insulin resistance.

The cellular mechanisms contributing to the accumulation of muscle lipids in response to fatty acid overload is a multifactorial process. One contributing factor is an increase in fatty acid uptake resulting from the redistribution of fatty acid transporters (i.e. FAT/CD36) from the cytosol to the plasma membrane (13). Bonen et al. showed a 4-fold increase in rate of fatty acid uptake in muscle from obese and type 2 diabetics that was associated with an increase in FAT/CD36 content at the membrane, leading to an increase in muscle lipid content (13). Additionally, impaired rates of fatty acid oxidation may also play a role in muscle lipid accumulation; however, the evidence is inconsistent. Exercise-induced increases in fatty acid oxidation are associated with improvements in insulin sensitivity (50) and a blunted rate of palmitate oxidation has been reported in muscle from extremely obese individuals (62). Obese

muscle is also resistant to both adiponectin (108) and leptin's (154) stimulatory effects on fatty acid oxidation. Despite this, recent studies generally concede that rates of fatty acid oxidation in obese muscle are similar (16, 154), if not increased (168), perhaps as a compensatory mechanism. Consequently, the relative contribution of reduced rates of oxidation to skeletal muscle lipid accumulation remains unclear. Finally, a more recent body of evidence also suggests a role for increased production of mitochondrial reactive oxygen species in response to fatty acid overload in the etiology of skeletal muscle insulin resistance (5).

1.3.3 Activation of intracellular inflammatory pathways

Chronic exposure to a high SFA diet evokes a systemic inflammatory response that is characterized not only by an increase in circulating inflammatory cytokines but also by the activation of cellular inflammatory pathways in tissues including skeletal muscle. TLR4 represents a ligand-activated membrane receptor that plays an important role in innate immunity (165). Although lipopolysaccharides are considered classical activators of TLR4 (165), evidence suggests that SFA also serve as TLR4 ligands (144). SFA activate TLR4 to initiate a downstream signalling cascade involving the activation of IKK, subsequent inactivation of I κ B α and translocation of NF- κ B to the nucleus to upregulate inflammatory gene expression (144) (**Figure 1.5**). As such, both lipid infusion (131) and high SFA diets (196) have been demonstrated to activate the TLR4/NF- κ B pathway in skeletal muscle. Importantly, unsaturated fatty acids are incapable of stimulating TLR4 (88, 146) and both DHA and EPA have been shown to actually inhibit TLR signalling (87, 102), suggesting a role for n-3 PUFA in preventing SFA-induced activation of skeletal muscle inflammation.

Figure 1.5: Proposed mechanism for the activation of TLR4/NF-κB signalling pathway by SFA. SFA activate TLR4 to stimulate IKK, which decreases IκBα and allows for the translocation of NF-κB to the nucleus to upregulate the expression of inflammatory genes. n-3 PUFA act to inhibit the activation of this pathway. Saturated fatty acids (SFA), n-3 polyunsaturated fatty acids (PUFA), toll-like receptor (TLR)4, IκB kinase (IKK), inhibitor of kappa B alpha (IκBα), nuclear factor (NF)-κB.

In addition to intramuscular lipid accumulation, activation of the aforementioned inflammatory pathways in skeletal muscle may represent an underlying mechanism by which excess dietary lipid consumption contributes to insulin resistance. Sinta et al. found that inhibition of NF-κB either directly or by inhibiting its upstream activator, IKK effectively prevents palmitate-induced impairments in insulin-stimulated glucose transport in myocytes

(146). Similarly, blocking TLR4 activity has been shown to prevent reductions in glucose uptake in cultured L6 myotubes treated with palmitate (131). These findings are complemented by *in vivo* studies demonstrating that TLR4 knockout mice display partial protection against insulin resistance induced by both lipid infusion (131, 144) and administration of a high fat diet (144). Lastly, muscle from obese insulin resistant humans exhibits both elevated TLR4 expression and I κ B/NF- κ B signalling compared to muscle from lean, insulin sensitive humans (133). Collectively, these studies support a role for inflammation as an important underlying mechanism by which lipid oversupply impairs skeletal muscle insulin response.

Section 1.4: Adipose tissue-skeletal muscle cross-talk

Given the strong causal link between obesity and skeletal muscle insulin resistance, the role of adipose-derived factors in communicating with skeletal muscle to affect its function is of interest. The following section will summarize the research conducted to date on the cross-talk that occurs between adipocytes and myocytes, as well as highlight some of the limitations of this current body of work.

1.4.1 Regulation of myocyte insulin signalling by adipocyte-derived factors

In 2002, Dietze and colleagues developed a novel *in vitro* co-culture system to study the paracrine communication between adipocytes and myocytes and its role in the development of skeletal muscle insulin resistance (30). Co-culture of human myocytes with isolated human adipocytes for 48 hours was shown to impair myocyte insulin signalling, as evidenced by blunted insulin-stimulated phosphorylation of both IRS-1 and Akt (30). Thus, it appears that adipocyte-

derived factors may communicate with myocytes to interfere with insulin signalling, thereby contributing to the development of insulin resistance.

As the adipocyte secretory profile is known to change in response to expanding adipose tissue mass (148), Skurk et al. assessed the role of both BMI and adipocyte size in mediating the communication between adipose tissue and skeletal muscle (147). In keeping with the results of Dietze et al. (30), exposure of human myocytes to adipocyte-conditioned medium significantly blunted Akt phosphorylation by insulin (147). Importantly, this impairment was significantly exacerbated with both an increasing BMI and adipocyte volume (147). It is also noteworthy that adipocytes obtained from the omental depot induced a more pronounced blunting in Akt phosphorylation compared to subcutaneous adipocytes (147). Therefore, the communication between adipocytes and myocytes appears to be regulated by adiposity, adipocyte size, and anatomical location of fat, providing a potential underlying mechanism by which obesity contributes to skeletal muscle insulin resistance.

1.4.2 MCP-1 and activation of NF- κ B as regulators of adipocyte-myocyte cross-talk

In an attempt to identify key factors responsible for the observed impairments in myocyte insulin signalling, levels of inflammatory cytokines in the co-culture medium were quantified and compared to those required to reduce insulin signalling as determined by dose-response studies (30). Levels of TNF- α and resistin in the co-culture medium were found to be considerably lower than those required to impair muscle insulin signalling, suggesting that these adipokines are not responsible for the observed loss of insulin response (30). Similarly, in a follow-up study, levels of IL-6, IL-8, and macrophage inflammatory protein-1 α/β released from human adipocytes were found to be insufficient to induce impairments in insulin signalling in

isolated muscle cells (141). However, it must be acknowledged that although the concentration of each cytokine in isolation was inadequate to impair insulin signalling in accordance with the dose-response studies, it is possible that their cumulative effects would indeed be sufficient. In contrast to the abovementioned cytokines, MCP-1 concentration in the medium was representative of that required to impair both insulin signalling and glucose uptake in myocytes, suggesting MCP-1 may represent a key mediator of adipose-muscle cross-talk and ensuing changes in insulin sensitivity (141).

Furthermore, there is also evidence to suggest that adipose-derived signals may mediate changes in insulin signalling in skeletal muscle via inflammatory pathways involving activation of IKK and its downstream target, NF- κ B. In support of this, decreased Akt phosphorylation in response to acute exposure of human myocytes to adipose-conditioned medium for 30 minutes was paralleled by an activation of NF- κ B in myocytes (31). Notably, both NF- κ B activation and impairments in insulin signalling were prevented by co-administration of an IKK inhibitor (31), further substantiating an important role for inflammation in mediating the communication between adipocytes and myocytes. As activation of NF- κ B has been previously shown to induce MCP-1 expression (157), it is also possible that activation of these intracellular inflammatory pathways further perpetuates impairments in myocyte insulin signalling induced by MCP-1 in a feedforward manner, but this requires further study. Importantly, the role of dietary fatty acids known to alter the adipokine secretory profile (42, 43) and induce changes in inflammation (7), in regulating the communication between adipose tissue and skeletal muscle has yet to be examined and also merits investigation.

1.4.3 Prevention and reversibility of adipocyte-induced changes in myocyte function

Studies have addressed the ability of factors to prevent adipocyte-myocyte co-culture induced impairments in insulin signalling. In a co-culture of human myocytes and adipocytes, the addition of troglitazone to the culture medium prevented the decrease in insulin-stimulated IRS-1 and Akt phosphorylation in the myocytes (30). Given the experimental design, however, it was impossible to determine if troglitazone exerted this protective effect by acting on the adipocytes or the myocytes or both, limiting the mechanistic interpretations that can be drawn from this work. In a subsequent study by the same group, the addition of synthetic adiponectin to a co-culture of human adipocytes and myocytes also effectively prevented impairments in myocyte insulin signalling (32). To advance understanding of the mechanisms involved, myocytes were concomitantly incubated with adipose-conditioned medium and synthetic adiponectin to determine if the same protective effect would be observed in the absence of adipocyte exposure to adiponectin (32). Interestingly, Akt phosphorylation remained blunted, indicating that the prevention of impairments in insulin signalling was attributable to an effect of adiponectin on the adipocytes themselves (32).

Given that the adipokine-induced disruption of myocyte insulin signalling can be prevented by exposure to sensitizing factors such as troglitazone and adiponectin, Sell and colleagues subsequently addressed the potential reversibility of co-culture induced insulin resistance in myocytes (142). To this end, human myocytes were exposed to adipose-conditioned medium for 24 hours at which point the adipose-conditioned medium was removed and replaced with normal, adipose-free culture medium for an additional 24 or 48 hours (142). Regeneration of the myocytes in adipose-free medium effectively restored insulin signalling as evidenced by normalization of Akt phosphorylation (142). However, it is noteworthy that while

impairments in insulin signalling were reversed, the adipose-induced increase in MCP-1 secretion from the myocytes remained elevated at both 24 and 48 hours post-removal of the adipose-conditioned medium (142). Therefore, while the withdrawal of signals from adipocytes may reverse impairments in myocyte insulin signalling, it is possible that changes induced in the myocyte secretory profile may persist in an irreversible manner.

1.4.4 Regulation of adipose tissue by muscle-derived factors

Although most adipose-muscle cross-talk research to date is adipocentric in nature, myocyte-derived factors (or myokines) are also known to regulate the function of adipocytes as well. IL-6 is a classic example of a cytokine that is released from both myocytes and adipocytes (170) and, therefore, represents a factor involved in the bi-directional communication between adipose tissue and skeletal muscle. Secretion of IL-6 from skeletal muscle increases during exercise (152), and has been shown to stimulate lipolysis in adipose tissue (170). As such, IL-6 appears to serve an important role in the acute liberation of fatty acids for oxidation during exercise. Conversely, plasma IL-6 levels are positively correlated with obesity (173) and chronic exposure of skeletal muscle to IL-6 is associated with impairments in insulin responsiveness (115). Therefore, while IL-6 exerts favourable effects acutely during exercise, chronic elevation of circulating IL-6 levels may contribute to the development of insulin resistance, illustrating an interesting paradoxical role of IL-6 in the communication between adipose tissue and skeletal muscle.

Furthermore, IL-15 represents a second myokine known to communicate with adipose tissue to regulate its function. In addition to its important immunological role, IL-15 is highly expressed in skeletal muscle where it functions in an autocrine manner to stimulate muscle

growth by upregulating expression of contractile proteins (19). Interestingly, IL-15 has also been shown to regulate adiposity (19). Subcutaneous injection of IL-15 for 7 days led to a 33% decrease in adipose tissue mass in male Wistar rats and this was associated with both a decrease in lipoprotein lipase activity and a decreased rate of lipogenesis (19). Therefore, while IL-15 functions in an autocrine manner to stimulate muscle growth, it may also decrease adiposity in an endocrine fashion. In support of this, a negative association between plasma IL-15 levels and both total fat mass and trunk fat mass has been demonstrated in humans (114). To summarize, although research investigating the role of myocyte-derived factors in mediating muscle-adipose cross-talk is limited, IL-6 and IL-15 represent two myokines involved in the regulation of adiposity, thus further substantiating the significance of cross-talk between these two tissues.

1.4.5 Summary and limitations of current adipose tissue-skeletal muscle cross-talk research

In summary, a significant body of literature exists at the *in vitro* level suggesting that adipocytes communicate with myocytes to impair insulin signalling, ultimately contributing to the development of insulin resistance. However, as adipose tissue is comprised of multiple cell types, the role of non-adipocyte cells in mediating the cross-talk between adipose tissue and skeletal muscle requires examination. This is especially true of adipose tissue resident macrophages given their abundant secretion of inflammatory cytokines including MCP-1, which has been implicated as a key mediator of adipose-muscle cross-talk with regards to insulin sensitivity (141). It is also important to acknowledge that in addition to adipokines, free fatty acids are also released from adipose tissue and likely play an important role in mediating adipose-muscle cross-talk. Therefore, while the current evidence derived from adipocyte-myocyte co-culture studies at the cellular level is indeed intriguing, additional work at the whole

adipose tissue-skeletal muscle level is warranted to better understand the role of adipose tissue-skeletal muscle communication in mediating changes in insulin sensitivity. In addition, the role of dietary fatty acids known to modify the adipose tissue secretory profile (42, 43) has not been evaluated in adipose tissue-skeletal muscle cross-talk studies to date and also requires examination.

Section 1.5: Conclusions

Adipose tissue represents an active endocrine organ that can communicate with skeletal muscle to regulate lipid metabolism and insulin sensitivity. Adipose-derived adiponectin is an anti-inflammatory adipokine that stimulates skeletal muscle fatty acid oxidation, thereby improving insulin responsiveness. However, both the secretion of adiponectin from adipose tissue and its stimulatory effects on skeletal muscle are impaired in obesity and in response to a high SFA diet. In contrast, dietary long-chain n-3 PUFA are thought to increase adiponectin secretion from adipocytes, possibly through a PPAR γ -dependent mechanism, although this has not been studied in human cells. In addition, the role of n-3 PUFA in mediating the response of skeletal muscle to adiponectin, as well as any associated functional effects on insulin response, are completely unknown. Finally, despite convincing evidence at the cellular level to suggest that adipocytes communicate with myocytes to regulate insulin signalling, the role of adipocyte-derived factors in regulating muscle insulin response at the whole tissue level has yet to be directly examined. Investigation of n-3 PUFA-mediated modulation of adiponectin secretion and ensuing changes in skeletal muscle adiponectin and insulin responsiveness would enhance our understanding of a promising nutritional approach to aid in the management of obesity and related pathologies.

Chapter 2: Aims of the Thesis

Obesity is characterized by dysregulated adipokine secretion from adipose tissue, contributing to a state of whole body low-grade inflammation and impairments in lipid metabolism and insulin response in peripheral tissues, including skeletal muscle. Adiponectin is a beneficial adipokine that has been shown to exert peripheral effects on skeletal muscle to stimulate fatty acid oxidation (36); however, this effect is blunted in obesity (16, 154) and in response to consumption of a high SFA diet (108). Whereas high SFA diets are closely linked to obesity and the development of insulin resistance, fish-oil derived n-3 PUFA are thought to exert protective effects, possibly by modulating adiponectin, an anti-inflammatory and insulin-sensitizing adipokine.

To date, studies examining dietary fat-mediated modulation of adiponectin secretion from rodent adipocytes have yielded inconsistent findings (17, 64, 95, 113) and, importantly, no such studies have been conducted with human adipocytes. Additionally, given the strong causal link between obesity and insulin resistance, the role of adipose-derived factors in communicating with skeletal muscle to affect its function is of importance. Despite a reported positive association between n-3 PUFA and circulating adiponectin (3, 64, 76, 78, 116), the role of n-3 PUFA in mediating skeletal muscle response to adiponectin, as well as any associated functional effects in muscle, such as insulin responsiveness, is unknown. In a co-culture of human adipocytes and myocytes, adipocyte-derived factors impair myocyte insulin signalling (30), suggesting that cross-talk between these two tissues may play a key role in regulating insulin sensitivity. However, the communication between adipose and muscle, as well as its modulation by dietary fatty acids, had never been studied at the whole tissue level. Given the important contribution of non-adipocyte cells such as macrophages to the secretory profile of adipose tissue

(156), as well as the association between dietary fatty acid intake and circulating adipokine levels (43), such investigation is warranted.

The primary objective of the thesis was to investigate the role of n-3 PUFA in the modulation of adiponectin secretion and ensuing changes in skeletal muscle inflammation and adiponectin and insulin responsiveness. To this end, three studies were conducted.

The specific objectives of study 1 were:

- 1) To examine the modulation of adiponectin secretion and cellular protein content by long-chain n-3 PUFA and rosiglitazone (a TZD) in cultured primary human adipocytes.
- 2) To determine if n-3 PUFA mediate their effects on adiponectin via a PPAR γ -dependent mechanism.

The specific objectives of study 2 were:

- 1) To determine if long-chain n-3 PUFA could prevent and/or restore high SFA diet-induced impairments in adiponectin and insulin response in rodent soleus muscle.
- 2) To elucidate the potential mechanisms by which n-3 PUFA exert these effects.

The specific objectives of study 3 were:

- 1) To develop an *ex vivo* model to study the communication between adipose and skeletal muscle at the whole tissue level.
- 2) To determine the effects of a high SFA diet, with or without long-chain n-3 PUFA, on the adipose tissue secretory profile and ensuing changes in skeletal muscle inflammation and insulin sensitivity in rodents.

The overall hypotheses of the thesis were:

- 1) Treatment with n-3 PUFA, as well as rosiglitazone, would increase adiponectin secretion and cellular protein content and this would occur via a PPAR γ -dependent mechanism.
- 2) Dietary n-3 PUFA would prevent and restore high SFA diet-induced impairments in both adiponectin and insulin response via reduced activation of inflammatory pathways.
- 3) Consumption of a high SFA diet would decrease adiponectin and increase pro-inflammatory cytokine release from adipose tissue, leading to skeletal muscle inflammation and impaired insulin sensitivity, and this would be prevented by dietary n-3 PUFA.

Chapter 3: Eicosapentaenoic acid and rosiglitazone increase adiponectin in an additive and PPAR γ -dependent manner in cultured human adipocytes.

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3.1 Abstract

Adiponectin, an anti-inflammatory and insulin-sensitizing protein secreted from adipose tissue, may be modulated by dietary fatty acids, although the mechanism is not fully known. Our objective was to investigate the effect of long-chain n-3 polyunsaturated fatty acids (PUFA) on adiponectin in cultured human adipocytes, and to elucidate the role of PPAR γ in this regulation. Isolated human adipocytes were cultured for 48 h with 100 μ M eicosapentaenoic acid (C20:5n-3, EPA), docosahexaenoic acid (C22:6n-3, DHA), palmitic acid (C16:0), 100 μ M EPA plus 100 μ M DHA or bovine serum albumin (control). Additionally, adipocytes were treated for 48 h with a PPAR γ antagonist (BADGE) or agonist (rosiglitazone) in isolation or in conjunction with either EPA or DHA. At 48 h, EPA and DHA increased ($P < 0.05$) adiponectin secretion by 88% and 47%, respectively, while EPA, but not DHA, also increased (136%, $P < 0.001$) cellular adiponectin protein. Interestingly, PPAR γ antagonism completely abolished the DHA-mediated increase in secreted adiponectin, but only partially attenuated the EPA-mediated response. Thus, EPA's effects on adiponectin do not appear to be entirely PPAR γ mediated. Rosiglitazone increased ($P < 0.001$) secreted and cellular adiponectin protein (90% and 582%, respectively).

Finally, the effects of EPA and rosiglitazone on adiponectin secretion were additive (+230% at 48 h combined, compared to 121% and 124% by EPA or rosiglitazone alone, respectively).

Overall, our findings emphasize the therapeutic importance of long-chain n-3 PUFA alone, or in combination with a PPAR γ agonist, as a stimulator of adiponectin, a key adipokine involved in obesity and related diseases.

3.2 Introduction

Obesity, characterized by an excess accumulation of adipose tissue, and obesity-related diseases are increasing at an alarming rate (51). It is now widely accepted that adipose tissue represents an active endocrine organ that secretes a multitude of proteins, collectively termed adipokines (1). More specifically, excess adipose tissue in sedentary states is characterized by an elevated secretion of pro-inflammatory adipokines, such as interleukin-6, resistin, tumour necrosis factor- α , and monocyte chemoattractant protein-1 (132). In contrast, adiponectin, an anti-inflammatory and insulin-sensitizing adipokine, is negatively correlated with adiposity (184). The precise regulation of adiponectin remains unclear; however, evidence suggests that dietary fatty acids are involved (42).

The long-chain n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA) are well-known for their anti-inflammatory properties (135). One mechanism by which EPA and DHA may exert such anti-inflammatory effects is by modulating adipokine production and secretion (18). Recent studies examining the effects of PUFA on adiponectin secretion from isolated adipocytes have been inconsistent, reporting an increase (9), decrease (17, 95) and no change (64, 113); importantly, studies have not yet been extended to cultured human adipocytes. Given the increased availability of n-3

PUFA-containing foods in today's society, examination of how these fatty acids may impact adiponectin in a human model is warranted.

The potential mechanism by which n-3 PUFA modulate adiponectin is unclear, but may involve PPAR γ , a nuclear receptor protein that regulates the transcription of several genes, including adiponectin (20). Since long-chain n-3 PUFA are thought to represent natural ligands for PPAR γ , this suggests their potential usefulness as a nutrition strategy to modulate adiponectin. In addition, synthetic ligands for PPAR γ , including the insulin-sensitizing drugs known as thiazolidinediones (TZD) have also been demonstrated to increase circulating levels of adiponectin (97). Thus, both long-chain n-3 PUFA and TZD may upregulate adiponectin by a mechanism involving PPAR γ , suggesting that both nutritional and pharmaceutical interventions may be useful therapeutic strategies in the treatment of obesity-related diseases. To the best of our knowledge, the regulation of adiponectin by long-chain n-3 PUFA and TZD such as rosiglitazone, has yet to be examined using a human adipocyte model.

Our objective in the current study was to determine the effects of long-chain n-3 PUFA on the secretion and cellular protein content of adiponectin in cultured human adipocytes, as well as to elucidate the role of PPAR γ in this regulation. We hypothesized that EPA and DHA, as well as rosiglitazone, would increase adiponectin and that this would occur via a PPAR γ -dependent mechanism.

3.3 Methods

3.3.1 Adipocyte culture and differentiation

Isolated primary subcutaneous human pre-adipocytes from a pooled sample of 5 overweight female donors were obtained from Zen-Bio Inc. (Research Triangle Park, NC). The donors were non-smokers without type 2 diabetes, with a mean BMI and age of 27.9 kg/m² (25.7-28.9 kg/m²) and 45.6 y (37-57 y), respectively. Pre-adipocytes were incubated with preadipocyte medium (Zen-Bio Inc.) in a humidified incubator at 37 °C with 5% CO₂ until fully confluent. The medium was then removed, replaced with 7 mL adipocyte differentiation medium (Zen-Bio Inc.) and after 7 d, 4.2 mL of medium was removed from each flask and replaced with 5.6 mL of adipocyte maintenance medium (Zen-Bio Inc.). After 2 d, 4.2 mL of medium was removed and replaced with 4.2 mL of adipocyte maintenance medium. This process was repeated for a subsequent 5 d until the adipocytes were fully differentiated and exhibited marked lipid accumulation as assessed by microscopy, at which time treatments were initiated as described below.

3.3.2 Fatty acid treatments

14 d after induction of differentiation, adipocytes were rinsed with phosphate buffer saline (PBS) (Thermo Fisher Scientific, Ottawa, Canada) and serum starved for 12 h in Dulbecco's Modified Eagle's Medium (DMEM) prior to fatty acid treatment. Fatty acid treatment solutions were prepared by complexing fatty acids with bovine-serum albumin (BSA) in DMEM. Adipocytes were treated for 48 h with 100 µM of EPA, DHA, palmitic acid, or 100 µM of EPA plus 100 µM of DHA, all complexed with 20 µM BSA. BSA alone served as the

control. Fatty acid dosages and duration of treatment were chosen in accordance with pilot work, with DHA used as a representative fatty acid of interest (**Fig. 3.1**). Samples of the culture medium were obtained every 12 h throughout the 48 h treatment period. After 48 h, adipocytes were lysed for protein isolation and all samples were stored at -80 °C until analysis. All treatments were performed in triplicate and the entire experiment was replicated 3 separate times.

Figure 3.1: Fatty acid dose response and timecourse pilot work. Cells were treated with 50, 100, 125, or 250 μ mol/l of DHA for 12, 24, 36, 48, 60, or 72 h. Bovine serum albumin alone acted as the control condition. Secreted adiponectin values are means $\pm$ s.e.m., $n = 3$ replicates. DHA, docosahexaenoic acid.

3.3.3 PPAR γ antagonist (BADGE) and agonist (rosiglitazone) treatment

PPAR γ antagonist solutions were prepared by dissolving 100 mg of bisphenol A diglycidyl ether (BADGE, Cayman Chemical) in 1 mL ethanol and diluting to 100 μ M in DMEM. PPAR γ agonist solutions were prepared by dissolving 0.5 mg/mL of rosiglitazone (Cayman Chemical, MI, USA) in a 1:3 v/v dimethyl sulfoxide:PBS solution, diluting to 10 nM in DMEM. Doses of rosiglitazone and BADGE were selected in accordance with pilot work (data not shown). The PPAR γ agonist and antagonist solutions were added to the adipocytes alone or in combination with 100 μ M EPA or DHA, according to the methods described above. Adipocytes were incubated for a period of 48 h, during which time the culture medium was sampled at 24 h and 48 h. At 48 h, the adipocytes were lysed for protein isolation and all samples were stored at -80 °C until analysis. All treatments were performed in triplicate and the entire experiment was replicated 3 separate times.

3.3.4 Protein isolation

Medium was aspirated and the adipocytes were released in 0.25% 1 x Trypsin-ethylenediamine tetraacetic acid (EDTA) (Invitrogen, Burlington, Canada) and combined with DMEM. All subsequent steps were performed on ice. Samples were centrifuged (Microfuge 22R, Beckman Coulter) and the cell pellets were washed twice by resuspension in PBS. Cell pellets were then resuspended in PBS with protease inhibitor (Roche Diagnostics) and stored at -80 °C for subsequent analysis.

3.3.5 Cellular fatty acid composition

Total lipids were extracted from cell lysates as described by Folch et al (45), then fatty acid methyl esters were prepared for analysis by gas liquid chromatography as previously described in Lau et al (83). Fatty acids were identified by comparing the retention times of the samples with those of a known standard (GLC463; Nu-Chek Prep, Elysian, MN). Fatty acid composition values are expressed as a percent of total fatty acids.

3.3.6 Secreted and cellular adiponectin protein measurement

Adiponectin in the cell culture medium was quantified by ELISA (Human Adiponectin ELISA, B-Bridge International, Inc., Mountain View, CA) according to the manufacturer's instructions. Cell lysates were centrifuged to pellet the cells and resuspended in PBS. Total protein was quantified using a bicinchoninic acid protein assay (Fisher Scientific) according to the manufacturer's instructions. Volumes of cell lysate assayed were standardized by total protein. Cellular adiponectin protein was quantified by ELISA in accordance with procedures described above. A subset of samples consisting of three samples per group per time point was analyzed for both secreted and cellular high molecular weight (HMW) adiponectin protein by ELISA (Human High Molecular Weight Adiponectin ELISA, Millipore, Billerica, MA)

3.3.7 Statistical analysis

Adiponectin protein concentration in the medium was analysed for the effect of treatment, time, and treatment by time interaction using a two-way analysis of variance (ANOVA) for repeated measures. Cellular adiponectin protein data (obtained at 48 h only) was analysed using a one-way ANOVA for effect of treatment. A Tukey post-hoc test was used to

identify significant differences. Total secreted adiponectin protein data for the fatty acid treatment experiments, as well as cellular adiponectin protein data for the PPAR γ antagonist and agonist experiments, were not normally distributed and were log-transformed prior to statistical analysis. All other data were normally distributed. Data are expressed as mean $\pm$ SEM, unless otherwise indicated. Statistical significance was set at $P < 0.05$ and all statistical analysis was performed using Sigma Stat version 2.03.

3.4 Results

3.4.1 Cellular fatty acid composition

Adipocytes treated with EPA, DHA, and EPA plus DHA exhibited a significant ($P < 0.05$) increase in the fatty acid corresponding to the specific treatment (**Table 3.1**). In contrast, adipocytes treated with palmitic acid did not contain a higher amount of palmitic acid compared to control ($P = 0.820$). The fatty acid composition of the adipocytes from the PPAR γ antagonist (BADGE) and agonist (rosiglitazone) experiments are not shown, as these did not affect cellular fatty acid composition.

Table 3.1: Fatty acid composition of total lipids in human adipocytes cultured for 48 h

	Control	Palmitic Acid	EPA	DHA	EPA+DHA
Fatty acid	% of total fatty acids¹				
C14:0	1.7 ± 0.5	1.8 ± 0.7	1.9 ± 0.2	1.5 ± 0.6	2.0 ± 1.1
C16:0	25.1 ± 1.8	29.0 ± 2.4	24.6 ± 2.3	20.2 ± 1.5	20.2 ± 2.8
C16:1c9	12.6 ± 3.7	11.3 ± 3.7	13.9 ± 3.0	8.9 ± 1.4	9.6 ± 3.7
C18:0	15.1 ± 3.3	15.3 ± 3.4	11.9 ± 2.5	13.3 ± 2.1	12.9 ± 3.9
C18:1c9+c11	39.0 ± 1.9	35.8 ± 1.3	33.7 ± 2.2	28.8 ± 4.1	26.3 ± 4.9
C18:2n6	1.2 ± 0.4	1.2 ± 0.4	0.9 ± 0.2	1.0 ± 0.2	1.2 ± 1.6
C19:0	1.5 ± 0.3	1.6 ± 0.6	1.1 ± 0.3	1.2 ± 0.5	1.2 ± 0.9
C20:4n-6	1.0 ± 0.5	1.1 ± 0.4	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.7
C20:5n-3	ND ²	ND	5.3 ± 0.8* ³	0.9 ± 0.2	4.6 ± 1.8*
C22:5n-6	0.8 ± 0.2	0.9 ± 0.4	1.0 ± 1.4	0.7 ± 0.4	0.8 ± 0.8
C22:5n-3	1.2 ± 0.5	1.2 ± 0.6	4.4 ± 2.5*	0.8 ± 0.4	4.4 ± 1.6*
C22:6n-3	0.6 ± 0.4	0.7 ± 0.4	0.5 ± 0.3	21.9 ± 7.1*	20.1 ± 5.9*
Total saturates	43.5 ± 5.9	47.7 ± 7.2	39.6 ± 5.3	36.2 ± 4.7	36.3 ± 8.7
Total monounsaturates	51.6 ± 5.6	47.2 ± 4.9	47.6 ± 5.2	37.7 ± 5.5	35.8 ± 8.6
Total polyunsaturates	4.9 ± 2.0	5.2 ± 2.3	12.8 ± 5.5*	26.1 ± 8.6*	31.7 ± 12.4*
Total n-6 polyunsaturates	3.1 ± 1.1	3.3 ± 1.3	2.6 ± 1.9	2.6 ± 0.9	2.7 ± 3.1
Total n-3 polyunsaturates	1.8 ± 0.9	1.9 ± 1.0	10.1 ± 3.6*	23.5 ± 7.7*	29.0 ± 9.3*
n6/n3 ratio	1.7 ± 1.2	1.7 ± 1.3	0.3 ± 0.5*	0.1 ± 0.1*	0.1 ± 0.3*

¹ Values are percentages of total fatty acids and are expressed as mean ± SD, n = 3 replicates in triplicate.

² ND, not detected

³ Asterisk (*) indicates statistically different than control (P < 0.05) as determined by one-way ANOVA for effect of treatment.

3.4.2 Both EPA and DHA increase adiponectin concentration human adipocyte medium

The concentration of adiponectin protein in the cell medium significantly increased over time (from 12 to 48 h) in all treatment groups (**Fig. 3.1A**, statistics not shown for clarity).

Compared to control, secreted adiponectin protein was increased ($P < 0.05$) by EPA at 24 h, 36 h and 48 h by 44%, 86% and 88%, respectively (**Fig. 3.2A**). Adiponectin concentration in the medium was also increased ($P < 0.05$) by DHA at 36 h and 48 h by 39% and 47%, respectively (**Fig. 3.2A**). It is noteworthy that EPA increased secreted adiponectin protein to a greater extent than DHA at 36 h (+47%, $P < 0.05$) and also at 48 h (+41%, $P = 0.09$). In contrast, treatment with either palmitic acid or the combination of EPA plus DHA, did not significantly affect the concentration of adiponectin in the medium (**Fig. 3.2A**). HMW adiponectin secretion exhibited similar trends to total adiponectin in response to the fatty acid treatments (**Fig 3.2B**).

A **B**

Figure 3.2: Effect of fatty acids on (A) total and (B) high molecular weight (HMW) adiponectin protein secreted from cultured human adipocytes. Cells were treated with 100 $\mu\text{mol/l}$ of palmitic acid, EPA, DHA, or 100 $\mu\text{mol/l}$ EPA plus 100 $\mu\text{mol/l}$ DHA, all complexed with bovine serum albumin (BSA; 5:1 ratio) for a total of 48 h with medium samples taken every 12 h. BSA alone acted as the control condition. Total adiponectin values were log transformed before statistical analysis and are presented as geometric means $\pm$ pooled s.e.m., $n = 3$ replicates in triplicate. HMW values are means $\pm$ s.e.m., $n = 3$ replicates. Secreted adiponectin protein increased over time in all treatment groups (statistics not shown for clarity). Statistical significance is represented as $*P < 0.05$ or $**P < 0.01$. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

3.4.3 EPA, but not DHA, increases cellular adiponectin protein content in human adipocytes

At 48 h, EPA increased ($P < 0.05$) cellular adiponectin protein content by 136% compared to control (**Fig. 3.3A**). Interestingly, although DHA increased secreted adiponectin protein (Fig. 3.2A,B), it did not affect cellular adiponectin protein (Fig. 3.3A). Similarly, neither palmitic acid nor the combination of EPA and DHA significantly affected the cellular content of adiponectin protein at 48 h (Fig. 3.3A). Once more, the effects of the fatty acids on HMW adiponectin protein content mirrored that of total adiponectin protein content (**Fig 3.3B**).

A

B

Figure 3.3: Effect of fatty acids on (A) total and (B) high molecular weight (HMW) cellular adiponectin protein content in cultured human adipocytes. Cells were treated with 100 $\mu\text{mol/l}$ of palmitic acid, EPA, DHA, or 100 $\mu\text{mol/l}$ EPA and 100 $\mu\text{mol/l}$ DHA, all complexed with bovine serum albumin (BSA; 5:1 ratio) for 48 h. BSA alone acted as the control condition. Values are means $\pm$ s.e.m., $n = 3$ replicates in triplicate for total and $n = 3$ replicates for HMW. Statistical significance is represented as * $P < 0.05$ or ** $P < 0.01$. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

3.4.4 PPAR γ antagonism inhibits n-3 PUFA-induced increase in adiponectin, but to varying degrees

To determine if EPA and DHA increase adiponectin via a PPAR γ -dependent mechanism, human adipocytes were incubated with a PPAR γ antagonist (BADGE) alone or in combination with EPA or DHA. BADGE completely attenuated the DHA-mediated increase in secreted adiponectin protein, but only partially attenuated the EPA-mediated increase (**Fig. 3.4A**). However, the EPA-mediated increase in cellular adiponectin protein content was completely abolished in the presence of BADGE (**Fig. 3.4B**). BADGE, alone or in combination with DHA, had no effect on cellular adiponectin protein content (Fig. 3.4B).

Figure 3.4: Effect of peroxisome proliferator-activated receptor- γ (PPAR γ) antagonism on the fatty acid-mediated regulation of (A) secreted and (B) cellular adiponectin protein in cultured human adipocytes. Cells were treated with 100 μ mol/l BADGE (a PPAR γ antagonist) alone or in combination with 100 μ mol/l of EPA or DHA for 48 h. Bovine serum albumin alone acted as the control condition. Secreted adiponectin values are means $\pm$ S.E.M., $n = 3$ replicates in triplicate.

Cellular adiponectin protein values were log transformed before statistical analysis and are presented as geometric means $\pm$ pooled S.E.M., $n = 3$ replicates in triplicate for total and $n = 3$ replicates for high molecular weight. Statistical significance is represented as $*P < 0.05$ or $**P < 0.01$. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

3.4.5 Rosiglitazone, a PPAR γ agonist, increases both secreted and cellular adiponectin protein in human adipocytes

To further examine the regulation of adiponectin by PPAR γ , human adipocytes were treated with rosiglitazone, a known PPAR γ agonist. Rosiglitazone increased ($P < 0.001$) secreted adiponectin protein by 79% and 90% at 24 h and 48 h, respectively (**Fig. 3.5A**). Rosiglitazone also increased ($P < 0.001$) the cellular protein content of adiponectin by 582% at 48 h (**Fig. 3.5B**).

3.4.6 EPA plus rosiglitazone elicits additive effects on adiponectin secretion from human adipocytes

To determine if two different classes of PPAR γ agonists (n-3 PUFA and TZD) have an additive effect on adiponectin, adipocytes were treated with rosiglitazone alone or in combination with either EPA or DHA. The combined treatment of rosiglitazone and EPA was additive, increasing the concentration of adiponectin in the medium to a greater extent (+230% at 48 h; $P < 0.001$) than either rosiglitazone (124%, $P < 0.001$) or EPA (121%, $P < 0.001$) alone (**Fig. 3.5A**). Compared to DHA alone, the combined treatment of rosiglitazone and DHA also increased the concentration of adiponectin in the medium (74% at 48 h, $P < 0.001$), but this was not significantly different than rosiglitazone on its own (Fig. 3.5A, $P = 0.228$). Combining

rosiglitazone with either EPA or DHA failed to elicit an additive effect on cellular adiponectin protein (**Fig. 3.5B**).

Figure 3.5: Effect of rosiglitazone (a peroxisome proliferator-activated receptor γ (PPAR γ) agonist) alone or in combination with EPA or DHA on (a) secreted and (b) cellular adiponectin protein cultured human adipocytes. Cells were treated with 10 nmol/l rosiglitazone (a PPAR γ agonist) alone or in combination with 100 μ mol/l of EPA or DHA for 48 h. Bovine serum albumin alone acted as the control condition. Secreted adiponectin values are means $\pm$ S.E.M., n

= 3 replicates in triplicate. Cellular adiponectin protein values were log transformed before statistical analysis and are presented as geometric means $\pm$ pooled s.e.m., n = 3 replicates in triplicate. Statistical significance is represented as *P < 0.05 or **P < 0.01. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

3.5 Discussion

A reduced adiponectin concentration in obesity is likely to be an important contributing factor to the various pathologies related to this state. Consequently, nutritional and pharmaceutical strategies to increase adiponectin are of great interest. Our study is the first to investigate the effects of n-3 PUFA on adiponectin production and secretion in a human model of cultured adipocytes. We demonstrate in human adipocytes that EPA and DHA, as well as rosiglitazone, increase the secretion of adiponectin. Importantly, we show for the first time that the stimulatory effects of EPA and rosiglitazone on adiponectin secretion from human adipocytes are additive, indicating that this may be an extremely valuable therapeutic strategy.

3.5.1 EPA and rosiglitazone increase adiponectin in an additive manner.

Our findings are consistent with those of Kuda et al. (80) who reported an additive effect of rosiglitazone and a long-chain n-3 PUFA concentrate (46% DHA, 14% EPA) on plasma adiponectin levels in mice. It is well established that rosiglitazone, a PPAR γ agonist, increases plasma concentrations of adiponectin *in vivo* (103); however, much less is known about the ability of rosiglitazone to stimulate adiponectin in adipocytes in culture. Our results are consistent with findings in murine 3T3-L1 adipocytes (178), but contradict observations that rosiglitazone induces an increase in adiponectin secreted from human omental, but not

subcutaneous adipocytes (107). In Motoshima et al. subcutaneous adipocytes were obtained from morbidly obese donors (mean BMI of 40.6 kg/m²), whereas the adipocytes used in the current study were obtained from an overweight population (mean BMI of 27.9 kg/m²) (107). This difference in degree of overweight or obesity and possible differences in the adipocytes themselves may account for the discrepancies between the studies, but this requires further study.

3.5.2 EPA and DHA increase adiponectin partially and completely via a PPAR γ -dependent mechanism, respectively.

The combination of DHA and rosiglitazone did not increase secreted adiponectin concentration to a greater extent than DHA on its own. The disparity between the effects of EPA and DHA on adiponectin might be explained by the involvement of PPAR γ . To elucidate this as a possible mechanism, we combined EPA or DHA with BADGE, a PPAR γ antagonist with a dissociation constant (K_d) of 100 μ M (186). In the presence of BADGE, the EPA-mediated increase in adiponectin secretion was only partially attenuated, whereas the DHA-mediated increase in adiponectin secretion was completely abolished. This suggests that both EPA and DHA elicit their effects on adiponectin at least partially via PPAR γ , which is consistent with the observations of Neschen et al (113), as well as with our own work in murine adipocytes (119). Overall, our results suggest that rosiglitazone and DHA upregulate adiponectin secretion exclusively via PPAR γ activation, whereas EPA likely mediates adiponectin through a combination of PPAR γ activation and an unknown additional mechanism. Taken together, it seems that two different PPAR γ agonists (n-3 PUFA and TZD) may work additively.

3.5.3 EPA and DHA increase adiponectin secretion, and EPA (but not DHA) increases cellular adiponectin protein content.

Our finding that long-chain n-3 PUFA upregulated adiponectin in human adipocytes is consistent with those of Banga et al. who observed an increase in adiponectin secretion from rat adipocytes treated overnight with 25 μ M EPA or DHA (9). Additionally, Yamazaki et al. demonstrated that 10, 30 and 50 μ M of DHA enhanced the phenolamide-induced increase in adiponectin from human preadipocytes over 14 d; however, the effect of DHA on adiponectin in the absence of phenolamide treatment was not examined in this study (195). In contrast, Lorente-Cebrian et al. (95) found decreased adiponectin secretion in response to 96 h treatment with 100 and 200 μ M of EPA in rat adipocytes, while Itoh et al. (64) found no effect of 200 μ M EPA on adiponectin secretion from 3T3-L1 adipocytes at 48 h. Our pilot work (Figure 3.2) showed that the effect of fatty acids on adiponectin is dependent on both fatty acid dose and length of exposure, factors that may account for the array of inconsistencies in reported findings.

Adipocytes incubated with EPA, DHA, and their combination contained significantly more of each respective fatty acid, compared to control. Since EPA-treated adipocytes also contained a discernible increase in the EPA derivative, docosapentaenoic acid (DPA), it is possible that observed changes in adiponectin may be partly attributable to DPA, although this requires further study. Also, the percent composition of EPA was markedly lower than DHA (**Table 1**), possibly attributable to oxidation or lower half-life relative to DHA. Despite this, EPA had a greater impact on adiponectin secretion and cellular protein content, suggesting that EPA is more potent than DHA. Unlike EPA and DHA, adipocytes treated with palmitic acid did not contain higher amounts of this fatty acid compared to control. It is possible that since adipocytes are already enriched in palmitic acid, further increases in this saturated fatty acid are

difficult to attain. The lack of palmitic acid incorporation into adipocytes *in vitro* may help to explain why this fatty acid had no effect on adiponectin protein content compared to control.

One unexpected, but interesting finding was that DHA increased secreted adiponectin concentration, but had no effect on cellular adiponectin protein content. Recently, Wang et al. (179) showed that adiponectin is confined to the cell by chaperone proteins in the endoplasmic reticulum and that its secretion is highly dependent on the posttranslational modification of adiponectin. It has also been shown that TZDs enhance the secretion of adiponectin by upregulating endoplasmic reticulum oxidoreductase 1-L α , a molecular chaperone protein responsible adiponectin release from the cell (181). Therefore, it is possible that DHA may also act to facilitate the appropriate posttranslational modifications necessary for the release of adiponectin from the cell in the absence of any increase in cellular protein content. Alternatively, we must acknowledge the possibility that DHA did increase cellular protein content, but that this occurred transiently and prior to 48 hours when the cells were lysed.

The differential effects of EPA and DHA on cellular adiponectin protein further emphasize the importance of studying each of these fatty acids on their own, as their mechanisms of action appear to differ. Until recently, many studies examining the effects of n-3 PUFA on adiponectin regulation have used fish oil or a combination of EPA and DHA (78, 80, 113), and therefore have failed to distinguish which specific n-3 PUFA is responsible for an observed effect. By examining EPA and DHA separately, we showed firstly that the long-chain n-3 PUFA are only partially regulated by a common mechanism, and secondly that EPA is a more potent inducer of adiponectin than DHA.

Surprisingly, although separately EPA and DHA increased secreted adiponectin protein, the combination of EPA plus DHA failed to elicit such an effect. It is important to recognize that

EPA and DHA together provided a total of 200 μM of fatty acids, whereas EPA or DHA on its own provided only 100 μM of fatty acid. Therefore, it is plausible that the combination of EPA and DHA may have elicited a toxic effect on the cells that might explain the failure of this treatment to increase adiponectin. Furthermore, it has been demonstrated that elevated levels of fatty acids can result in oxidative stress in cultured adipocytes (47), which is associated with dysregulated secretion of adiponectin (150). Therefore, the exposure to higher fatty acid concentrations may have disrupted normal adipocyte function, resulting in an inability of the n-3 PUFA to stimulate adiponectin secretion.

3.5. Summary and perspectives

In conclusion, we found that EPA and DHA increase the amount of adiponectin secreted from human adipocytes, *in vitro*, and that EPA elicits this effect only partly via PPAR γ whereas DHA elicits this effect exclusively via PPAR γ . Additionally, we demonstrated that combining an n-3 PUFA (EPA) with a TZD used in type 2 diabetes management (rosiglitazone), increases adiponectin in an additive manner. Together, these results suggest that long-chain n-3 PUFA, alone or in combination with a pharmaceutical agent, represent a potential treatment strategy to upregulate adiponectin, an anti-inflammatory and insulin-sensitizing adipokine that is classically downregulated in obesity and related diseases.

Chapter 4: Fish oil prevents high saturated fat diet-induced impairments in adiponectin and insulin response in rodent skeletal muscle.

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4.1 Abstract

High saturated fatty acid (SFA) diets contribute to the development of insulin resistance, whereas fish oil-derived n-3 polyunsaturated fatty acids (PUFA) increase the secretion of adiponectin, an adipocyte-derived protein that stimulates fatty acid oxidation (FAO) and improves skeletal muscle insulin response. We sought to determine if fish oil could prevent and/or restore high SFA diet-induced impairments in adiponectin and insulin response in soleus muscle. Sprague-Dawley rats were fed: (1) low fat control diet (CON), (2) high SFA diet (SFA) or (3) high SFA with n-3 PUFA diet (SFA/n-3 PUFA). At 4 weeks, CON and SFA/n-3 PUFA animals were terminated, and SFA animals were either terminated or fed SFA or SFA/n-3 PUFA for an additional 2 or 4 wk. The effect of diet on adiponectin-stimulated FAO, insulin-stimulated glucose transport, and expression of adiponectin, insulin and inflammatory signalling proteins was determined in soleus muscle. Adiponectin stimulated FAO in CON and 4wk SFA/n-3 PUFA (+36%, +39%, respectively $p \leq 0.05$) only. Insulin increased glucose transport in CON, 4wk SFA/n-3 PUFA, and 4wk SFA + 4wk SFA/n-3 PUFA (+82%, +33%, +25%, respectively $p \leq 0.05$); this effect was lost in all other groups. TLR4 expression was increased with 4wk of SFA feeding (+24%, $p \leq 0.05$) and this was prevented in 4wk SFA/n-3 PUFA. SOCS3

expression was increased in SFA and SFA/n-3 PUFA (+33, +18%, respectively $p \leq 0.05$). Our results demonstrate that fish oil can prevent high SFA diet-induced impairments in both adiponectin and insulin response in soleus muscle.

4.2 Introduction

Adiponectin, a 30 kDa adipokine secreted almost exclusively from adipose tissue (AT), is known to exert insulin-sensitizing effects that are largely attributed to its role in glucose and lipid metabolism in skeletal muscle and liver (68). The consumption of a diet high in saturated fatty acids (SFA), results in a marked decrease in circulating adiponectin concentrations, which may contribute to impairments in fatty acid metabolism and insulin responsiveness (68). In contrast, fish oil-derived n-3 polyunsaturated fatty acids (PUFA) have been shown to stimulate adiponectin secretion (119), which may serve as an underlying mechanism by which dietary n-3 PUFA can improve insulin sensitivity (4, 84, 155).

High SFA diets have been shown to activate inflammatory pathways that interfere with insulin signalling (144). SFA act as ligands for Toll-like receptor (TLR)4, which initiates a downstream signalling cascade resulting in the activation of nuclear factor (NF)- κ B and upregulation of inflammatory gene expression (144). Importantly, the deletion of TLR4 in skeletal muscle has been demonstrated to protect against high SFA diet-induced insulin resistance, emphasizing the important connection between inflammation and insulin signalling (131). Fish oil-derived n-3 PUFA are thought to elicit their anti-inflammatory effects in part by downregulating NF- κ B (102); however, the effects of n-3 PUFA on the expression of inflammatory signalling mediators in soleus muscle has yet to be examined.

In addition to dysregulated adipokine synthesis and secretion, research suggests that tissue response to adiponectin may also be impaired in obesity (36). Both adiponectin and leptin are known to stimulate fatty acid oxidation (FAO) in rodent and human skeletal muscle (36). However, this effect is blunted in an obese state (16, 154), which may contribute to the accumulation of skeletal muscle lipids that are known to interfere with insulin-stimulated glucose uptake (37). Additionally, Mullen et al. recently demonstrated that adiponectin resistance not only develops rapidly in response to a high SFA diet, but also precedes both the accumulation of muscle lipid species and development of insulin resistance in soleus muscle (108). Interestingly, while fish oil-derived n-3 PUFA have been shown to stimulate adiponectin secretion (119), their effects on the response of skeletal muscle to adiponectin, as well as any associated functional effects on insulin-stimulated glucose transport, are currently unknown.

Our objectives were to determine (1) if fish oil-derived n-3 PUFA could prevent and/or restore high SFA diet-induced impairments in adiponectin-stimulated FAO and maximal insulin-stimulated glucose transport in rodent soleus muscle, and (2) the potential mechanisms by which fish oil exerts these effects. We hypothesized that (1) fish oil-derived n-3 PUFA would prevent and restore high SFA diet-induced impairments in both adiponectin response and insulin response, and (2) fish oil-derived n-3 PUFA would mediate these effects via reduced activation of inflammatory pathways.

4.3 Methods

4.3.1 Animals and diets

Female Sprague-Dawley rats were purchased from Charles River (Charles River Laboratories, St-Constant, QC, Canada) at approximately 50 days of age and weighed 151 ± 0.67 g upon arrival. The animals were housed in groups of 6 with a 12:12 h reverse light dark cycle and allowed unlimited access to water. Rats were fed a standard Teklad (Harlan Laboratories, Mississauga, ON, Canada) rodent chow diet for 3 d, at which point they were randomly assigned to receive (1) low fat control diet (6% kcal from soybean oil, 4% kcal from lard, 4wk CON), (2) high SFA diet (55% kcal from lard, 5% kcal from soybean oil, 4wk SFA) known to induce both adiponectin resistance and insulin resistance (109), or (3) high SFA with n-3 PUFA diet (40% kcal from lard, 15% kcal from menhaden oil, 5% kcal from soybean oil, 4wk SFA/n-3 PUFA) for a period of 4 weeks (**Table 4.1**). The SFA/n-3 diet contained a total of 7.9% long-chain n-3 PUFA in the form of eicosapentaenoic acid (4.1%), docosapentaenoic acid (0.8%), and docosahexaenoic acid (3.0%) (**Table 4.2**). At 4 wk, CON and SFA/n-3 PUFA animals were terminated, and SFA animals were either terminated or fed SFA or SFA/n-3 PUFA for an additional 2 (6wk SFA; 4wk SFA + 2wk SFA/n-3 PUFA) or 4 (8wk SFA; 4wk SFA + 4wk SFA/n-3 PUFA) wk. All diets were purchased from Research Diets (Research Diets Inc., New Brunswick, NJ, USA), stored at -20 °C, and administered fresh daily to the animals *ad libitum*. Body mass measurements were taken twice per week, and the animals were fasted overnight prior to experiments. Approval for all experimental procedures was obtained from the University of Guelph Animal Care Committee (Guelph, ON, Canada).

Table 4.1: Composition of Experimental Diets

	CON	SFA	SFA/n-3 PUFA
	kcal %	kcal %	kcal %
Protein	20	20	20
Carbohydrate	70	20	20
Fat	10	60	60
Total	100	100	100
kcal/g			
	kcal	kcal	kcal
Casein, 80 Mesh	800	800	800
L-Cysteine	12	12	12
Corn Starch	1260	0	0
Maltodextrin 10	140	500	500
Sucrose	1400	275.2	275.2
Cellulose BW200	0	0	0
Soybean Oil	225	225	225
Lard	180	2205	1593
Menhaden Oil	0	0	612
Mineral Mix, S10026	0	0	0
Dicalcium Phosphate	0	0	0
Calcium Carbonate	0	0	0
Potassium Citrate, 1 H₂O	0	0	0
Vitamin Mix, V10001	40	40	40
Choline Bitartrate	0	0	0
Total	4057	4057	4057

Table 4.2: Fatty Acid Composition of Experimental Diets

Fatty Acid	Percentage of Total Fatty Acids		
	CON	SFA	SFA/n-3 PUFA
C14:0	0.4	0.9	2.8
C14:1	0.2	0.5	0.3
C16:0	16.8	23.2	21.2
C16:1	1.8	3.7	5.5
C18:0	8.4	13.2	10.4
C18:1 n-9	32.9	41.9	33.5
C18:2 n-6	33.9	13.3	12.0
C18:3	4.8	1.7	2.3
C18:4			1.3
C20:0			0.1
C20:1			0.4
C20:4 n-6	0.8	1.6	1.8
C20:5 n-3			4.1
C22:0			0.1
C22:1			0.1
C22:4			0.1
C22:5 n-3			0.8
C22:6 n-3			3.0
C24:0			0.2
Saturated	25.6	37.3	34.8
Monounsaturated	34.9	46.1	39.8
n-6 Polyunsaturated	34.7	14.9	13.8
n-3 Polyunsaturated	4.8	1.7	10.2

4.3.2 Tissue and blood sampling

Rats were anaesthetized by an intraperitoneal injection with sodium pentobarbital at a dose of 6 mg per 100 g body mass. Intact soleus muscle was isolated and stripped longitudinally from tendon to tendon into 3 strips of approximate equal size. The outside 2 strips were used for either glucose transport or FAO assays and the remaining inside strip was used for insulin-stimulated signalling incubations or frozen immediately in liquid nitrogen for subsequent total protein analysis. Blood was obtained by cardiac puncture, transferred to heparinized tubes and centrifuged (10,000 x g, 5 min, 4 °C) to isolate plasma for analysis of fasting insulin (Rat Insulin RIA kit, Millipore, Billerica, MA, USA), free fatty acids (Wako Chemicals Inc., Richmond, VA, USA), and adiponectin (Rat Adiponectin ELISA, Millipore, Billerica, MA, USA). Fasting plasma interleukin (IL)-6, leptin, monocyte chemoattractant protein (MCP)-1, and tumour necrosis factor (TNF)- α (Rat Cytokine/Chemokine Milliplex kit, Millipore, Billerica, MA, USA) were analyzed by Luminex xMAP technology (Bioplex-200, Bio-Rad Laboratories, Mississauga, ON, Canada). Fasting whole blood glucose values were obtained using a OneTouch Ultra2 glucometer (LifeScan Inc., Milpitas, CA, USA).

4.3.3 Fatty acid oxidation

Soleus strips were incubated in 2 mL of pregassed (95% O₂-5% CO₂) Krebs-Henseleit buffer (KHB), 4% BSA, at 30 °C, with 1 mM palmitate and 5 mM glucose in a gentle shaking water bath for 30 min to equilibrate. Soleus strips were then incubated for an additional 60 min, in the presence or absence of recombinant globular adiponectin (2.5 μ g/mL, Peprotech, Rocky Hill, NJ, USA) with the addition of 0.5 μ Ci/ml [1-¹⁴C]palmitate (Amersham, Oakville, ON, Canada) to determine palmitate oxidation as previously outlined in detail (38). In brief, after the incubations the soleus strips were blotted, tendons removed, weighed, and placed in a 14 mL

tubes containing 5 mL of cold 2:1 chloroform methanol. Muscles were homogenized using a polytron (PT1200, Brinkman Institute, Mississauga, ON, Canada) and centrifuged (10,000 x g, 10 min, 4 °C). The supernatant was extracted and combined with 2 mL of water, shaken for 10 min, and centrifuged (10,000 x g, 10 min, 4 °C) to isolate the aqueous phase. The amount of ^{14}C in 1 mL of the aqueous phase was quantified by liquid scintillation counting. Additionally, the $^{14}\text{CO}_2$ remaining in the buffer was released by transferring 1 mL of buffer into a sealed flask, acidifying the buffer with 1 mL of 1 M sulfuric acid and trapping it in 250 μL benzethonium hydroxide contained in a microcentrifuge tube suspended in the sealed flask. The tubes containing benzethonium hydroxide and the trapped $^{14}\text{CO}_2$ were quantified using liquid scintillation counting. Total palmitate oxidation was determined by summing the aqueous and gaseous ^{14}C -labelled oxidation intermediates.

4.3.4 Glucose transport.

Soleus strips were incubated in 2 mL of pregassed (95% O_2 -5% CO_2) KHB (0.1% BSA, 30 °C) containing 8 mM glucose and 32 mM mannitol, for 30 min in the absence or presence of insulin (10 mU/mL) in a gently shaking water bath to equilibrate. The strips were washed twice in 2 mL glucose-free KHB (4 mM pyruvate, 36 mM mannitol) for 10 min each, and then incubated for 40 min (basal) or 20 min (insulin-stimulated) in KHB (4 mM pyruvate, 8 mM 3-*O*-[^3H]methyl-D-glucose, 28 mM [^{14}C]mannitol). Strips were blotted, tendons removed, weighed and then digested in 1 mL sodium hydroxide for 10 min at 95 °C. 200 μL of muscle digest was sampled and quantified by liquid scintillation counting, and glucose transport was determined from intracellular 3-*O*-[^3H]methyl-D-glucose as previously described (174).

4.3.5 Insulin-stimulated signalling proteins.

Soleus strips were incubated in 2 mL of pregassed (95% O₂-5% CO₂) KHB (0.1% BSA, 30 °C) containing 8 mM glucose and 32 mM mannitol, for 30 min to equilibrate. Strips were then incubated in the same KHB for a subsequent 10 min in the presence of 10 mU/mL insulin and immediately frozen in liquid nitrogen for subsequent western blotting analysis.

4.3.6 Western blot analysis.

Soleus muscle strips were homogenized in ice cold buffer suitable for whole cell protein extraction, sonicated for 5 sec, and then centrifuged (1500 x g, 15 min, 4 °C) to isolate the supernatant. Total protein was quantified using a bicinchoninic acid protein assay (Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer's instructions. Whole tissue lysate protein (50 µg) was solubilized in 4x Laemmli's buffer, boiled (5 min, 95 °C), resolved by SDS-PAGE, and wet transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature, washed, and then incubated overnight at 4 °C with specific primary antibodies for total Akt (Upstate, Billerica, MA, USA), Thr³⁰⁸ phosphorylated Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA), adaptor protein phosphotyrosine interaction pH domain and leucine zipper containing 1 (APPL1), 5' AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), TLR4, total and Ser³² phosphorylated IκBα, total and Ser⁵³⁶ phosphorylated NF-κB, and suppressor of cytokine signalling (SOCS)-3 (see Appendix A for detailed western blotting conditions). All antibodies were purchased from Cell Signalling (Danvers, MA, USA) unless otherwise stated. The immune complexes were washed, incubated with the appropriate secondary antibody for 1 h at room temperature, detected using the enhanced chemiluminescence method (Syngene Chemigenius2; PerkinElmer, Waltham, MA,

USA) and then quantified with densitometry (Gene Tools software, PerkinElmer). Equal loading was confirmed by probing for α -tubulin (Abcam Cambridge, MA) and using nonspecific protein staining with Ponceau-S (Sigma Aldrich, Oakville, ON, Canada).

4.3.7 Statistical analysis.

Body mass, blood measurements, and protein expression data were analyzed using a one-way ANOVA. FAO and glucose transport data was analyzed using a two-way repeated measures ANOVA for dietary treatment and time. A Tukey post-hoc test was used to identify significant differences between diet treatments within each time point. All data are expressed as mean $\pm$ SEM. Statistical significance was set at $p \leq 0.05$ and all statistical analysis was performed using Sigma Stat version 2.03.

4.4 Results

4.4.1 Body mass and bloods.

Initial body mass did not differ between any of the groups (**Table 4.3**). Terminal body mass was higher (+18%, $p \leq 0.05$) in 4wk SFA compared to CON (Table 4.3). Fasting whole blood glucose, plasma insulin, plasma free fatty acids, and plasma TNF- α did not differ in any of the groups (Table 4.3). Fasting plasma adiponectin was elevated in 4wk SFA/n-3 PUFA compared to both CON (+38%, $p \leq 0.05$) and 4wk SFA (+58%, $p \leq 0.05$) (Table 4.3). Fasting plasma leptin and MCP-1 levels were increased in 4wk SFA (+74% and +23%, respectively $p \leq 0.05$) and 4wk SFA/n-3 PUFA (+65%, +23%, respectively $p \leq 0.05$) (Table 4.3) compared to

CON. Fasting plasma IL-6 levels were elevated in 4wk SFA (+30%, $p \leq 0.05$) compared to CON, but this increase was not seen in 4wk SFA/n-3 PUFA (Table 4.3).

Table 4.3: Body Mass and Blood Measurements

	4wk CON	4wk SFA	4wk SFA/n-3 PUFA	6wk SFA	4wk SFA + 2wk SFA/n-3 PUFA	8wk SFA	4wk SFA + 4wk SFA/n-3 PUFA
Arrival body mass (g)	153.1 ± 1.5	153.0 ± 2.1	153.2 ± 1.9	151.3 ± 1.9	149.2 ± 1.8	149.4 ± 1.8	150.3 ± 2.4
Terminal body mass (g)	254.8 ± 9.0	299.9 ± 10.5*	289.5 ± 7.7	330.3 ± 6.4	337.3 ± 10.2	345.8 ± 7.5	342.4 ± 9.6
Fasting blood glucose (mmol/L)	8.9 ± 0.3	9.8 ± 0.4	9.9 ± 0.4	8.8 ± 0.2	9.3 ± 0.3	8.5 ± 0.2	8.3 ± 0.3
Fasting plasma insulin (ng/ml)	5.2 ± 0.7	6.2 ± 0.5	4.8 ± 0.8	5.2 ± 0.6	4.3 ± 0.6	5.7 ± 0.5	4.3 ± 0.5
Fasting plasma free fatty acids (mmol/L)	0.64 ± 0.04	0.70 ± 0.04	0.72 ± 0.04	0.74 ± 0.05	0.74 ± 0.06	0.84 ± 0.07	0.88 ± 0.06
Fasting plasma adiponectin (µg/ml)	9.5 ± 0.4	8.3 ± 0.4	13.1 ± 0.6**	8.4 ± 0.7	8.5 ± 0.5	8.2 ± 0.6	9.6 ± 0.4
Fasting plasma IL-6 (pg/mL)	13.4 ± 0.7	17.4 ± 0.7*	14.3 ± 0.7	19.6 ± 0.9	17.5 ± 0.7	17.8 ± 0.8	15.2 ± 0.6
Fasting plasma leptin (ng/mL)	3.1 ± 0.07	5.4 ± 0.4*	5.1 ± 0.1*	5.9 ± 0.2	5.9 ± 0.2	6.6 ± 0.4	6.1 ± 0.3
Fasting plasma MCP-1 (pg/mL)	22.8 ± 0.6	28.0 ± 1.3*	28.1 ± 1.3*	27.7 ± 1.0	26.7 ± 0.9	25.7 ± 1.0	27.1 ± 0.8
Fasting plasma TNF-α (pg/mL)	24.3 ± 0.7	24.0 ± 0.8	25.3 ± 0.7	23.8 ± 0.7	23.3 ± 0.5	21.7 ± 0.6	22.4 ± 0.7

Data are mean ± SEM, n = 10-12 * = significantly different from CON within the same time point, $p \leq 0.05$; ** = significantly different from SFA within the same time point, $p \leq 0.05$.

4.4.2 Fatty acid oxidation.

In CON, globular adiponectin increased FAO compared to the basal condition (+36%, $P \leq 0.05$) (**Fig. 4.1**). The stimulatory effect of adiponectin was preserved in 4wk SFA/n-3 PUFA (+39%, $P \leq 0.05$) but completely attenuated in all other groups (Fig. 4.1). There were no differences in either basal or adiponectin-stimulated FAO between diet groups for a given time point (Fig. 4.1).

Figure 4.1: Basal and globular adiponectin-stimulated palmitate oxidation in soleus muscle.

Values are expressed as means $\pm$ SE; $n = 12$. *Significantly different from basal, $P \leq 0.05$. Gray bars denote basal, while solid bars denote globular adiponectin-stimulated (2.5 $\mu\text{g/mL}$). CON, control diet; SFA, high saturated fatty acid diet; SFA/n-3 PUFA, high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

4.4.3 Glucose transport.

Insulin increased glucose transport compared to basal in CON (+82%, $P \leq 0.05$), 4wk SFA/n-3 PUFA (+33%, $P \leq 0.05$), and 4wk SFA + 4wk SFA/n-3 PUFA (+25%, $P \leq 0.05$) (**Fig. 4.2**). Insulin's stimulatory effect was lost in all other diet groups (Fig. 4.2). There were no differences in either basal or insulin-stimulated glucose transport between diet groups for a given time point (Fig. 4.2).

Figure 4.2: Basal and insulin-stimulated glucose transport in soleus muscle. Values are expressed as means $\pm$ SE; $n = 12$. *Significantly different from basal; $P \leq 0.05$. Gray bars denote basal, while solid bars denote insulin-stimulated (10 mU/mL). CON, control diet; SFA, high saturated fatty acid diet; SFA/n-3 PUFA, high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

4.4.4 Adiponectin signalling proteins.

There was no significant effect of diet on the total protein content of APPL1 (Fig. 4.3a), AMPK (Fig 4.3b), or ACC (Fig. 4.3c).

Figure 4.3: The effect of high-SFA diet and fish oil supplementation on total protein content of adiponectin signalling intermediates in soleus muscle. A) APPL1, B) AMPK, and C) ACC.

Alpha-tubulin was used as a loading control. Values are expressed as means $\pm$ SE; n = 12. CON, control diet; SFA, high saturated fatty acid diet; SFA/n-3 PUFA, high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

4.4.5 Insulin signalling proteins.

There was no significant effect of diet on the total protein content of Akt (**Fig. 4.4a**). Thr³⁰⁸ phosphorylation of Akt was impaired (-29%, $P \leq 0.05$) in response to 10 min of insulin exposure in 4wk SFA compared to CON, and this impairment was not seen in 4wk SFA/n-3 PUFA (**Fig. 4.4b**).

Figure 4.4: The effect of high SFA diet and fish oil supplementation on total protein content of insulin signalling intermediates in soleus muscle. A) Total Akt, and B) Acute effect of 10-min insulin exposure (10 mU/mL) on protein content of Thr³⁰⁸-phosphorylated Akt. Alpha tubulin was used as a loading control. Values are means $\pm$ SE; n = 12. *Significantly different from CON, $P \leq 0.05$. CON, control diet; SFA, high saturated fatty acid diet; SFA/n-3 PUFA, high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

4.4.6 Inflammatory signalling proteins.

TLR4 protein expression in soleus muscle was elevated in 4wk SFA compared to CON (+24%, $p \leq 0.05$) (**Fig. 4.5a**). There was no significant effect of diet on total protein content or phosphorylation of I κ B α (**Fig. 4.5b,c**) or NF- κ B (**Fig. 4.5d,e**). SOCS3 protein content was increased in both 4wk SFA and 4wk SFA/n-3 PUFA relative to CON (+33%, +18%, respectively $P \leq 0.05$) (**Fig. 4.5f**).

Figure 4.5: The effect of high SFA diets and fish oil supplementation on total protein content of inflammatory signalling intermediates in soleus muscle. A) TLR4, B) IκBα, C) Ser³² phosphorylated IκBα, D) NF-κB, E) Ser⁵³⁶ phosphorylated NF-κB, and F) SOCS3. Alpha-tubulin was used as a loading control. Values are means ± SE; n = 12. *Significantly different from CON, $P \leq 0.05$. CON, control diet; SFA, high saturated fatty acid diet; SFA/n-3 PUFA, high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

4.5 Discussion

This study is the first to demonstrate that the partial substitution of long-chain n-3 PUFA into a high SFA diet can modulate the response of rodent soleus muscle, an important tissue in the regulation of insulin sensitivity, to adiponectin. Specifically, we demonstrate for the first time that fish oil-derived n-3 PUFA can prevent, but not restore, impairments in adiponectin-stimulated FAO, and both prevent and restore impairments in insulin-stimulated glucose

transport, in response to a high SFA diet. In addition, protein content of both TLR4 and SOCS3 was increased in soleus muscle in response to the high SFA diet, and the increase in TLR4 expression was prevented by the partial substitution of long-chain n-3 PUFA in the diet. Taken together, these results suggest that dietary consumption of fish oil, rich in long-chain n-3 PUFA, confers beneficial effects on lipid metabolism and insulin response in soleus muscle.

4.5.1 n-3 PUFA prevent, but not restore, high SFA diet-induced impairments in soleus muscle adiponectin response.

Recently, Mullen et al. demonstrated that a high SFA diet impairs the acute increase in FAO in isolated soleus muscle in response to globular adiponectin in as little as 3 days (108). In the current study, we show that the high SFA diet-induced impairment in adiponectin response can be prevented, but not restored, by the partial dietary substitution of fish oil. While the inclusion of long-chain n-3 PUFA in the diet successfully prevented the development of adiponectin resistance in soleus muscle, it was unable to restore it. It is possible that if rats had been fed fish oil for longer than 4 weeks, adiponectin responsiveness may have been recovered, but this requires further study. It is also interesting to note that feeding the high SFA diet containing fish oil for 4 weeks significantly increased circulating adiponectin concentrations; however, when fish oil was supplemented into the diet subsequent to an initial 4 weeks of high SFA feeding, no change in circulating adiponectin concentrations was observed. Ultimately, the ability of fish oil to prevent, but not restore, impairments in adiponectin-stimulated FAO suggests that fish oil supplementation may be of greater therapeutic importance as a prevention, rather than treatment, for obesity-induced impairments in soleus muscle lipid metabolism.

4.5.2 n-3 PUFA both prevent and restore high SFA diet-induced impairments in soleus muscle insulin response.

High SFA diets are known to impair insulin response in skeletal muscle (75, 108, 109, 185). In this study, we demonstrate that fish oil can prevent high SFA diet-induced impairments in insulin-stimulated glucose transport in isolated soleus muscle. This observation is consistent with Kim et al. who found that replacing 18% (of a 50% energy high fat diet) of corn oil, high in linoleic acid, with menhaden oil partially prevents high SFA diet-induced blunting in insulin-stimulated glucose transport in both epitrochlearis and soleus muscles (75). Additionally, our findings are supported by several groups (69, 84, 155) who report that fish oil supplementation can prevent high SFA diet-induced skeletal muscle insulin resistance in rodents as assessed by the hyperinsulinemic euglycemic, clamp technique.

Importantly, our study is the first to demonstrate that the SFA diet-induced impairment in insulin response in soleus muscle can be effectively restored with 4 weeks of incorporation of long-chain n-3 PUFA into a high SFA diet. To the best of our knowledge, the ability of fish oil to reverse high SFA diet-induced impairments in insulin response has not been previously examined in skeletal muscle specifically. However, both Muurling et al. (111) and Podolin et al. (128) report that the inclusion of fish oil in a high fat diet is insufficient to reverse whole body insulin resistance as assessed by the hyperinsulinemic euglycemic, clamp technique. The inability of fish oil to reverse whole body insulin resistance in these studies suggests that improvements in muscle insulin response may actually precede changes in systemic insulin sensitivity. In contrast to the aforementioned studies, Kalupahana et al. (69) demonstrated that 5 weeks of EPA supplementation into a high fat diet restored glucose tolerance in mice, suggesting that EPA may be of particular importance in n-3 PUFA-mediated improvements in whole body

insulin sensitivity. Although we observed diet-induced changes in soleus muscle insulin response, fasting glucose and insulin concentrations were unaltered by the diets, suggesting that whole body insulin sensitivity was unchanged. However, it must be recognized that a more sensitive measure of systemic insulin sensitivity (glucose and insulin tolerance tests, and/or a hyperinsulinemic euglycemic clamp) may have revealed diet-induced changes that existed; however, the focus of this study was on the response of soleus muscle to adiponectin and insulin. Furthermore, future work should examine the ability of n-3 PUFA to prevent and restore impairments in insulin response in glycolytic muscle types, such as the extensor digitorum longus, as well.

It is noteworthy that the n-3 PUFA-induced restoration of soleus muscle insulin response occurred independently of a restoration in adiponectin response. This suggests that although high SFA diet-induced impairments in adiponectin-stimulated FAO precede the loss in insulin responsiveness (108), its restoration is not required for the restoration of insulin-stimulated glucose transport by n-3 PUFA. These findings are consistent with Ritchie et al. (134) who report an exercise-induced restoration of insulin-stimulated glucose transport prior to a restoration in leptin-stimulated FAO. Overall, our findings emphasize the importance of considering fish oil-derived long-chain n-3 PUFA as a therapeutic strategy in both the prevention and treatment of soleus muscle insulin resistance.

4.5.3 Total protein content of adiponectin signalling mediators are unaffected by high fat feeding.

Given our findings that dietary fat composition altered adiponectin and insulin responses in soleus muscle, we analyzed the content of several proteins involved in the adiponectin, insulin

and inflammatory signalling cascades to investigate potential underlying mechanisms. Globular adiponectin signals by binding to its receptor, AdipoR1 that then associates with APPL1, thereby increasing FAO (100, 191). Despite diet-induced functional changes in adiponectin-stimulated FAO, we found no effect of diet on the total protein expression of APPL1, AMPK, or ACC. This suggests that high SFA-induced impairments in adiponectin-stimulated FAO are not attributable to changes in total protein content of these adiponectin signalling intermediates and is consistent with previous findings (108, 110). It is possible that the impairments observed with the high SFA diet are a result of changes in the phosphorylated state or cellular location of these proteins, as well as their co-localization with other proteins (176).

4.5.4 n-3 PUFA prevent and restore impairments in insulin-stimulated phosphorylation of Akt in soleus muscle.

Our observation that the Thr³⁰⁸ phosphorylation of Akt is impaired in response to a high SFA diet is consistent with previous findings (108). Diet had no effect on total Akt protein expression, which is also consistent with previous findings (108). Taouis et al. (159) demonstrated that skeletal muscle insulin-stimulated insulin receptor and insulin receptor substrate (IRS)-1 tyrosine phosphorylation was restored in rodents fed a high SFA diet supplemented with fish oil. To the best of our knowledge, the effects of dietary fish oil supplementation into a high SFA diet on the protein expression of insulin signalling mediators downstream of IRS-1 have not been previously examined. In the current study we found that the partial substitution of fish oil into a high SFA diet was able to prevent and restore impairments in phosphorylation of Thr³⁰⁸ Akt induced by the high SFA diet. This finding is consistent with

reports that EPA, one of the bioactive n-3 PUFA in fish oil, stimulates phosphorylation of Akt in 3T3-L1 adipocytes (94), and rodent adipose tissue (85).

4.5.5 n-3 PUFA prevent high SFA diet-induced upregulation of TLR4 protein content in soleus muscle.

Activation of TLR4 by SFA and subsequent stimulation of the NF- κ B inflammatory pathway has been demonstrated in skeletal muscle and is thought to contribute to the development of insulin resistance (131, 144). We observed an increase in soleus muscle TLR4 and SOCS3 protein content with the high SFA diet, which is consistent with several other reports (131, 134, 144, 167, 196). Importantly, we demonstrated for the first time that the high SFA diet-induced upregulation of TLR4 expression in soleus muscle was completely prevented by the partial substitution of SFA with fish oil. This observation is consistent with Lee et al. who reported that saturated, but not unsaturated, fatty acids stimulated TLR4 in macrophages (87). Although there was a trend towards a decrease in the expression of SOCS3 with the partial substitution of n-3 PUFA into the high SFA diet, SOCS3 protein remained elevated compared to CON animals. Although SOCS3 is known to interfere with insulin signalling in muscle (196), it also acts to suppress inflammatory cytokine signalling pathways (15). Therefore, it is possible that n-3 PUFA induced SOCS3 expression to feedback and downregulate cytokine signalling, which is consistent with the observed decrease in plasma IL-6 concentrations in the SFA/n-3 PUFA fed animals, as well as reports of DHA-induced increases in SOCS3 expression in 3T3-L1 adipocytes (15).

Despite changes in TLR4 and SOCS3 expression, we found no effect of any of our diets on the total protein content or phosphorylation of I κ B α or NF- κ B in soleus muscle. This is in

contrast to Yaspelkis et al. who recently demonstrated that feeding a high SFA diet for 12 weeks significantly decreased total protein content and increased serine phosphorylation of I κ B α (196). It is possible that if we had extended our high SFA diet feeding for an additional 4 weeks, we may have observed a similar result. Furthermore, although long-chain n-3 PUFA have been shown to inhibit NF- κ B activation (102), we found no effect of dietary fish oil on NF- κ B protein content or phosphorylation in soleus muscle. It is conceivable that any fish oil-induced inhibition of NF- κ B was masked by the high SFA diet, although this could not be resolved with the current study design. Finally, we must also acknowledge the possibility that, although there were no changes in total protein content or phosphorylation of I κ B α and NF- κ B, the cellular localization of these proteins between the cytoplasm and nucleus may have been altered.

4.5.6 Summary and perspectives

In summary, we found that partial substitution of fish oil into a high SFA diet prevents impairments in adiponectin-stimulated FAO, and importantly, both prevents and restores impairments in maximal insulin-stimulated glucose transport in rodent soleus muscle. Additionally, high SFA diet stimulates TLR4 expression and this can be prevented, but not restored, by fish oil supplementation. Overall, our findings emphasize the importance of considering fish oil-derived n-3 PUFA as a therapeutic strategy in the prevention of high SFA diet-induced impairments in soleus muscle lipid metabolism and insulin responsiveness.

Chapter 5: A new rodent model to study adipose-muscle cross-talk: dietary fatty acids modulate visceral fat adipokine secretion and induce skeletal muscle inflammatory changes without affecting insulin response, *ex vivo*.

In preparation for submission to *International Journal of Obesity*:

Tishinsky JM, De Boer AA, Dyck DJ, and Robinson LE. A new rodent model to study adipose-muscle cross-talk: dietary fatty acids modulate visceral fat adipokine secretion and induce skeletal muscle inflammatory changes without affecting insulin response, *ex vivo*.

5.1 Abstract

Adipokines are thought to communicate with skeletal muscle to regulate inflammation and insulin response, yet their role in adipose-muscle cross-talk, as well as the influence of dietary fat in such cross-talk, has never been studied in isolated whole tissue. We sought to determine if (1) high fat diets can modulate adipokine secretion from visceral adipose tissue, and (2) such changes exert direct effects on skeletal muscle inflammation and insulin response. Sprague-Dawley rats were fed (1) low fat control diet (LF), (2) high saturated fatty acid diet (SFA), or (3) high SFA with n-3 polyunsaturated fatty acid diet (SFA/n-3 PUFA) for 4 wk. Perigonadal adipose tissue was isolated and cultured for 72 h, to determine adipokine concentrations in adipose conditioned medium (ACM). ACM from SFA and SFA/n-3 PUFA fed rats contained more IL-6 (+26 to 37%, $P \leq 0.05$) and MCP-1 (+30%, $P \leq 0.05$) than LF animals. Adiponectin was decreased (-29%, $P \leq 0.05$) in ACM from SFA compared to LF, and this was prevented in SFA/n-3 PUFA. A second experiment used a newly developed adipose-muscle cross-talk model to test direct effects mediated by the altered adipokine profile on isolated skeletal muscle. To this end, soleus and extensor digitorum longus (EDL) muscles were isolated

from LF-fed Sprague-Dawley rats and incubated for 8 h in: (1) control buffer (CON), (2) CON with 2 mM palmitate (PALM, positive control), (3) ACM from LF, (4) ACM from SFA, and (5) ACM from SFA/n-3 PUFA. TLR4 gene expression was increased in soleus muscle incubated in PALM (+356%, $P \leq 0.05$) and less so in all ACM groups (+175-191%, $P \leq 0.05$). MCP-1 gene expression was elevated in soleus muscle from PALM (+163%, $P \leq 0.05$) and similarly with ACM from SFA (+159%, $P \leq 0.05$) and SFA/n-3 PUFA (+151%, $P \leq 0.05$). ACM LF induced an increase in IL-6 expression in soleus and EDL muscles (+182% and +175%, respectively, $p \leq 0.05$). Insulin-stimulated glucose transport was impaired in PALM soleus and EDL muscles (-13% and -36%, respectively $P \leq 0.05$), but preserved in all ACM groups. Our findings suggest that dietary SFA and n-3 PUFA modulate the release of adipose-derived factors from visceral adipose tissue, and acute exposure to this altered adipokine profile can induce changes in inflammatory gene expression without affecting insulin sensitivity in skeletal muscle in a short 8 h timeframe.

5.2 Introduction

Adipose tissue, an active endocrine organ, secretes a multitude of factors called adipokines that are known to regulate whole body inflammation, lipid metabolism and insulin-sensitivity (48). These adipose-derived factors include classic pro-inflammatory cytokines such as tumour necrosis factor (TNF)- α , interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1, as well as adiponectin, an anti-inflammatory and insulin-sensitizing adipokine (164). It is well established that the secretory profile of adipose tissue changes in response to an expansion in adipose tissue mass, with increasing BMI associated with elevated pro-inflammatory adipokine secretion and decreased adiponectin (164). Furthermore, although dietary fatty acid intake has been correlated with circulating adipokine levels (43), the effect of

dietary fatty acids on adipokine secretion *per se* has been less studied. Furthermore, since adipose tissue is comprised of multiple non-adipocyte cells, including macrophages that secrete an abundance of inflammatory cytokines such as MCP-1 (183), it is vital to consider the contribution of non-adipocyte cells to the secretory profile of adipose tissue. Given that a diet high in saturated fatty acids (SFA) contributes to obesity and insulin resistance (88), while a diet rich in fish oil-derived n-3 polyunsaturated fatty acids (PUFA) may confer a protective effect (162), there is growing interest in elucidating the role of these dietary fatty acids in modulating adipokine secretion from adipose tissue.

Skeletal muscle is responsible for approximately 80% of insulin-stimulated glucose disposal (10) and, as such, represents a key tissue governing whole body insulin sensitivity. Insulin signalling in skeletal muscle is impaired by pro-inflammatory adipokines, including TNF- α (198), IL-6 (73), and MCP-1 (141). In contrast, adiponectin is known to stimulate fatty acid oxidation in skeletal muscle, thereby improving insulin responsiveness (191); however, this stimulatory effect is lost following consumption of a high SFA diet (108). SFA are known ligands for toll-like receptor (TLR)4, a membrane receptor that, upon activation, initiates an intracellular signalling cascade culminating in the activation of nuclear factor (NF)- κ B and the upregulation of inflammatory gene expression (144). Evidence suggests that activation of such inflammatory pathways may serve as an underlying mechanism by which adipose-derived free fatty acids (FFA) and pro-inflammatory cytokines induce insulin resistance (31). In contrast to SFA, n-3 PUFA have been shown to inhibit activation of these inflammatory pathways (87, 88) but this has not been studied in whole skeletal muscle.

To investigate the role of adipose-derived factors in communication with skeletal muscle, previous work using a co-culture of human myocytes and isolated human adipocytes for 48 hours

showed impaired insulin-stimulated phosphorylation of both IRS-1 and Akt (30). Overall, while there is strong evidence at the cellular level that adipose-derived factors interfere with insulin signalling in myocytes (30-32, 141, 143), the endocrine communication between adipose tissue and skeletal muscle, and the regulation of this cross-talk by dietary fatty acids, has never been examined at the whole tissue level.

The objective of our study was to develop an *ex vivo* model to study the communication between adipose and skeletal muscle at the whole tissue level. More specifically, we aimed to (1) elucidate the effects of a high SFA diet, with or without n-3 PUFA, on the secretory profile of visceral adipose tissue, and (2) determine if such changes in the adipose tissue secretory profile could exert direct effects on inflammatory gene expression and insulin response in rodent skeletal muscle. We hypothesized that a high SFA diet would decrease adiponectin and increase pro-inflammatory cytokine release from adipose tissue and, in turn, induce skeletal muscle inflammation and impairments in insulin response, and this would be prevented by the inclusion of n-3 PUFA in the diet.

5.3 Methods

5.3.1 Animals and Diets

Female Sprague-Dawley rats were purchased from Charles River (Charles River Laboratories, St-Constant, QC, Canada) at approximately 50 days of age and weighed 142.5 ± 1.3 g upon arrival. The animals were housed in groups of 6 with a 12:12 h reverse light dark cycle and allowed unlimited access to water. Rats were fed a standard Teklad (Harlan Laboratories, Mississauga, ON, Canada) rodent chow diet for 3 days, at which point they were randomly assigned to receive (1) low fat control diet (6% kcal from soybean oil, 4% kcal from

lard, LF), (2) high SFA diet (55% kcal from lard, 5% kcal from soybean oil, SFA) known to induce insulin resistance (108), or (3) high SFA with n-3 PUFA diet (40% kcal from lard, 15% kcal from menhaden oil, 5% kcal from soybean oil, SFA/n-3 PUFA) for a period of 4 weeks (**Table 4.1**). The SFA/n-3 diet contained a total of 7.9% long-chain n-3 PUFA in the form of eicosapentaenoic acid (4.1%), docosapentaenoic acid (0.8%), and docosahexaenoic acid (3.0%) (**Table 4.2**). All diets were purchased from Research Diets (Research Diets Inc., New Brunswick, NJ, USA), stored at -20 °C, and administered fresh daily to the animals *ad libitum*. Body mass measurements were taken twice per week, and the animals were fasted overnight prior to experiments. Approval for all experimental procedures was obtained from the University of Guelph Animal Care Committee (Guelph, ON, Canada).

5.3.2 Adipose Tissue Organ Culture

Rats were anaesthetized by an intraperitoneal injection with sodium pentobarbital at a dose of 6 mg per 100 g body mass. Perigonadal fat pads were isolated and immediately placed in 50 mL sterile conical tubes containing phosphate buffer saline supplemented with 1% Penicillin-Streptomycin (Fisher Scientific, Ottawa, ON, Canada). Total protein was quantified using a bicinchoninic acid protein assay (Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer's instructions. Under sterile conditions, 500-700 mg of tissue (normalized by total protein content of adipose tissue isolated from LF fed animals – **Appendix D**) were placed into culture dishes containing 15 mL of medium 199 (M199) supplemented with 1% Penicillin-Streptomycin, 50 µU insulin, 2.5 nM dexamethasone, and 1% bovine serum albumin. The tissue was then minced into 5-10 mg pieces and cultured in a humidified incubator for 72 hours at 37 °C with 5% CO₂. 72 hours was chosen in accordance with data from a pilot experiment

assessing the timecourse of adipokine secretion into culture medium (**Appendix E**). After the incubation period, the adipose tissue minces were removed using 70 μ m cell strainers (BD Biosciences, Bedford, MA, USA), and the adipose conditioned medium (ACM) was stored at -80°C until further analysis. Secreted adiponectin in the medium was quantified by ELISA (Rat Adiponectin ELISA, Millipore, Billerica, MA, USA), and secreted IL-6, leptin, MCP-1, and TNF- α were analyzed by Luminex xMAP technology (Bioplex-200, Bio-Rad Laboratories, Mississauga, ON, Canada; Rat Cytokine/Chemokine Milliplex kit, Millipore, Billerica, MA, USA). Free fatty acid (FFA) concentrations were determined using an HR series non-esterified FFA kit. (Wako Chemicals Inc., Richmond, VA, USA).

5.3.3 Blood Sampling

Blood was obtained by cardiac puncture, transferred to heparinized tubes and centrifuged (10,000 x g, 5 min, 4°C) to isolate plasma. Fasting insulin (Rat/Mouse Insulin ELISA, Millipore, Billerica, MA, USA), and adiponectin (Rat Adiponectin ELISA, Millipore, Billerica, MA, USA) were determined by ELISA. Fasting plasma IL-6, leptin, MCP-1, and tumour necrosis factor (TNF)- α (Rat Cytokine/Chemokine Milliplex kit, Millipore, Billerica, MA, USA) were analyzed by Luminex xMAP technology (Bioplex-200, Bio-Rad Laboratories, Mississauga, ON, Canada). Fasting whole blood glucose values were obtained using a OneTouch Ultra2 glucometer (LifeScan Inc., Milpitas, CA, USA).

5.3.4 Skeletal Muscle Incubations

Female Sprague-Dawley rats were purchased from Charles River (Charles River Laboratories, St-Constant, QC, Canada) at approximately 18 days of age and weighed 45 ± 0.21

g upon arrival. The animals were housed in groups of 6 with a 12:12 h reverse light dark cycle, allowed unlimited access to water, and fed a standard Teklad (Harlan Laboratories, Mississauga, ON, Canada) rodent chow diet. After a 3 day acclimation period, rats were anaesthetized by an intraperitoneal injection with sodium pentobarbital at a dose of 6 mg per 100 g body mass, and intact soleus and extensor digitorum longus (EDL) (as representative of oxidative and glycolytic muscles, respectively) were isolated. Soleus and EDL strips were incubated in a 30 °C gently shaking water bath for 8 hours in glass vials containing 2mL of pre-gassed (95% O₂-5% CO₂) buffer. The conditions were as follows: (1) M199 containing 0.1% BSA and 8 mM glucose (CON) (2) M199 containing 4% BSA, 8 mM glucose, and supplemented with 2mM palmitate (PALM, positive control) (3) ACM from LF fed animals (ACM LF) (4) ACM from SFA fed animals (ACM SFA) (5) ACM from SFA/n-3 PUFA fed animals (ACM SFA/n-3 PUFA). After 8 hours, soleus and EDL strips were used for determination of basal or insulin-stimulated glucose transport rates or immediately frozen in liquid nitrogen for subsequent gene expression analysis.

5.3.5 Glucose Transport Assay

Following the 8 hour incubation, soleus and EDL muscle strips were incubated in the absence or presence of insulin (0.5 mU/mL) for 40 min (basal) or 20 min (insulin-stimulated) in M199 containing 4 mM pyruvate, 8 mM 3-*O*-[³H]methyl-D-glucose (American Radiolabeled Chemicals, St. Louis, MO, USA), and 28 mM [¹⁴C]mannitol (GE Healthcare, Baie d'Urfe, QC, Canada). Strips were blotted, tendons removed, weighed and then digested in 1 mL sodium hydroxide for 10 min at 95 °C. 200 µL of muscle digest was sampled and quantified by liquid

scintillation counting, and glucose transport rates were determined from intracellular 3-*O*-[³H]methyl-D-glucose as previously described (174).

5.3.6 Real-Time PCR

RNA was isolated from soleus and EDL muscles using an RNeasy kit (Qiagen, Toronto, ON, Canada) as per the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg of extracted RNA. Real-time PCR analysis was performed using a 7900HT Fast Real Time PCR system (Applied Biosystems, Forest City, CA, USA). Taqman gene expression assays (Applied Biosystems, Forest City, CA, USA) were used to determine the expression of β-actin (endogenous control) and the following genes of interest: TLR4, NF-κB, TNF-α, IL-6, and MCP-1. Samples were run in duplicate in 96-well plate format, and each 20 µL reaction contained 1 µL gene expression assay, 1 µL cDNA template, 10 µL Taqman Fast Universal PCR Master Mix, and 8 µL of RNase free water. All results were normalized to β-actin mRNA expression (C_t standard deviation = 0.045), and relative differences in gene expression between treatment groups were determined using the $\Delta\Delta CT$ method calculated by RQ Manager Software v 1.2 (Applied Biosystems, Forest City, CA, USA).

5.3.7 Statistical Analysis

All data are expressed as mean ± SEM. Body mass, blood measurement, adipose tissue organ culture, and gene expression data were all analyzed using a one-way analysis of variance (ANOVA). A two-way ANOVA test was conducted (treatment x condition) for comparison of basal and insulin-stimulated glucose transport. A Tukey post-hoc test was used to identify

significant differences. Statistical significance was set at $p \leq 0.05$ and all statistical analysis was performed using SigmaPlot version 12.0.

5.4 Results

5.4.1 Body Mass and Bloods

Initial body mass did not differ between any of the groups (**Table 5.1**). Terminal body mass was higher in SFA (+15%, $p \leq 0.05$) and SFA/n-3 PUFA (+11%, $p \leq 0.05$) compared to LF (Table 5.1). Fasting plasma adiponectin was decreased (-16%, $p \leq 0.05$) in SFA compared to LF and elevated in SFA/n-3 PUFA compared to both LF (+31%, $p \leq 0.05$) and SFA (+56%, $p \leq 0.05$) (Table 5.1). Fasting plasma IL-6 was increased in SFA/n-3 PUFA (+25%, $p \leq 0.05$) (Table 5.1) compared to LF but remained unchanged in SFA. Fasting plasma MCP-1 was elevated in both SFA and SFA/n-3 PUFA (+34%, +26%, respectively, $p \leq 0.05$) compared to LF (Table 5.1). There were no significant differences in fasting whole blood glucose, plasma insulin, leptin, or TNF- α between any of the diet groups (Table 5.1).

Table 5.1: Body Mass and Fasting Blood Measurements

	LF	SFA	SFA/n-3 PUFA
Arrival body mass (g)	142.5 ± 1.3	142.6 ± 1.6	143.0 ± 1.3
Terminal body mass (g)	257.1 ± 5.2	295.2 ± 4.7*	286.4 ± 4.4*
Whole blood glucose (mmol/L)	7.9 ± 0.2	8.4 ± 0.2	8.5 ± 0.2
Plasma insulin (ng/ml)	3.4 ± 0.2	3.4 ± 0.1	3.6 ± 0.1
Plasma adiponectin (µg/ml)	8.1 ± 0.2	6.8 ± 0.1*	10.6 ± 0.3* [‡]
Plasma IL-6 (pg/mL)	20.3 ± 0.6	23.5 ± 1.1	25.3 ± 1.2*
Plasma leptin (ng/mL)	7.4 ± 0.3	7.8 ± 0.3	7.8 ± 0.3
Plasma MCP-1 (pg/mL)	31.6 ± 1.2	42.2 ± 2.0*	39.9 ± 1.5*
Plasma TNF-α (pg/mL)	32.0 ± 1.4	31.0 ± 1.8	31.5 ± 2.0

Measurements were taken following 4 weeks of feeding.

Data are mean ± SEM, n = 11-12.

* = significantly different from LF, $p \leq 0.05$; [‡] = significantly different from SFA, $p \leq 0.05$.

5.4.2 Adipose Tissue Secretory Profile

Secreted adiponectin protein concentration was decreased in ACM from SFA fed animals (-30%, $p \leq 0.05$) compared to LF (**Figure 5.1A**) and this was prevented in SFA/n-3 PUFA ACM. Compared to LF, ACM from SFA and SFA/n-3 PUFA fed animals contained more IL-6 (+35%, +58%, respectively, $p \leq 0.05$) and MCP-1 (+40%, +44%, respectively, $p \leq 0.05$) (**Figures 5.1B,D**). Dietary fat composition did not affect secreted leptin concentration or FFA levels in ACM (**Figures 5.1C,E**). Secreted TNF-α protein concentration was below the detectable limit (6.6 pg/mL) in ACM from all three diet groups.

Fig. 5.1: Concentrations of A) adiponectin, B) IL-6, C) leptin, D) MCP-1, E) FFA in adipose-conditioned medium from LF, SFA, and SFA/n-3 PUFA fed animals. Values are means $\pm$ SEM, n=12. * = significantly different from LF, $p \leq 0.05$. LF = low-fat control diet, SFA = high saturated fatty acid diet, SFA/n-3 PUFA = high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

5.4.3 Inflammatory Signalling Gene Expression

Gene expression of TLR4 was increased in both soleus (+356%, $p \leq 0.05$) and EDL (+143%, $p \leq 0.05$) muscles incubated in PALM (**Figure 5.2A**). ACM from LF, SFA and SFA/n-3 PUFA fed animals also induced an increase in TLR4 expression in soleus (+175%, +179%, +191%, respectively, $p \leq 0.05$), but not EDL, muscle (Figure 5.2A). NF- κ B expression was elevated in PALM-incubated soleus and EDL muscles (+173%, +125%, respectively, $p \leq 0.05$) but remained unchanged in all ACM groups (**Figure 5.2B**).

Fig. 5.2: Gene expression of inflammatory signalling intermediates in i) soleus and ii) EDL muscle. A) TLR4, B) NF- κ B. β -actin was used as an endogenous control. Values are means $\pm$ SEM, n=8. * = significantly different from CON, $p \leq 0.05$. CON = control buffer; PALM = control buffer supplemented with 2mM palmitate; ACM LF = adipose conditioned medium from animals fed a low-fat diet; ACM SFA = adipose conditioned medium from animals fed a high saturated fatty acid diet; ACM SFA/n-3 PUFA = adipose conditioned medium from animals fed a high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

5.4.4 Inflammatory Cytokine Gene Expression

TNF- α gene expression was elevated in PALM-incubated soleus (+498%, $p \leq 0.05$) and EDL (+709%, $p \leq 0.05$) muscles but was unaffected by ACM incubation (**Figure 5.3A**). ACM LF induced an increase in IL-6 expression in soleus and EDL muscles (+182%, +175%, respectively, $p \leq 0.05$) (**Figure 5.3B**). MCP-1 expression was elevated in soleus muscle incubated with PALM, ACM SFA, and ACM SFA/n-3 PUFA (+163%, +159%, +151%, respectively, $p \leq 0.05$) but was unchanged in soleus muscle incubated with ACM LF and all EDL groups (**Figure 5.3C**).

A i

B i

C i

Fig. 5.3: Gene expression of inflammatory cytokines in i) soleus and ii) EDL muscle. A) TNF- α B) IL-6 C) MCP-1. β -actin was used as an endogenous control. Values are means $\pm$ SEM, n=8. * = significantly different from CON, $p \leq 0.05$. CON = control buffer; PALM = control buffer supplemented with 2mM palmitate; ACM LF = adipose conditioned medium from animals fed a low-fat diet; ACM SFA = adipose conditioned medium from animals fed a high saturated fatty acid diet; ACM SFA/n-3 PUFA = adipose conditioned medium from animals fed a high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

5.4.5 Glucose Transport

In both soleus and EDL muscles, insulin significantly increased glucose uptake compared to basal in CON (+67%, +72%, respectively, $P \leq 0.05$), ACM LF (+61%, +62%, respectively, $P \leq 0.05$), ACM SFA (+80%, +87%, respectively, $P \leq 0.05$), and ACM SFA/n-3 PUFA (+53%, +86%, respectively, $P \leq 0.05$) (**Figure 5.4A,B**). Insulin's stimulatory effect was lost in PALM incubated soleus and EDL muscles (Figure 5.4A,B). Basal and insulin-stimulated glucose transport did not differ between any groups in soleus or EDL muscles (Figure 5.4A,B).

Fig. 5.4: Basal and insulin-stimulated glucose transport in A) soleus muscle, and B) EDL muscle. Values are means $\pm$ SEM, n=8. * = significantly different from basal within the same treatment group, $p \leq 0.05$. White bars = basal, Black bars = insulin-stimulated (0.5 mU/mL). CON = control buffer; PALM = control buffer supplemented with 2mM palmitate; ACM LF = adipose conditioned medium from animals fed a low-fat diet; ACM SFA = adipose conditioned medium from animals fed a high saturated fatty acid diet; ACM SFA/n-3 PUFA = adipose conditioned medium from animals fed a high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

5.5 Discussion

This study is the first to demonstrate that dietary fatty acids can modulate the secretory profile of rodent adipose tissue explants and that exposure of isolated skeletal muscle to adipose-derived factors can induce inflammatory changes without impairing insulin response. More specifically, we demonstrated that consumption of a high SFA diet induced an increase in the secretion of pro-inflammatory adipokines and a decrease in adiponectin from cultured rodent adipose tissue. Importantly, the SFA-mediated decrease in adiponectin secretion was prevented by inclusion of fish oil-derived n-3 PUFA in the high SFA diet. In addition, skeletal muscle incubation in medium with an altered adipokine profile induced an increase in gene expression of TLR4 and MCP-1 in soleus (but not EDL) muscle and had no effect on insulin-stimulated glucose transport in either muscle type. Overall, these results suggest that dietary SFA and n-3 PUFA can modulate the secretory profile of visceral adipose tissue and affect the cross-talk between adipose tissue and skeletal muscle to induce changes in skeletal muscle inflammatory gene expression without affecting insulin sensitivity in an acute 8 hour timeframe.

5.5.1 Exposure to a high SFA diet, with or without n-3 PUFA, alters visceral fat adipokine secretion.

To the best of our knowledge, we are the first to demonstrate that high fat diets can alter the secretory profile of cultured adipose tissue explants from rodents. The observed increase in pro-inflammatory adipokine secretion and decrease in adiponectin after the high SFA diet is consistent with previous findings (17, 169). Although the high SFA diet, regardless of n-3 PUFA content, resulted in elevated IL-6 and MCP-1 protein concentrations, it is interesting to note that inclusion of n-3 PUFA in the high SFA diet completely prevented the SFA-induced decrease in secreted adiponectin concentration. This protective effect of n-3 PUFA is consistent with reports that n-3 PUFA prevent high fat diet-induced elevations in inflammatory gene expression in mouse adipose tissue (162). Furthermore, it is noteworthy that the high SFA diet, with or without n-3 PUFA, had no effect on total FFA release from cultured adipose tissue, which was unanticipated given the association between high fat feeding and increased plasma FFA levels (88). Taking into consideration that the medium was collected 72 hours after initiation of adipose tissue culture, it is possible that some FFA may have been degraded and/or oxidized. Furthermore, although there was no difference in total FFA concentration in the ACM, we must also acknowledge that the fatty acid composition of the ACM may have differed between diet groups.

One advantage of using a whole adipose tissue model to examine the cross-talk between adipose tissue and skeletal muscle is the inclusion of non-adipocyte cells of the stroma-vascular fraction that also contribute to the secretory profile of adipose tissue. Of particular importance are resident macrophages within adipose tissue that are a main source of inflammatory cytokines and have been shown to exert paracrine effects on adipokine release from adipocytes (156).

More specifically, MCP-1 is a key mediator of the negative cross-talk between adipocytes and myocytes in culture (141). Given that MCP-1 is secreted in large part by macrophages (70), the use of a model such as ours that includes macrophage-derived factors in the examination of adipose-muscle cross-talk is of value. Additionally, macrophage infiltration into adipose tissue is increased in response to 12 weeks of high fat feeding in mice (183), lending support to our observed increases in IL-6 and MCP-1 in ACM from SFA and SFA/n-3 PUFA fed animals.

5.5.2 Acute exposure of skeletal muscle to adipose-derived factors does not affect insulin sensitivity.

Since dietary fatty acids changed the adipokine secretory profile of visceral adipose tissue explants, a second objective of this study was to examine if the altered adipokine milieu in the ACM could modulate skeletal muscle glucose transport, a functional measure indicative of changes in insulin sensitivity. The effects of ACM on insulin response in whole muscle has not been previously examined, although co-culture of myocytes with adipocytes or incubation of myocytes with ACM has been demonstrated to impair both myocyte insulin signalling (30-32, 141, 142, 147) and glucose uptake (81), providing good basis for the current work. We report for the first time that although dietary fatty acids changed the adipokine milieu of the ACM, this had no downstream functional effect as measured by insulin-stimulated glucose transport in isolated soleus and EDL muscles. As expected, incubation of both soleus and EDL muscle with 2mM palmitate impaired insulin-stimulated glucose transport and is consistent with the work of others (2, 160).

While it is possible that ACM exposure may have altered insulin signalling, as has been shown by others using myocytes (30-32, 141, 142, 147), our finding that insulin-stimulated rates

of glucose transport remained unchanged in response to diet-induced changes in ACM indicates that muscle insulin response was unaffected by adipose-derived factors. This is likely attributable to several factors. Firstly, levels of MCP-1 in the ACM were approximately 60% lower than those reported to induce decreased glucose transport in isolated muscle cells treated with synthetic adipokines (141), suggesting that the concentration of adipokines in the ACM may have been insufficient to affect insulin response. Along a similar line, it is possible that isolated myocytes and intact whole muscle strips differ in terms of sensitivity to adipose-derived factors. It is also noteworthy that resistin has been demonstrated to impair insulin-stimulated glucose transport in rodent soleus muscle in the presence, but not absence, of 2 mM palmitate (67), signifying that the concomitant presence of elevated fatty acids may be necessary for adipokine-induced impairments in whole muscle insulin response but, as we only studied 2 mM palmitate on its own, this requires further study. Lastly, it is plausible that 8 hours was simply an insufficient duration for ACM to induce changes in skeletal muscle insulin response, as previous work has typically incubated myocytes in ACM for approximately 24-48 hours (30, 32, 141, 142). In our isolated whole muscle model, 8 hours was the maximum incubation time that could be used without comprising muscle strip viability as determined by quantification of muscle phosphagens (**Appendix G**).

5.5.3 Acute exposure of skeletal muscle to adipose-derived factors induces fibre type-specific changes in inflammatory gene expression.

Although acute exposure to the diet-induced changes in adipokine milieu did not affect insulin sensitivity in skeletal muscle, changes in inflammatory gene expression in muscle were found. TLR4 gene expression was significantly increased in soleus muscle after incubation with

ACM from LF, SFA, and SFA/n-3 PUFA fed animals. SFA are known activators of TLR4, a membrane receptor that can initiate an intracellular inflammatory signalling cascade resulting in the translocation of NF- κ B to the nucleus and the upregulation of inflammatory gene expression. The fact that TLR4 expression was induced to a similar degree in all three diet groups is likely attributable to the similar levels of FFA in the ACM from each. Despite ACM-induced upregulation of TLR4 gene expression in soleus muscle, we observed no change in NF- κ B expression in soleus or EDL muscles incubated in ACM. In contrast to our findings, Dietze and colleagues found that ACM exposure activates the IKK/NF- κ B pathway in cultured myocytes and is necessary for ACM-mediated impairments in myocyte insulin signalling (31). Given that a concentrated adipocyte supernatant was used in the aforementioned study (31), it is likely that the adipokine concentrations in the ACM in our study were simply too low to induce a similar upregulation of NF- κ B expression. Alternatively, although we observed no detectable changes in NF- κ B gene expression, it is possible that the protein expression and/or activity of NF- κ B may have been altered, but this requires further study. Finally, we are also the first to demonstrate that incubation of isolated whole muscle, both soleus and EDL, with palmitate significantly increased gene expression of inflammatory mediators and cytokines. This is in agreement with reports at the muscle cell level that palmitate increases inflammatory cytokine gene expression by activation of NF- κ B in C2C12 myocytes (23) and L6 myotubes (182) and further emphasizes the ability of this SFA to induce inflammation.

It is interesting to note that MCP-1 gene expression was significantly increased in soleus muscle exposed to ACM from high fat, but not LF, fed animals, suggesting diet-induced changes in adipose-muscle communication. Although the relationship between adipokine concentrations and inflammatory changes has not been previously examined in whole adipose-muscle cross-talk

models, there is evidence that altered adipokine concentrations correlate directly with changes in myocyte insulin signalling (32, 147). The addition of synthetic adiponectin to a co-culture of human adipocytes and myocytes effectively prevented impairments in myocyte insulin signalling (32). Furthermore, an inverse relationship between concentrations of IL-6, IL-8, and leptin in ACM and the degree of co-culture-induced impairments in insulin-stimulated Akt phosphorylation are reported in human skeletal muscle cells (147). Curiously, we observed an increase in IL-6 gene expression in soleus and EDL muscles exposed to ACM from LF fed animals only. Although plasma IL-6 is positively correlated with BMI and closely linked with type 2 diabetes and CVD risk (201), muscle-derived IL-6 secretion increases during exercise and has been shown to exert an inhibitory effect on inflammatory cytokine production (122). As such, it is possible the elevated IL-6 gene expression in muscle incubated in LF ACM is reflective of an anti-inflammatory feedback response and is in keeping with the high fat diet-induced changes in MCP-1 expression in soleus muscle.

Finally, we chose to examine the response of both soleus and EDL, an oxidative and glycolytic muscle type, respectively, to adipose-derived factors to elucidate any fiber-type specific effects in our adipose-muscle cross-talk model. While the trend in response was remarkably similar between soleus and EDL muscles, soleus muscle appears to exhibit heightened sensitivity to ACM-induced changes in inflammatory gene expression. As soleus muscle possesses a greater oxidative capacity compared to EDL muscle (125), this heightened inflammatory response to adipose-derived factors may be accredited to a greater propensity of soleus muscle to respond to FFA exposure, but this requires further study.

5.5.4 Summary and perspectives

In summary, we found that a high SFA diet, with or without n-3 PUFA, modulated the secretion of adipokines from rodent visceral adipose tissue. Additionally, using a newly developed model to study adipose tissue-skeletal muscle cross-talk at the whole tissue level, we demonstrated that exposure of isolated skeletal muscle to ACM originating from different diet groups resulted in changes in inflammatory gene expression without affecting insulin-stimulated glucose transport. Overall, our findings suggest that dietary fatty acids can affect the communication between adipose tissue and skeletal muscle resulting in inflammatory changes, but that longer exposure time may be required to affect insulin sensitivity.

Chapter 6: Integrative Discussion

The overall objective of this thesis was to examine the modulation of adipose-derived factors by long-chain n-3 PUFA and to characterize their effects on skeletal muscle fatty acid metabolism, inflammation and insulin response. In contrast to SFA, which are positively correlated with impaired lipid metabolism and insulin resistance (35), n-3 PUFA are well-known for their anti-inflammatory and insulin-sensitizing properties and are associated with improved circulating adipokine concentrations, including increased adiponectin (130). Prior to this thesis, the ability of n-3 PUFA to directly stimulate adiponectin secretion from human adipocytes, as well as to affect the responsiveness of peripheral skeletal muscle to circulating adiponectin had not been addressed. Furthermore, previous work had demonstrated that consumption of a high SFA diet impairs the ability of adiponectin to stimulate skeletal muscle fatty acid oxidation which, in turn, is associated with blunted insulin responsiveness (108). However, the ability of dietary n-3 PUFA to prevent and/or restore such skeletal muscle impairments, as well as any potential underlying changes in inflammation, had never been studied. Finally, despite strong evidence at the cellular level that adipose-derived factors communicate with myocytes to regulate insulin signalling (30-32, 141, 143), the endocrine communication between adipose tissue and skeletal muscle, and the regulation of this cross-talk by dietary fatty acids, had never been examined at the whole tissue level.

6.1 Summary of results

Consistent with our hypothesis, study one of this thesis demonstrated for the first time that both EPA and DHA increase the secretion of adiponectin from cultured primary human adipocytes. Interestingly, EPA, but not DHA, also elicited an increase in cellular adiponectin

protein content, and the upregulation of adiponectin by EPA and DHA occurred partially and exclusively via a PPAR γ -dependent mechanism, respectively. In addition, the EPA-induced increase in adiponectin secretion was further augmented when combined with rosiglitazone, indicating that n-3 PUFA and TZDs may exert additive effects on adiponectin in a human adipocyte model.

Given that n-3 PUFA can increase adiponectin secretion from adipocytes, and that adiponectin is known to affect skeletal muscle function, the second study of this thesis aimed to examine if dietary n-3 PUFA could in fact enhance the response of skeletal muscle to circulating adiponectin. More specifically, study two sought to determine if impairments in adiponectin responsiveness induced by a high SFA diet would be prevented and/or restored by n-3 PUFA and, in turn, if this would be associated with improvements in muscle insulin response. We showed for the first time that incorporation of n-3 PUFA into a high SFA diet effectively prevented impairments in adiponectin response, and both prevented and restored impairments in insulin response, in isolated rodent soleus muscle. Additionally, the high SFA diet increased both TLR4 and SOCS3 protein expression in soleus muscle, and the increase in TLR4 expression was prevented by the inclusion of n-3 PUFA in the diet. Collectively, these findings suggest that changes in skeletal muscle inflammation may underlie diet-induced effects on skeletal muscle adiponectin and insulin response.

Lastly, the third study in this thesis involved the development of a novel *ex vivo* model to study the communication between adipose tissue and skeletal muscle, as well as its regulation by dietary fatty acids at the whole tissue level. Consumption of a high SFA diet, with or without n-3 PUFA, induced changes in the profile of adipokines secreted from rodent visceral adipose tissue explants. In turn, acute exposure of isolated skeletal muscle to the altered adipokine

milieu elicited fibre-type specific changes in inflammatory gene expression, indicating that cross-talk between adipose tissue and skeletal muscle can induce alterations in muscle inflammation. However, in contrast to our hypothesis, insulin sensitivity in both soleus and EDL muscles remained unaffected by acute exposure to adipose tissue-derived factors, suggesting an ability of healthy skeletal muscle to sustain its insulin sensitivity over 8 hours.

6.2 Dietary consumption of long-chain n-3 PUFA as a therapeutic strategy in obesity and type 2 diabetes

The prevalence of obesity and obesity-related pathologies such as type 2 diabetes is increasing at an astounding rate; consequently, identifying strategies to prevent and treat these diseases is of utmost importance. The work presented herein strongly suggests that dietary n-3 PUFA intake represents a promising nutritional strategy to improve the metabolic disturbances underlying these conditions. Both EPA and DHA, the bioactive fatty acids in fish oil, increased the secretion of adiponectin from isolated adipocytes, while the incorporation of fish oil into a high SFA diet prevented impairments in adiponectin secretion from rodent adipose tissue explants, clearly demonstrating the ability of n-3 PUFA to exert direct favourable effects on the profile of adipose tissue-derived factors that play a prominent role in obesity-related disease. This thesis also establishes that the benefits of dietary n-3 PUFA extend to peripheral tissues, as fish oil was shown to prevent SFA-induced impairments in both adiponectin and insulin response in isolated soleus muscle. As obesity is characterized by decreased adiponectin secretion from adipose tissue and resistance of skeletal muscle to circulating adiponectin and insulin, it stands to reason that the consumption of long-chain n-3 PUFA represents a viable therapeutic strategy to aid in the management of these diseases.

A strength of study one was that the n-3 PUFA doses used to treat human adipocytes were comparable to typical plasma concentrations of EPA and DHA (175). That said, plasma levels of palmitic acid are typically much higher than those used (175), and this may explain why no change in adiponectin secretion from the cultured adipocytes was observed with palmitic acid. A limitation of studies two and three also pertains to the amount and composition of dietary fatty acids. In these studies, both high fat diets contained 60% of total kcal derived from fat which exceeds that of the typical North American diet. Additionally, the SFA/n-3 PUFA diet contained 15% of kcal from menhaden oil, 8% of which was derived from long-chain n-3 PUFA. Based on average food intakes, this translates to the rodents receiving approximately 0.6 g/day of fish oil which, considering their body mass, represents a supraphysiological dose as the recommended dietary intake of n-3 PUFA for humans does not exceed 2-4 g/day (79). While the diets used in this thesis were chosen to induce rapid changes in adipose tissue and skeletal muscle metabolism in an effort to evaluate the potential regulatory role of dietary fatty acids and underlying mechanisms by which they function, future studies using diets that are more characteristic of typical dietary intake patterns would be valuable. Lastly, though the current findings cannot easily be directly extrapolated to humans, they serve an important purpose in developing our current understanding of dietary fatty acid-induced changes in adipokine secretion, inflammation, and insulin responsiveness in skeletal muscle.

The combination of EPA and rosiglitazone treatment in study one provoked an additive increase in the secretion of adiponectin from adipocytes and this was consistent with the recent findings of Banga and colleagues (9). As hypoadiponectinemia is a common feature of type 2 diabetes, this carries notable therapeutic implications for diabetics currently receiving TZD

treatment. The value of combining TZD therapy with EPA supplementation has yet to be examined at the *in vivo* level and should be the focus of future clinical studies.

Furthermore, although EPA and DHA both stimulated adiponectin secretion from isolated adipocytes, only EPA treatment resulted in an increase in cellular adiponectin protein content. It is possible that DHA elicited an increase in adiponectin secretion by regulating post-transcriptional modifications of adiponectin necessary for its release from adipocytes (179, 181); however, this cannot be determined from this current body of work. Moreover, DHA was also found to regulate adiponectin secretion exclusively via PPAR γ , whereas EPA functioned by PPAR γ and an additional unknown mechanism. The differential regulation of adiponectin by EPA and DHA emphasizes the importance of considering the independent properties of these n-3 PUFA, as they are typically evaluated in combination in the form of fish oil supplementation. Future work should also aim to elucidate the PPAR γ -independent mechanisms by which EPA modulates adiponectin. In light of the finding that n-3 PUFA consumption prevented SFA-induced TLR4 expression in study two, it seems possible that EPA may increase adiponectin secretion by downregulating inflammatory pathways in adipocytes, but this requires further study.

6.3 Adiponectin as a regulator of skeletal muscle function

Previous work has demonstrated that adiponectin stimulates fatty acid oxidation in both rodent (109) and human (16) muscle. Importantly, the response of skeletal muscle to adiponectin is blunted in obesity (16) and as a consequence of high SFA consumption (109). As the accumulation of muscle lipids species can interfere with insulin signalling (129, 199), impaired adiponectin-stimulated oxidation of fatty acids may be a determinant of skeletal muscle insulin sensitivity. Indeed, a recent study by Mullen et al. revealed that adiponectin resistance occurs

prior to both accumulation of muscle lipids and blunted insulin-stimulated glucose uptake in response to high fat feeding in rodents (108). In study two of this thesis we demonstrated for the first time that the loss of both adiponectin and insulin response in soleus muscle can be prevented by incorporation of fish oil into a high SFA diet.

Interestingly, after 4 weeks of high SFA intake, fish oil incorporation into the high SFA diet led to the restoration of high SFA diet-induced impairments in insulin response, without rescuing adiponectin response in soleus muscle. Thus, although adiponectin resistance precedes the development of insulin resistance (108), it does not appear to be required for its restoration. These findings are consistent with reports that exercise can restore muscle insulin response despite continued resistance to adiponectin (Gulli et al., unpublished findings) or leptin (134). It is possible that if the dietary intervention in study two had extended longer than 8 weeks adiponectin response may have been restored as well, but this cannot be determined from the current work. Regardless, the observation that adiponectin response is not required for the restoration of insulin response is an interesting point, emphasizing that resistance to adiponectin is merely one factor that contributes to impairments in lipid metabolism and, ultimately, diet-induced muscle insulin resistance. Future studies should aim to characterize other potential benefits of fish oil on skeletal muscle lipid metabolism that may account for the observed restoration of insulin response. For example, leptin has been shown to downregulate triglyceride esterification in muscle and this effect is blunted in response to high SFA consumption but maintained with fish oil supplementation (153). Therefore, while it is possible that fish oil decreased muscle lipid accumulation by targeting storage rather than oxidation, this requires further study.

Finally, although this thesis has considered resistance to adiponectin-stimulated fatty acid oxidation to be a metabolic disadvantage, it is possible that loss of adiponectin response actually occurs as a protective mechanism with regards to the production of reactive oxygen species (ROS). Consumption of a high SFA diet has been shown to increase skeletal muscle ROS production in rodents, coinciding with the development of insulin resistance (5). Remarkably, if ROS production is attenuated via mitochondrial-targeted antioxidant administration, insulin sensitivity is preserved (5), suggesting that oxidative stress is a key factor in the etiology of high SFA diet-induced insulin resistance. Therefore, it is possible that during exposure to excess dietary fat, skeletal muscle sacrifices its responsiveness to factors such as adiponectin that stimulate fatty acid oxidation to defend itself against increased ROS production and oxidative stress. As fish oil may possess antioxidant properties (177), its role in the convergence of adiponectin resistance, oxidative stress and insulin resistance should be examined.

6.4 Are changes in inflammation an underlying mechanism by which dietary fatty acids affect skeletal muscle function?

A common objective in studies two and three was to evaluate the role of inflammation as an underlying mechanism by which dietary fatty acids regulate skeletal muscle function. Study two revealed that a high SFA diet induced an increase in TLR4 and SOCS3 expression in skeletal muscle and that n-3 PUFA prevented the increase in TLR4, but not SOCS3. In study three, exposure of soleus muscle to adipose-derived factors led to an increase in TLR4, IL-6, and MCP-1 gene expression; however, the effect of dietary fatty acids was minimal. Although these findings are intriguing, it must be acknowledged that both gene and protein expression of numerous additional inflammatory signalling mediators and cytokines were unaffected by dietary fatty acids in studies two and three. Consequently, it would be premature to conclude that

changes in skeletal muscle inflammation represent a mechanism by which dietary fatty acids, including n-3 PUFA, mediate adiponectin and insulin response. Likewise, the observation that changes in both skeletal muscle inflammation and adiponectin and insulin response occur concomitantly in response to dietary fatty acids does not necessitate or even suggest causality between these events; the possibility that these changes are unrelated cannot be excluded. Nonetheless, given that inflammation is considered a hallmark of obesity and type 2 diabetes (135), a more thorough exploration of the role of inflammatory changes underlying diet-induced changes in muscle metabolism is certainly justified.

Various limitations regarding the methods by which we investigated the effects of dietary fatty acids on skeletal muscle inflammation must be recognized in this thesis. Firstly, quantification of mRNA and protein expression represent static measurements that fail to capture the intricacies of complex cellular pathways. For instance, recent work has demonstrated that consumption of a high SFA diet results in co-localization of SOCS3 with the insulin receptor and IRS-1, resulting in impaired downstream insulin signalling via steric hindrance (196). In accordance with these findings, determination of the activity and cellular localization of inflammatory signalling mediators (methods not widely available) would have significantly enhanced our characterization of diet-induced changes in muscle inflammation in studies two and three and should be included in future work. Secondly, it is possible that since the animals were fasted overnight prior to tissue isolation, changes in gene and protein expression induced by the different high fat diets may have been masked. Although the overnight fast was necessary to eliminate potential confounding variables associated with the acute effects of a last meal, its limitation with respect to unveiling transient diet-induced changes in gene and protein expression of inflammatory mediators represents a shortcoming in studies two and three.

6.5 The development and use of an adipose tissue-skeletal muscle crosstalk model

A major objective of study three was to design and characterize an *ex vivo* model to investigate the communication between adipose and skeletal muscle at the whole tissue level. By utilizing a whole tissue model, the influence of non-adipocyte cells, such as macrophages, residing in adipose tissue on its secretory function was effectively addressed and represents a major strength of this study. Macrophages secrete numerous cytokines including MCP-1, and also communicate in a paracrine fashion to regulate the release of adipokines from adipocytes (156), thus playing an integral role in mediating the secretory profile of whole adipose tissue. With the notable exception of adiponectin, the adipokines examined in the adipose tissue organ culture model are secreted from both adipocytes and macrophages, making it difficult to interpret the extent to which macrophages and other non-adipocyte cells were contributing to the overall secretory profile of the tissue, as well as whether they too were altered by high fat feeding. Investigation of the potential differences in skeletal muscle response to medium generated from isolated adipocytes versus medium generated from whole tissue explants would be a helpful future study to elucidate the degree to which non-adipocyte cells residing in adipose tissue contribute to adipose-muscle communication.

Additionally, the perigonadal visceral adipose tissue depot was selected for culture in our crosstalk model as visceral adipose has been demonstrated to secrete higher concentrations of adipokines compared to subcutaneous adipose (40). However, it would be worthwhile to compare the responses of different visceral adipose tissue depots (i.e. perigonadal vs. retroperitoneal fat pads) to dietary interventions to investigate any potential depot-specific effects. Similarly, given that research suggests that male rodents exhibit a heightened susceptibility to high fat diet-induced changes in adipose tissue metabolism (39, 63), future work

should also aim to characterize any potential sex differences in the dietary modulation of adipokine secretion and ensuing effects on skeletal muscle metabolism.

Using an *in vitro* co-culture model, Dietze and colleagues determined that exposure of cultured myocytes to adipocyte-conditioned medium induced impairments in IRS-1 and Akt phosphorylation (30-32), suggesting that adipose-derived factors are able to directly communicate with myocytes to activate inflammatory pathways and induce impairments in insulin signalling. Furthermore, incubation of L6 myotubes with conditioned medium from human visceral fat impaired rates of insulin-stimulated glucose uptake (81), a key functional measurement indicative of changes in insulin sensitivity. Given that myocytes tend to exhibit a different (less dramatic) response to insulin compared to a whole muscle preparation (104, 108, 109), the effects of adipose-derived factors on insulin-stimulated glucose transport in whole muscle warranted investigation. Study three in this thesis demonstrated for the first time that exposure of healthy whole skeletal muscle to adipose-derived factors for an acute period of 8 hours was insufficient to perturb insulin sensitivity. In development of our adipose-muscle crosstalk model, one important consideration associated with the use of whole muscle strips as opposed to isolated myocytes was tissue viability. Whereas myocytes are commonly exposed to adipose-conditioned medium for upwards of 48 hours (30), the viability of isolated skeletal muscle strips is much more time sensitive. In accordance with the phosphogens data (see Appendix G), 8 hours was the maximum length of time that muscle strips could be incubated *ex vivo* without comprising the quality and viability of the tissue. As such, the inability of adipose-derived factors to inflict changes in insulin-stimulated glucose transport is likely attributable in part to the acute exposure time of the current model compared to previous work (30-32, 81, 81, 141). Furthermore, it is conceivable that intact muscle strips are simply less sensitive to

inflammatory mediators than isolated myocytes and may have required higher concentrations of pro-inflammatory adipokines to elicit an effect on insulin sensitivity. It would be interesting to expose skeletal muscle to a concentrated adipose-conditioned medium (achieved by ultracentrifugation) to determine if higher concentrations of pro-inflammatory adipokines might elicit a different response. Lastly, examining the effects of whole adipose tissue-derived factors on isolated myocytes also represents a logical future study to clarify any potential discrepancies in sensitivity between isolated myocytes and whole muscle strips.

In study three, consumption of a high SFA diet, with or without a fish oil-derived long-chain n-3 PUFA, resulted in discernible changes in the adipokine milieu produced from visceral adipose tissue explants. However, changes in skeletal muscle inflammatory gene expression were similar in response to exposure to ACM from animals fed different diets. Two notable exceptions were the increase in soleus and EDL IL-6 expression elicited by ACM from LF fed animals only, and the increase in soleus MCP-1 expression produced by exposure to ACM from the high fat, but not LF, diets. It should be emphasized that although not statistically significant, LF ACM treatment resulted in a trend towards an increase in MCP-1 gene expression as well. The similarity in skeletal muscle response to ACM despite the differences in adipokine concentrations may be credited to several factors. Firstly, it is likely that the changes in adipokine concentrations induced by the different diets were simply too modest to translate to functional changes in skeletal muscle response to the adipose-derived factors. Moreover, FFA concentrations in the ACM were unaffected by diet and, therefore, it is possible that these FFA levels masked the effects of the altered adipokine profiles on skeletal muscle function. Lastly, it must be acknowledged that adipose tissue secretes hundreds of different adipokines and hormones, and the adipokines quantified in these experiments represent only a snapshot of those

actually present in the ACM. Therefore, although the high fat diets resulted in changes in adiponectin, IL-6 and MCP-1 secretion from adipose tissue explants, it is possible that dietary effects, or lack thereof, on other adipose-derived factors not measured in this thesis may have superseded or counteracted those that were altered.

6.6 Concluding remarks

In summary, the current thesis highlights the role of n-3 PUFA in modulating adipokine secretion and ensuing functional changes in skeletal muscle inflammation, lipid metabolism, and insulin response. Given that the prevalence of obesity and type 2 diabetes is escalating at an alarming rate, the importance of identifying potential strategies for the prevention and treatment of these pathologies cannot be understated. The ability of n-3 PUFA to counteract several of the metabolic impairments induced by a high SFA diet is also worth highlighting in view of the increase in consumption of calorically dense, high saturated fat foods in today's society. The mechanisms by which n-3 PUFA exert their beneficial health effects are numerous and multifactorial. The studies presented in this thesis suggest that modulation of adipokine secretion and response, as well as changes in intracellular inflammatory signalling mediators, represent targets by which n-3 PUFA may affect insulin responsiveness in skeletal muscle. Future studies should aim to further elucidate potential underlying mechanisms to gain a more comprehensive understanding of the means by which dietary n-3 PUFA may aid in the management of obesity and its related pathologies.

References:

- 1. Ahima RS. Adipose tissue as an endocrine organ. *Obesity (Silver Spring)* 14 Suppl 5: 242S-249S, 2006.**
- 2. Alkhateeb H, Chabowski A, Glatz JF, Luiken JF and Bonen A. Two phases of palmitate-induced insulin resistance in skeletal muscle: impaired GLUT4 translocation is followed by a reduced GLUT4 intrinsic activity. *Am.J.Physiol.Endocrinol.Metab.* 293: 3: E783-93, 2007.**
- 3. An WS, Son YK, Kim SE, Kim KH, Bae HR, Lee S, Park Y, Kim HJ and Vaziri ND. Association of adiponectin and leptin with serum lipids and erythrocyte omega-3 and omega-6 fatty acids in dialysis patients. *Clin.Nephrol.* 75: 3: 195-203, 2011.**
- 4. Andersen G, Harnack K, Erbersdobler HF and Somoza V. Dietary eicosapentaenoic acid and docosahexaenoic acid are more effective than alpha-linolenic acid in improving insulin sensitivity in rats. *Ann.Nutr.Metab.* 52: 3: 250-256, 2008.**
- 5. Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin CT, Price JW,3rd, Kang L, Rabinovitch PS, Szeto HH, Houmard JA, Cortright RN, Wasserman DH and Neuffer PD. Mitochondrial H₂O₂ emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J.Clin.Invest.* 119: 3: 573-581, 2009.**
- 6. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T and Matsuzawa Y. Paradoxical decrease of an**

adipose-specific protein, adiponectin, in obesity. *Biochem.Biophys.Res.Commun.* 257: 1: 79-83, 1999.

7. Baeuerle PA and Henkel T. Function and activation of NF-kappa B in the immune system. *Annu.Rev.Immunol.* 12: 141-179, 1994.

8. Bajaj M, Suraamornkul S, Piper P, Hardies LJ, Glass L, Cersosimo E, Pratipanawatr T, Miyazaki Y and DeFronzo RA. Decreased plasma adiponectin concentrations are closely related to hepatic fat content and hepatic insulin resistance in pioglitazone-treated type 2 diabetic patients. *J.Clin.Endocrinol.Metab.* 89: 1: 200-206, 2004.

9. Banga A, Unal R, Tripathi P, Pokrovskaya I, Owens RJ, Kern PA and Ranganathan G. Adiponectin translation is increased by the PPARgamma agonists pioglitazone and omega-3 fatty acids. *Am.J.Physiol.Endocrinol.Metab.* 296: 3: E480-9, 2009.

10. Baron AD, Brechtel G, Wallace P and Edelman SV. Rates and tissue sites of non-insulin- and insulin-mediated glucose uptake in humans. *Am.J.Physiol.* 255: 6 Pt 1: E769-74, 1988.

11. Berg AH, Combs TP, Du X, Brownlee M and Scherer PE. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat.Med.* 7: 8: 947-953, 2001.

12. Berg AH, Combs TP and Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol.Metab.* 13: 2: 84-89, 2002.

13. Bonen A, Parolin ML, Steinberg GR, Calles-Escandon J, Tandon NN, Glatz JF, Luiken JJ, Heigenhauser GJ and Dyck DJ. Triacylglycerol accumulation in human obesity and

type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36. *FASEB J.* 18: 10: 1144-1146, 2004.

14. Bouskila M, Pajvani UB and Scherer PE. Adiponectin: a relevant player in PPARgamma-agonist-mediated improvements in hepatic insulin sensitivity? *Int.J.Obes.(Lond)* 29 Suppl 1: S17-23, 2005.

15. Bradley RL, Fisher FF and Maratos-Flier E. Dietary fatty acids differentially regulate production of TNF-alpha and IL-10 by murine 3T3-L1 adipocytes. *Obesity (Silver Spring)* 16: 5: 938-944, 2008.

16. Bruce CR, Mertz VA, Heigenhauser GJ and Dyck DJ. The stimulatory effect of globular adiponectin on insulin-stimulated glucose uptake and fatty acid oxidation is impaired in skeletal muscle from obese subjects. *Diabetes* 54: 11: 3154-3160, 2005.

17. Bueno AA, Oyama LM, de Oliveira C, Pisani LP, Ribeiro EB, Silveira VL and Oller do Nascimento CM. Effects of different fatty acids and dietary lipids on adiponectin gene expression in 3T3-L1 cells and C57BL/6J mice adipose tissue. *Pflugers Arch.* 455: 4: 701-709, 2008.

18. Calder PC. Polyunsaturated fatty acids and inflammation. *Prostaglandins Leukot.Essent.Fatty Acids* 75: 3: 197-202, 2006.

19. Carbo N, Lopez-Soriano J, Costelli P, Alvarez B, Busquets S, Baccino FM, Quinn LS, Lopez-Soriano FJ and Argiles JM. Interleukin-15 mediates reciprocal regulation of adipose

and muscle mass: a potential role in body weight control. *Biochim.Biophys.Acta* 1526: 1: 17-24, 2001.

20. Chambrier C, Bastard JP, Rieusset J, Chevillotte E, Bonnefont-Rousselot D, Therond P, Hainque B, Riou JP, Laville M and Vidal H. Eicosapentaenoic acid induces mRNA expression of peroxisome proliferator-activated receptor gamma. *Obes.Res.* 10: 6: 518-525, 2002.

21. Chen MB, McAinch AJ, Macaulay SL, Castelli LA, O'brien PE, Dixon JB, Cameron-Smith D, Kemp BE and Steinberg GR. Impaired activation of AMP-kinase and fatty acid oxidation by globular adiponectin in cultured human skeletal muscle of obese type 2 diabetics. *J.Clin.Endocrinol.Metab.* 90: 6: 3665-3672, 2005.

22. Civitarese AE, Jenkinson CP, Richardson D, Bajaj M, Cusi K, Kashyap S, Berria R, Belfort R, DeFronzo RA, Mandarino LJ and Ravussin E. Adiponectin receptors gene expression and insulin sensitivity in non-diabetic Mexican Americans with or without a family history of Type 2 diabetes. *Diabetologia* 47: 5: 816-820, 2004.

23. Coll T, Eyre E, Rodriguez-Calvo R, Palomer X, Sanchez RM, Merlos M, Laguna JC and Vazquez-Carrera M. Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. *J.Biol.Chem.* 283: 17: 11107-11116, 2008.

24. Combs TP, Berg AH, Obici S, Scherer PE and Rossetti L. Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J.Clin.Invest.* 108: 12: 1875-1881, 2001.

25. Combs TP, Wagner JA, Berger J, Doebber T, Wang WJ, Zhang BB, Tanen M, Berg AH, O'Rahilly S, Savage DB, Chatterjee K, Weiss S, Larson PJ, Gottesdiener KM, Gertz BJ, Charron MJ, Scherer PE and Moller DE. Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization. *Endocrinology* 143: 3: 998-1007, 2002.
26. Corbetta S, Redaelli A, Pozzi M, Bovo G, Ratti L, Redaelli E, Pellegrini C, Beck-Peccoz P and Spada A. Fibrosis is associated with adiponectin resistance in chronic hepatitis C virus infection. *Eur.J.Clin.Invest.* 41: 8: 898-905, 2011.
27. Day C. Thiazolidinediones: a new class of antidiabetic drugs. *Diabet.Med.* 16: 3: 179-192, 1999.
28. Debard C, Laville M, Berbe V, Loizon E, Guillet C, Morio-Liondore B, Boirie Y and Vidal H. Expression of key genes of fatty acid oxidation, including adiponectin receptors, in skeletal muscle of Type 2 diabetic patients. *Diabetologia* 47: 5: 917-925, 2004.
29. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J and Felber JP. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* 30: 12: 1000-1007, 1981.
30. Dietze D, Koenen M, Rohrig K, Horikoshi H, Hauner H and Eckel J. Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. *Diabetes* 51: 8: 2369-2376, 2002.

31. Dietze D, Ramrath S, Ritzeler O, Tennagels N, Hauner H and Eckel J. Inhibitor kappaB kinase is involved in the paracrine crosstalk between human fat and muscle cells. *Int.J.Obes.Relat.Metab.Disord.* 28: 8: 985-992, 2004.
32. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M and Eckel J. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 54: 7: 2003-2011, 2005.
33. Dimopoulos N, Watson M, Sakamoto K and Hundal HS. Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. *Biochem.J.* 399: 3: 473-481, 2006.
34. Dobrzyn A, Dobrzyn P, Miyazaki M and Ntambi JM. Polyunsaturated fatty acids do not activate AMP-activated protein kinase in mouse tissues. *Biochem.Biophys.Res.Comm.* 332: 3: 892-896, 2005.
35. Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF and Shulman GI. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J.Clin.Invest.* 103: 2: 253-259, 1999.
36. Dyck DJ. Adipokines as regulators of muscle metabolism and insulin sensitivity. *Appl.Physiol.Nutr.Metab.* 34: 3: 396-402, 2009.

37. Dyck DJ, Heigenhauser GJ and Bruce CR. The role of adipokines as regulators of skeletal muscle fatty acid metabolism and insulin sensitivity. *Acta Physiol.(Oxf)* 186: 1: 5-16, 2006.
38. Dyck DJ, Peters SJ, Glatz J, Gorski J, Keizer H, Kiens B, Liu S, Richter EA, Spriet LL, van der Vusse GJ and Bonen A. Functional differences in lipid metabolism in resting skeletal muscle of various fiber types. *Am.J.Physiol.* 272: 3 Pt 1: E340-51, 1997.
39. Estrany ME, Proenza AM, Llado I and Gianotti M. Isocaloric intake of a high-fat diet modifies adiposity and lipid handling in a sex dependent manner in rats. *Lipids Health.Dis.* 10: 52, 2011.
40. Fain JN, Madan AK, Hiler ML, Cheema P and Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 145: 5: 2273-2282, 2004.
41. Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J and Paschke R. Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem.Biophys.Res.Comm.* 301: 4: 1045-1050, 2003.
42. Fernandez-Quintela A, Churrua I and Portillo MP. The role of dietary fat in adipose tissue metabolism. *Public Health Nutr.* 10: 10A: 1126-1131, 2007.
43. Fernandez-Real JM, Vendrell J and Ricart W. Circulating adiponectin and plasma fatty acid profile. *Clin.Chem.* 51: 3: 603-609, 2005.

44. Flachs P, Mohamed-Ali V, Horakova O, Rossmeisl M, Hosseinzadeh-Attar MJ, Hensler M, Ruzickova J and Kopecky J. Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet. *Diabetologia* 49: 2: 394-397, 2006.
45. Folch J, Lees M and Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J.Biol.Chem.* 226: 1: 497-509, 1957.
46. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE and Lodish HF. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc.Natl.Acad.Sci.U.S.A.* 98: 4: 2005-2010, 2001.
47. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M and Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J.Clin.Invest.* 114: 12: 1752-1761, 2004.
48. Galic S, Oakhill JS and Steinberg GR. Adipose tissue as an endocrine organ. *Mol.Cell.Endocrinol.* 316: 2: 129-139, 2010.
49. Gonzalez-Periz A, Horrillo R, Ferre N, Gronert K, Dong B, Moran-Salvador E, Titos E, Martinez-Clemente M, Lopez-Parra M, Arroyo V and Claria J. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J.* 23: 6: 1946-1957, 2009.

50. Goodpaster BH, Katsiaras A and Kelley DE. Enhanced fat oxidation through physical activity is associated with improvements in insulin sensitivity in obesity. *Diabetes* 52: 9: 2191-2197, 2003.
51. Grundy SM. Obesity, metabolic syndrome, and cardiovascular disease. *J.Clin.Endocrinol.Metab.* 89: 6: 2595-2600, 2004.
52. Gulick T, Cresci S, Caira T, Moore DD and Kelly DP. The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. *Proc.Natl.Acad.Sci.U.S.A.* 91: 23: 11012-11016, 1994.
53. Gustafson B, Jack MM, Cushman SW and Smith U. Adiponectin gene activation by thiazolidinediones requires PPAR gamma 2, but not C/EBP alpha-evidence for differential regulation of the aP2 and adiponectin genes. *Biochem.Biophys.Res.Comm.* 308: 4: 933-939, 2003.
54. Haffner SM. Relationship of metabolic risk factors and development of cardiovascular disease and diabetes. *Obesity (Silver Spring)* 14 Suppl 3: 121S-127S, 2006.
55. Hammarstedt A, Sopasakis VR, Gogg S, Jansson PA and Smith U. Improved insulin sensitivity and adipose tissue dysregulation after short-term treatment with pioglitazone in non-diabetic, insulin-resistant subjects. *Diabetologia* 48: 1: 96-104, 2005.
56. Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H, Imai Y, Nagai R and Kadowaki T. Measurement of the high-molecular weight form of adiponectin in

plasma is useful for the prediction of insulin resistance and metabolic syndrome. *Diabetes Care* 29: 6: 1357-1362, 2006.

57. Hensler M, Bardova K, Jilkova ZM, Wahli W, Meztger D, Chambon P, Kopecky J and Flachs P. The inhibition of fat cell proliferation by n-3 fatty acids in dietary obese mice. *Lipids Health.Dis.* 10: 128, 2011.

58. Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC and Matsuzawa Y. Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes* 50: 5: 1126-1133, 2001.

59. Hu E, Liang P and Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J.Biol.Chem.* 271: 18: 10697-10703, 1996.

60. Huber J, Loffler M, Bilban M, Reimers M, Kadl A, Todoric J, Zeyda M, Geyeregger R, Schreiner M, Weichhart T, Leitinger N, Waldhausl W and Stulnig TM. Prevention of high-fat diet-induced adipose tissue remodeling in obese diabetic mice by n-3 polyunsaturated fatty acids. *Int.J.Obes.(Lond)* 31: 6: 1004-1013, 2007.

61. Hug C, Wang J, Ahmad NS, Bogan JS, Tsao TS and Lodish HF. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc.Natl.Acad.Sci.U.S.A.* 101: 28: 10308-10313, 2004.

62. Hulver MW, Berggren JR, Cortright RN, Dudek RW, Thompson RP, Pories WJ, MacDonald KG, Cline GW, Shulman GI, Dohm GL and Houmard JA. Skeletal muscle lipid metabolism with obesity. *Am.J.Physiol.Endocrinol.Metab.* 284: 4: E741-7, 2003.
63. Hwang LL, Wang CH, Li TL, Chang SD, Lin LC, Chen CP, Chen CT, Liang KC, Ho IK, Yang WS and Chiou LC. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. *Obesity (Silver Spring)* 18: 3: 463-469, 2010.
64. Itoh M, Suganami T, Satoh N, Tanimoto-Koyama K, Yuan X, Tanaka M, Kawano H, Yano T, Aoe S, Takeya M, Shimatsu A, Kuzuya H, Kamei Y and Ogawa Y. Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects. *Arterioscler.Thromb.Vasc.Biol.* 27: 9: 1918-1925, 2007.
65. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishima M and Shimomura I. Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes* 52: 7: 1655-1663, 2003.
66. Johnson JA, Trasino SE, Ferrante AW,Jr and Vasselli JR. Prolonged decrease of adipocyte size after rosiglitazone treatment in high- and low-fat-fed rats. *Obesity (Silver Spring)* 15: 11: 2653-2663, 2007.
67. Junkin KA, Dyck DJ, Mullen KL, Chabowski A and Thrush AB. Resistin acutely impairs insulin-stimulated glucose transport in rodent muscle in the presence, but not absence, of palmitate. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 296: 4: R944-51, 2009.

68. Kadowaki T and Yamauchi T. Adiponectin and adiponectin receptors. *Endocr.Rev.* 26: 3: 439-451, 2005.
69. Kalupahana NS, Claycombe K, Newman SJ, Stewart T, Siriwardhana N, Matthan N, Lichtenstein AH and Moustaid-Moussa N. Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation. *J.Nutr.* 140: 11: 1915-1922, 2010.
70. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K and Kasuga M. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J.Clin.Invest.* 116: 6: 1494-1505, 2006.
71. Kang ES, Park SY, Kim HJ, Ahn CW, Nam M, Cha BS, Lim SK, Kim KR and Lee HC. The influence of adiponectin gene polymorphism on the rosiglitazone response in patients with type 2 diabetes. *Diabetes Care* 28: 5: 1139-1144, 2005.
72. Kershaw EE and Flier JS. Adipose tissue as an endocrine organ. *J.Clin.Endocrinol.Metab.* 89: 6: 2548-2556, 2004.
73. Kim HJ, Higashimori T, Park SY, Choi H, Dong J, Kim YJ, Noh HL, Cho YR, Cline G, Kim YB and Kim JK. Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. *Diabetes* 53: 4: 1060-1067, 2004.

74. Kim HK, Della-Fera M, Lin J and Baile CA. Docosahexaenoic acid inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 preadipocytes. *J.Nutr.* 136: 12: 2965-2969, 2006.
75. Kim JY, Nolte LA, Hansen PA, Han DH, Ferguson K, Thompson PA and Holloszy JO. High-fat diet-induced muscle insulin resistance: relationship to visceral fat mass. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 279: 6: R2057-65, 2000.
76. Kondo K, Morino K, Nishio Y, Kondo M, Fuke T, Ugi S, Iwakawa H, Kashiwagi A and Maegawa H. Effects of a fish-based diet on the serum adiponectin concentration in young, non-obese, healthy Japanese subjects. *J.Atheroscler.Thromb.* 17: 6: 628-637, 2010.
77. Kratz M, Swarbrick MM, Callahan HS, Matthys CC, Havel PJ and Weigle DS. Effect of dietary n-3 polyunsaturated fatty acids on plasma total and high-molecular-weight adiponectin concentrations in overweight to moderately obese men and women. *Am.J.Clin.Nutr.* 87: 2: 347-353, 2008.
78. Krebs JD, Browning LM, McLean NK, Rothwell JL, Mishra GD, Moore CS and Jebb SA. Additive benefits of long-chain n-3 polyunsaturated fatty acids and weight-loss in the management of cardiovascular disease risk in overweight hyperinsulinaemic women. *Int.J.Obes.(Lond)* 30: 10: 1535-1544, 2006.
79. Kris-Etherton PM, Harris WS, Appel LJ and American Heart Association. Nutrition Committee. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* 106: 21: 2747-2757, 2002.

80. Kuda O, Jelenik T, Jilkova Z, Flachs P, Rossmeisl M, Hensler M, Kazdova L, Ogston N, Baranowski M, Gorski J, Janovska P, Kus V, Polak J, Mohamed-Ali V, Burcelin R, Cinti S, Bryhn M and Kopecky J. N-3 Fatty Acids and Rosiglitazone Improve Insulin Sensitivity through Additive Stimulatory Effects on Muscle Glycogen Synthesis in Mice Fed a High-Fat Diet. *Diabetologia* 52: 5: 941-951, 2009.
81. Lam YY, Hatzinikolas G, Weir JM, Janovska A, McAinch AJ, Game P, Meikle PJ and Wittert GA. Insulin-stimulated glucose uptake and pathways regulating energy metabolism in skeletal muscle cells: the effects of subcutaneous and visceral fat, and long-chain saturated, n-3 and n-6 polyunsaturated fatty acids. *Biochim.Biophys.Acta* 1811: 7-8: 468-475, 2011.
82. Lara JJ, Economou M, Wallace AM, Rumley A, Lowe G, Slater C, Caslake M, Sattar N and Lean ME. Benefits of salmon eating on traditional and novel vascular risk factors in young, non-obese healthy subjects. *Atherosclerosis* 193: 1: 213-221, 2007.
83. Lau BY, Ward WE, Kang JX and Ma DW. Femur EPA and DHA are correlated with femur biomechanical strength in young fat-1 mice. *J.Nutr.Biochem.* 20: 6: 453-461, 2009.
84. Lavigne C, Tremblay F, Asselin G, Jacques H and Marette A. Prevention of skeletal muscle insulin resistance by dietary cod protein in high fat-fed rats. *Am.J.Physiol.Endocrinol.Metab.* 281: 1: E62-71, 2001.
85. Le Foll C, Corporeau C, Le Guen V, Gouygou JP, Berge JP and Delarue J. Long-chain n-3 polyunsaturated fatty acids dissociate phosphorylation of Akt from

phosphatidylinositol 3'-kinase activity in rats. *Am.J.Physiol.Endocrinol.Metab.* 292: 4: E1223-30, 2007.

86. LeBrasseur NK, Kelly M, Tsao TS, Farmer SR, Saha AK, Ruderman NB and Tomas E. Thiazolidinediones can rapidly activate AMP-activated protein kinase in mammalian tissues. *Am.J.Physiol.Endocrinol.Metab.* 291: 1: E175-81, 2006.

87. Lee JY, Plakidas A, Lee WH, Heikkinen A, Chanmugam P, Bray G and Hwang DH. Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. *J.Lipid Res.* 44: 3: 479-486, 2003.

88. Lee JY, Sohn KH, Rhee SH and Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J.Biol.Chem.* 276: 20: 16683-16689, 2001.

89. Lefils J, Geloën A, Vidal H, Lagarde M and Bernoud-Hubac N. Dietary DHA: time course of tissue uptake and effects on cytokine secretion in mice. *Br.J.Nutr.* 104: 9: 1304-1312, 2010.

90. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM and Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J.Biol.Chem.* 270: 22: 12953-12956, 1995.

91. Lihn AS, Jessen N, Pedersen SB, Lund S and Richelsen B. AICAR stimulates adiponectin and inhibits cytokines in adipose tissue. *Biochem.Biophys.Res.Comm.* 316: 3: 853-858, 2004.

92. Lin HV, Kim JY, Poci A, Rossetti L, Shapiro L, Scherer PE and Accili D. Adiponectin resistance exacerbates insulin resistance in insulin receptor transgenic/knockout mice. *Diabetes* 56: 8: 1969-1976, 2007.
93. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Jr, Ory DS and Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc.Natl.Acad.Sci.U.S.A.* 100: 6: 3077-3082, 2003.
94. Lorente-Cebrian S, Bustos M, Marti A, Martinez JA and Moreno-Aliaga MJ. Eicosapentaenoic acid stimulates AMP-activated protein kinase and increases visfatin secretion in cultured murine adipocytes. *Clin.Sci.(Lond)* 117: 6: 243-249, 2009.
95. Lorente-Cebrian S, Perez-Matute P, Martinez JA, Marti A and Moreno-Aliaga MJ. Effects of eicosapentaenoic acid (EPA) on adiponectin gene expression and secretion in primary cultured rat adipocytes. *J.Physiol.Biochem.* 62: 2: 61-69, 2006.
96. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y and Matsubara K. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem.Biophys.Res.Comm.* 221: 2: 286-289, 1996.
97. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I and Matsuzawa Y. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 50: 9: 2094-2099, 2001.

98. Magnusson I, Rothman DL, Katz LD, Shulman RG and Shulman GI. Increased rate of gluconeogenesis in type II diabetes mellitus. A ^{13}C nuclear magnetic resonance study. *J.Clin.Invest.* 90: 4: 1323-1327, 1992.
99. Manickam E, Sinclair AJ and Cameron-Smith D. Suppressive actions of eicosapentaenoic acid on lipid droplet formation in 3T3-L1 adipocytes. *Lipids Health.Dis.* 9: 57, 2010.
100. Mao X, Kikani CK, Riojas RA, Langlais P, Wang L, Ramos FJ, Fang Q, Christ-Roberts CY, Hong JY, Kim RY, Liu F and Dong LQ. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. *Nat.Cell Biol.* 8: 5: 516-523, 2006.
101. Milanski M, Degasperi G, Coope A, Morari J, Denis R, Cintra DE, Tsukumo DM, Anhe G, Amaral ME, Takahashi HK, Curi R, Oliveira HC, Carvalheira JB, Bordin S, Saad MJ and Velloso LA. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. *J.Neurosci.* 29: 2: 359-370, 2009.
102. Mishra A, Chaudhary A and Sethi S. Oxidized omega-3 fatty acids inhibit NF-kappaB activation via a PPARalpha-dependent pathway. *Arterioscler.Thromb.Vasc.Biol.* 24: 9: 1621-1627, 2004.
103. Miyazaki Y and DeFronzo RA. Rosiglitazone and pioglitazone similarly improve insulin sensitivity and secretion, glucose tolerance and adipocytokines in type 2 diabetic patients. *Diabetes Obes.Metab.* 10: 12: 1204-1211, 2008.

104. Moon B, Kwan JJ, Duddy N, Sweeney G and Begum N. Resistin inhibits glucose uptake in L6 cells independently of changes in insulin signaling and GLUT4 translocation. *Am.J.Physiol.Endocrinol.Metab.* 285: 1: E106-15, 2003.
105. Moro C, Galgani JE, Luu L, Pasarica M, Mairal A, Bajpeyi S, Schmitz G, Langin D, Liebisch G and Smith SR. Influence of gender, obesity, and muscle lipase activity on intramyocellular lipids in sedentary individuals. *J.Clin.Endocrinol.Metab.* 94: 9: 3440-3447, 2009.
106. Motawi TM, Hashem RM, Rashed LA and El-Razek SM. Comparative study between the effect of the peroxisome proliferator activated receptor-alpha ligands fenofibrate and n-3 polyunsaturated fatty acids on activation of 5'-AMP-activated protein kinase-alpha1 in high-fat fed rats. *J.Pharm.Pharmacol.* 61: 10: 1339-1346, 2009.
107. Motoshima H, Wu X, Sinha MK, Hardy VE, Rosato EL, Barbot DJ, Rosato FE and Goldstein BJ. Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone. *J.Clin.Endocrinol.Metab.* 87: 12: 5662-5667, 2002.
108. Mullen KL, Pritchard J, Ritchie I, Snook LA, Chabowski A, Bonen A, Wright D and Dyck DJ. Adiponectin resistance precedes the accumulation of skeletal muscle lipids and insulin resistance in high-fat-fed rats. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 296: 2: R243-51, 2009.

109. Mullen KL, Smith AC, Junkin KA and Dyck DJ. Globular adiponectin resistance develops independently of impaired insulin-stimulated glucose transport in soleus muscle from high-fat-fed rats. *Am.J.Physiol.Endocrinol.Metab.* 293: 1: E83-90, 2007.
110. Mullen KL, Tishinsky JM, Robinson LE and Dyck DJ. Skeletal muscle inflammation is not responsible for the rapid impairment in adiponectin response with high-fat feeding in rats. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 299: 2: R500-8, 2010.
111. Muurling M, Mensink RP, Pijl H, Romijn JA, Havekes LM and Voshol PJ. A fish oil diet does not reverse insulin resistance despite decreased adipose tissue TNF-alpha protein concentration in ApoE-3*Leiden mice. *J.Nutr.* 133: 11: 3350-3355, 2003.
112. Nawrocki AR, Rajala MW, Tomas E, Pajvani UB, Saha AK, Trumbauer ME, Pang Z, Chen AS, Ruderman NB, Chen H, Rossetti L and Scherer PE. Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists. *J.Biol.Chem.* 281: 5: 2654-2660, 2006.
113. Neschen S, Morino K, Rossbacher JC, Pongratz RL, Cline GW, Sono S, Gillum M and Shulman GI. Fish oil regulates adiponectin secretion by a peroxisome proliferator-activated receptor-gamma-dependent mechanism in mice. *Diabetes* 55: 4: 924-928, 2006.
114. Nielsen AR, Hojman P, Erikstrup C, Fischer CP, Plomgaard P, Mounier R, Mortensen OH, Broholm C, Taudorf S, Krogh-Madsen R, Lindegaard B, Petersen AM, Gehl J and Pedersen BK. Association between interleukin-15 and obesity: interleukin-15 as a potential regulator of fat mass. *J.Clin.Endocrinol.Metab.* 93: 11: 4486-4493, 2008.

115. Nieto-Vazquez I, Fernandez-Veledo S, de Alvaro C and Lorenzo M. Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. *Diabetes* 57: 12: 3211-3221, 2008.
116. Nomura S, Shouzu A, Omoto S, Inami N, Ueba T, Urase F and Maeda Y. Effects of eicosapentaenoic acid on endothelial cell-derived microparticles, angiopoietins and adiponectin in patients with type 2 diabetes. *J.Atheroscler.Thromb.* 16: 2: 83-90, 2009.
117. Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiwara T, Horikoshi H, Yazaki Y and Kadowaki T. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *J.Clin.Invest.* 101: 6: 1354-1361, 1998.
118. Oller do Nascimento CM, Ribeiro EB and Oyama LM. Metabolism and secretory function of white adipose tissue: effect of dietary fat. *An.Acad.Bras.Cienc.* 81: 3: 453-466, 2009.
119. Oster RT, Tishinsky JM, Yuan Z and Robinson LE. Docosahexaenoic acid increases cellular adiponectin mRNA and secreted adiponectin protein, as well as PPARgamma mRNA, in 3T3-L1 adipocytes. *Appl.Physiol.Nutr.Metab.* 35: 6: 783-789, 2010.
120. Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA and Scherer PE. Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J.Biol.Chem.* 279: 13: 12152-12162, 2004.

121. Pan DA, Lillioja S, Kriketos AD, Milner MR, Baur LA, Bogardus C, Jenkins AB and Storlien LH. Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 46: 6: 983-988, 1997.
122. Pedersen BK, Steensberg A, Keller P, Keller C, Fischer C, Hiscock N, van Hall G, Plomgaard P and Febbraio MA. Muscle-derived interleukin-6: lipolytic, anti-inflammatory and immune regulatory effects. *Pflugers Arch.* 446: 1: 9-16, 2003.
123. Perez-Matute P, Perez-Echarri N, Martinez JA, Marti A and Moreno-Aliaga MJ. Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: role of apoptosis, adiponectin and tumour necrosis factor-alpha. *Br.J.Nutr.* 97: 2: 389-398, 2007.
124. Perusse L and Bouchard C. Gene-diet interactions in obesity. *Am.J.Clin.Nutr.* 72: 5 Suppl: 1285S-1290S, 2000.
125. Philippi M and Sillau AH. Oxidative capacity distribution in skeletal muscle fibers of the rat. *J.Exp.Biol.* 189: 1-11, 1994.
126. Phillips SA, Ciaraldi TP, Kong AP, Bandukwala R, Aroda V, Carter L, Baxi S, Mudaliar SR and Henry RR. Modulation of circulating and adipose tissue adiponectin levels by antidiabetic therapy. *Diabetes* 52: 3: 667-674, 2003.
127. Phillips SA, Kung J, Ciaraldi TP, Choe C, Christiansen L, Mudaliar S and Henry RR. Selective regulation of cellular and secreted multimeric adiponectin by antidiabetic therapies in humans. *Am.J.Physiol.Endocrinol.Metab.* 297: 3: E767-73, 2009.

128. Podolin DA, Gayles EC, Wei Y, Thresher JS and Pagliassotti MJ. Menhaden oil prevents but does not reverse sucrose-induced insulin resistance in rats. *Am.J.Physiol.* 274: 3 Pt 2: R840-8, 1998.
129. Powell DJ, Turban S, Gray A, Hajduch E and Hundal HS. Intracellular ceramide synthesis and protein kinase C ζ activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. *Biochem.J.* 382: Pt 2: 619-629, 2004.
130. Puglisi MJ, Hasty AH and Saraswathi V. The role of adipose tissue in mediating the beneficial effects of dietary fish oil. *J.Nutr.Biochem.* 22: 2: 101-108, 2011.
131. Radin MS, Sinha S, Bhatt BA, Dedousis N and O'Doherty RM. Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle. *Diabetologia* 51: 2: 336-346, 2008.
132. Rasouli N and Kern PA. Adipocytokines and the metabolic complications of obesity. *J.Clin.Endocrinol.Metab.* 93: 11 Suppl 1: S64-73, 2008.
133. Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, Cersosimo E, Defronzo RA, Coletta DK, Sriwijitkamol A and Musi N. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. *Diabetes* 57: 10: 2595-2602, 2008.
134. Ritchie IR, Gulli RA, Stefanyk LE, Harasim E, Chabowski A and Dyck DJ. Restoration of skeletal muscle leptin response does not precede the exercise-induced

recovery of insulin-stimulated glucose uptake in high-fat-fed rats.

Am.J.Physiol.Regul.Integr.Comp.Physiol. 300: 2: R492-500, 2011.

135. Robinson LE, Buchholz AC and Mazurak VC. Inflammation, obesity, and fatty acid metabolism: influence of n-3 polyunsaturated fatty acids on factors contributing to metabolic syndrome. *Appl.Physiol.Nutr.Metab.* 32: 6: 1008-1024, 2007.

136. Rossi AS, Lombardo YB, Lacorte JM, Chicco AG, Rouault C, Slama G and Rizkalla SW. Dietary fish oil positively regulates plasma leptin and adiponectin levels in sucrose-fed, insulin-resistant rats. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 289: 2: R486-R494, 2005.

137. Saravanan N, Haseeb A, Ehtesham NZ and Ghafoorunissa. Differential effects of dietary saturated and trans-fatty acids on expression of genes associated with insulin sensitivity in rat adipose tissue. *Eur.J.Endocrinol.* 153: 1: 159-165, 2005.

138. Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, Kopp A, Schoelmerich J and Falk W. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. *Immunology* 126: 2: 233-245, 2009.

139. Scherer PE, Williams S, Fogliano M, Baldini G and Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J.Biol.Chem.* 270: 45: 26746-26749, 1995.

140. Schoonjans K, Staels B and Auwerx J. The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. *Biochim.Biophys.Acta* 1302: 2: 93-109, 1996.
141. Sell H, Dietze-Schroeder D, Kaiser U and Eckel J. Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. *Endocrinology* 147: 5: 2458-2467, 2006.
142. Sell H, Eckardt K, Taube A, Tews D, Gurgui M, Van Echten-Deckert G and Eckel J. Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility. *Am.J.Physiol.Endocrinol.Metab.* 294: 6: E1070-7, 2008.
143. Sell H, Eckel J and Dietze-Schroeder D. Pathways leading to muscle insulin resistance--the muscle--fat connection. *Arch.Physiol.Biochem.* 112: 2: 105-113, 2006.
144. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H and Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J.Clin.Invest.* 116: 11: 3015-3025, 2006.
145. Shimizu A, Takamura T, Matsuzawa N, Nakamura S, Nabemoto S, Takeshita Y, Misu H, Kurita S, Sakurai M, Yokoyama M, Zen Y, Sasaki M, Nakanuma Y and Kaneko S. Regulation of adiponectin receptor expression in human liver and a hepatocyte cell line. *Metabolism* 56: 11: 1478-1485, 2007.
146. Sinha S, Perdomo G, Brown NF and O'Doherty RM. Fatty acid-induced insulin resistance in L6 myotubes is prevented by inhibition of activation and nuclear localization of nuclear factor kappa B. *J.Biol.Chem.* 279: 40: 41294-41301, 2004.

147. Skurk T, Alberti-Huber C and Hauner H. Effect of conditioned media from mature human adipocytes on insulin-stimulated Akt/PKB phosphorylation in human skeletal muscle cells: role of BMI and fat cell size. *Horm.Metab.Res.* 41: 3: 190-196, 2009.
148. Skurk T, Alberti-Huber C, Herder C and Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J.Clin.Endocrinol.Metab.* 92: 3: 1023-1033, 2007.
149. Slater J, Green C, Sevenhuysen G, O'Neil J and Edginton B. Socio-demographic and geographic analysis of overweight and obesity in Canadian adults using the Canadian Community Health Survey (2005). *Chronic Dis.Can.* 30: 1: 4-15, 2009.
150. Soares AF, Guichardant M, Cozzone D, Bernoud-Hubac N, Bouzaidi-Tiali N, Lagarde M and Geloën A. Effects of oxidative stress on adiponectin secretion and lactate production in 3T3-L1 adipocytes. *Free Radic.Biol.Med.* 38: 7: 882-889, 2005.
151. Staiger H, Kaltenbach S, Staiger K, Stefan N, Fritsche A, Guirguis A, Peterfi C, Weisser M, Machicao F, Stumvoll M and Haring HU. Expression of adiponectin receptor mRNA in human skeletal muscle cells is related to in vivo parameters of glucose and lipid metabolism. *Diabetes* 53: 9: 2195-2201, 2004.
152. Steensberg A, Keller C, Starkie RL, Osada T, Febbraio MA and Pedersen BK. IL-6 and TNF- α expression in, and release from, contracting human skeletal muscle. *Am.J.Physiol.Endocrinol.Metab.* 283: 6: E1272-8, 2002.

153. Steinberg GR and Dyck DJ. Development of leptin resistance in rat soleus muscle in response to high-fat diets. *Am.J.Physiol.Endocrinol.Metab.* 279: 6: E1374-82, 2000.
154. Steinberg GR, Parolin ML, Heigenhauser GJ and Dyck DJ. Leptin increases FA oxidation in lean but not obese human skeletal muscle: evidence of peripheral leptin resistance. *Am.J.Physiol.Endocrinol.Metab.* 283: 1: E187-92, 2002.
155. Storlien LH, Kraegen EW, Chisholm DJ, Ford GL, Bruce DG and Pascoe WS. Fish oil prevents insulin resistance induced by high-fat feeding in rats. *Science* 237: 4817: 885-888, 1987.
156. Suganami T, Nishida J and Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler.Thromb.Vasc.Biol.* 25: 10: 2062-2068, 2005.
157. Sun J, Ramnath RD, Zhi L, Tamizhselvi R and Bhatia M. Substance P enhances NF-kappaB transactivation and chemokine response in murine macrophages via ERK1/2 and p38 MAPK signaling pathways. *Am.J.Physiol.Cell.Physiol.* 294: 6: C1586-96, 2008.
158. Szkudelski T, Nogowski L and Szkudelska K. Short-term regulation of adiponectin secretion in rat adipocytes. *Physiol.Res.* 60: 3: 521-530, 2011.
159. Taouis M, Dagou C, Ster C, Durand G, Pinault M and Delarue J. N-3 polyunsaturated fatty acids prevent the defect of insulin receptor signaling in muscle. *Am.J.Physiol.Endocrinol.Metab.* 282: 3: E664-71, 2002.

160. Thrush AB, Harasim E, Chabowski A, Gulli R, Stefanyk L and Dyck DJ. A single prior bout of exercise protects against palmitate-induced insulin resistance despite an increase in total ceramide content. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 300: 5: R1200-8, 2011.
161. Thrush AB, Heigenhauser GJ, Mullen KL, Wright DC and Dyck DJ. Palmitate acutely induces insulin resistance in isolated muscle from obese but not lean humans. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 294: 4: R1205-12, 2008.
162. Todoric J, Loffler M, Huber J, Bilban M, Reimers M, Kadl A, Zeyda M, Waldhausl W and Stulnig TM. Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia* 49: 9: 2109-2119, 2006.
163. Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang Cc C, Itani SI, Lodish HF and Ruderman NB. Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc.Natl.Acad.Sci.U.S.A.* 99: 25: 16309-16313, 2002.
164. Trayhurn P and Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br.J.Nutr.* 92: 3: 347-355, 2004.
165. Trinchieri G and Sher A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat.Rev.Immunol.* 7: 3: 179-190, 2007.
166. Tsuchida A, Yamauchi T, Ito Y, Hada Y, Maki T, Takekawa S, Kamon J, Kobayashi M, Suzuki R, Hara K, Kubota N, Terauchi Y, Froguel P, Nakae J, Kasuga M, Accili D,

Tobe K, Ueki K, Nagai R and Kadowaki T. Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. *J.Biol.Chem.* 279: 29: 30817-30822, 2004.

167. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, Araujo EP, Vassallo J, Curi R, Velloso LA and Saad MJ. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* 56: 8: 1986-1998, 2007.

168. Turner N, Bruce CR, Beale SM, Hoehn KL, So T, Rolph MS and Cooney GJ. Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. *Diabetes* 56: 8: 2085-2092, 2007.

169. van Dijk SJ, Feskens EJ, Bos MB, Hoelen DW, Heijligenberg R, Bromhaar MG, de Groot LC, de Vries JH, Muller M and Afman LA. A saturated fatty acid-rich diet induces an obesity-linked proinflammatory gene expression profile in adipose tissue of subjects at risk of metabolic syndrome. *Am.J.Clin.Nutr.* 90: 6: 1656-1664, 2009.

170. van Hall G, Steensberg A, Sacchetti M, Fischer C, Keller C, Schjerling P, Hiscock N, Moller K, Saltin B, Febbraio MA and Pedersen BK. Interleukin-6 stimulates lipolysis and fat oxidation in humans. *J.Clin.Endocrinol.Metab.* 88: 7: 3005-3010, 2003.

171. Vargas ML, Almario RU, Buchan W, Kim K and Karakas SE. Metabolic and endocrine effects of long-chain versus essential omega-3 polyunsaturated fatty acids in polycystic ovary syndrome. *Metabolism* 2011.

172. Vessby B, Uusitupa M, Hermansen K, Riccardi G, Rivellese AA, Tapsell LC, Nalsen C, Berglund L, Louheranta A, Rasmussen BM, Calvert GD, Maffetone A, Pedersen E, Gustafsson IB, Storlien LH and KANWU Study. Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study. *Diabetologia* 44: 3: 312-319, 2001.
173. Vozarova B, Weyer C, Hanson K, Tataranni PA, Bogardus C and Pratley RE. Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. *Obes.Res.* 9: 7: 414-417, 2001.
174. Wallberg-Henriksson H, Zetan N and Henriksson J. Reversibility of decreased insulin-stimulated glucose transport capacity in diabetic muscle with in vitro incubation. Insulin is not required. *J.Biol.Chem.* 262: 16: 7665-7671, 1987.
175. Wander RC and Du SH. Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids. *Am.J.Clin.Nutr.* 72: 3: 731-737, 2000.
176. Wang C, Xin X, Xiang R, Ramos FJ, Liu M, Lee HJ, Chen H, Mao X, Kikani CK, Liu F and Dong LQ. Yin-Yang regulation of adiponectin signaling by APPL isoforms in muscle cells. *J.Biol.Chem.* 284: 46: 31608-31615, 2009.
177. Wang HH, Hung TM, Wei J and Chiang AN. Fish oil increases antioxidant enzyme activities in macrophages and reduces atherosclerotic lesions in apoE-knockout mice. *Cardiovasc.Res.* 61: 1: 169-176, 2004.

178. Wang P, Renes J, Bouwman F, Bunschoten A, Mariman E and Keijer J. Absence of an adipogenic effect of rosiglitazone on mature 3T3-L1 adipocytes: increase of lipid catabolism and reduction of adipokine expression. *Diabetologia* 50: 3: 654-665, 2007.
179. Wang Y, Lam KS, Yau MH and Xu A. Post-translational modifications of adiponectin: mechanisms and functional implications. *Biochem.J.* 409: 3: 623-633, 2008.
180. Wang Y, Xu A, Knight C, Xu LY and Cooper GJ. Hydroxylation and glycosylation of the four conserved lysine residues in the collagenous domain of adiponectin. Potential role in the modulation of its insulin-sensitizing activity. *J.Biol.Chem.* 277: 22: 19521-19529, 2002.
181. Wang ZV, Schraw TD, Kim JY, Khan T, Rajala MW, Follenzi A and Scherer PE. Secretion of the adipocyte-specific secretory protein adiponectin critically depends on thiol-mediated protein retention. *Mol.Cell.Biol.* 27: 10: 3716-3731, 2007.
182. Weigert C, Brodbeck K, Staiger H, Kausch C, Machicao F, Haring HU and Schleicher ED. Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-kappaB. *J.Biol.Chem.* 279: 23: 23942-23952, 2004.
183. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL and Ferrante AW,Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J.Clin.Invest.* 112: 12: 1796-1808, 2003.

184. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE and Tataranni PA. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J.Clin.Endocrinol.Metab.* 86: 5: 1930-1935, 2001.
185. Wilkes JJ, Bonen A and Bell RC. A modified high-fat diet induces insulin resistance in rat skeletal muscle but not adipocytes. *Am.J.Physiol.* 275: 4 Pt 1: E679-86, 1998.
186. Wright HM, Clish CB, Mikami T, Hauser S, Yanagi K, Hiramatsu R, Serhan CN and Spiegelman BM. A synthetic antagonist for the peroxisome proliferator-activated receptor gamma inhibits adipocyte differentiation. *J.Biol.Chem.* 275: 3: 1873-1877, 2000.
187. Xi L, Qian Z, Xu G, Zhou C and Sun S. Crocetin attenuates palmitate-induced insulin insensitivity and disordered tumor necrosis factor-alpha and adiponectin expression in rat adipocytes. *Br.J.Pharmacol.* 151: 5: 610-617, 2007.
188. Yamagishi K, Iso H, Yatsuya H, Tanabe N, Date C, Kikuchi S, Yamamoto A, Inaba Y, Tamakoshi A and JACC Study Group. Dietary intake of saturated fatty acids and mortality from cardiovascular disease in Japanese: the Japan Collaborative Cohort Study for Evaluation of Cancer Risk (JACC) Study. *Am.J.Clin.Nutr.* 92: 4: 759-765, 2010.
189. Yamamoto K, Itoh T, Abe D, Shimizu M, Kanda T, Koyama T, Nishikawa M, Tamai T, Ooizumi H and Yamada S. Identification of putative metabolites of docosahexaenoic acid as potent PPARgamma agonists and antidiabetic agents. *Bioorg.Med.Chem.Lett.* 15: 3: 517-522, 2005.

190. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R and Kadowaki T. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423: 6941: 762-769, 2003.
191. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB and Kadowaki T. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat.Med.* 8: 11: 1288-1295, 2002.
192. Yamauchi T, Kamon J, Waki H, Murakami K, Motojima K, Komeda K, Ide T, Kubota N, Terauchi Y, Tobe K, Miki H, Tsuchida A, Akanuma Y, Nagai R, Kimura S and Kadowaki T. The mechanisms by which both heterozygous peroxisome proliferator-activated receptor gamma (PPARgamma) deficiency and PPARgamma agonist improve insulin resistance. *J.Biol.Chem.* 276: 44: 41245-41254, 2001.
193. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P and Kadowaki T. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat.Med.* 7: 8: 941-946, 2001.

194. Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, Kawamoto S, Kubota N, Kubota T, Ito Y, Kamon J, Tsuchida A, Kumagai K, Kozono H, Hada Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Awazawa M, Takamoto I, Froguel P, Hara K, Tobe K, Nagai R, Ueki K and Kadowaki T. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nat.Med.* 13: 3: 332-339, 2007.
195. Yamazaki Y, Kawano Y and Uebayasi M. Induction of adiponectin by natural and synthetic phenolamides in mouse and human preadipocytes and its enhancement by docosahexaenoic acid. *Life Sci.* 82: 5-6: 290-300, 2008.
196. Yaspelkis BB,3rd, Kvasha IA and Figueroa TY. High-fat feeding increases insulin receptor and IRS-1 coimmunoprecipitation with SOCS-3, IKKalpha/beta phosphorylation and decreases PI-3 kinase activity in muscle. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 296: 6: R1709-15, 2009.
197. Yoon MJ, Lee GY, Chung JJ, Ahn YH, Hong SH and Kim JB. Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha. *Diabetes* 55: 9: 2562-2570, 2006.
198. Youd JM, Rattigan S and Clark MG. Acute impairment of insulin-mediated capillary recruitment and glucose uptake in rat skeletal muscle in vivo by TNF-alpha. *Diabetes* 49: 11: 1904-1909, 2000.

199. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW and Shulman GI. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J.Biol.Chem.* 277: 52: 50230-50236, 2002.
200. Yu JG, Javorschi S, Hevener AL, Kruszynska YT, Norman RA, Sinha M and Olefsky JM. The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes* 51: 10: 2968-2974, 2002.
201. Yudkin JS, Kumari M, Humphries SE and Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* 148: 2: 209-214, 2000.
202. Zappala G and Rechler MM. IGFBP-3, hypoxia and TNF-alpha inhibit adiponectin transcription. *Biochem.Biophys.Res.Comm.* 382: 4: 785-789, 2009.

Appendix A

Western Blotting Conditions:

Protein	Molecular Weight	Gel	Block	Primary Antibody	Secondary Antibody
APPL1	82	10%	5% NFM	1:1000 5% NFM	1:2000 rabbit
AMPK	64	10%	5% BSA	1:1000 2.5% BSA	1:2000 rabbit
ACC	260	6%	2.5% BSA	1:1000 2.5% BSA	1:2000 rabbit
Akt	60	10%	5% BSA	1:2000 5% BSA	1:2000 rabbit
pAkt	60	10%	5% BSA	1:2000 5% BSA	1:2000 rabbit
TLR4	110	10%	5% BSA	1: 250 5% BSA	1:2000 rabbit
IκBα	39	10%	5% NFM	1:500 5% NFM	1:2000 mouse
pIκBα	41	10%	5% BSA	1:500 5% BSA (48 hrs)	1:2000 rabbit
NF-κB	65	10%	5% BSA	1:2000 5% BSA	1:2000 rabbit
pNF-κB	65	10%	5% BSA	1:1000 5% BSA	1:2000 rabbit
SOCS3	26	10%	5% BSA	1:1000 5% BSA	1:2000 rabbit

NFM = non-fat milk; BSA = bovine serum albumin.

All primary antibody incubations are overnight unless otherwise stated.

Appendix B

Adiponectin concentrations in adipose-conditioned media from rodent adipose tissue explants treated with 500 μ M EPA or DHA for 48 hours.

Data are mean $\pm$ SEM, n = 6.

CON = control; EPA = eicosapentaenoic acid; DHA= docosahexaenoic acid.

EPA and DHA treatments were complexed with 100 μ M of bovine serum albumin (BSA). CON condition contained 100 μ M only.

Secreted adiponectin in the medium was quantified by ELISA.

Appendix C

Protein concentrations of A) adiponectin, B) leptin, C) IL-6, and D) MCP-1 in adipose-conditioned medium from rodent adipose tissue explants standardized by mass from animals fed CON, SFA, or SFA/n-3 PUFA for 4 weeks.

Data are mean $\pm$ SEM, n = 8.

* = significantly different from LF at the same timepoint, $p \leq 0.05$.

LF = low-fat diet; SFA = high saturated fatty acid diet; SFA/n-3 PUFA = high SFA with n-3 polyunsaturated fatty acids diet.

Adiponectin concentrations in the medium were quantified by ELISA, and secreted leptin, IL-6, and MCP-1 concentrations in the medium were quantified by

Appendix D

Total protein content of perigonadal adipose tissue explants from animals fed LF, SFA, or SFA/n-3 PUFA for 4 weeks.

Data are mean $\pm$ SEM, n = 12.

* = significantly different from LF, $p \leq 0.05$.

LF = low-fat diet; SFA = high saturated fatty acid diet; SFA/n-3 PUFA = high SFA with n-3 polyunsaturated fatty acids diet.

Total protein was determined by a bicinchoninic acid protein assay.

Appendix E

Timecourse of A) adiponectin, B) leptin, C) IL-6, and D) MCP-1 protein concentrations in adipose-conditioned medium from rodent adipose tissue explants from animals fed CON, SFA, or SFA/n-3 PUFA for 4 weeks.

Data are mean $\pm$ SEM, n = 6

* = significantly different from LF at the same timepoint, $p \leq 0.05$.

LF = low-fat diet; SFA = high saturated fatty acid diet; SFA/n-3 PUFA = high SFA with n-3 polyunsaturated fatty acids diet.

Adiponectin concentrations were quantified by ELISA; leptin, IL-6, and MCP-1 concentrations were quantified by Bio-Plex.

Appendix F

Basal and insulin-stimulated glucose transport in soleus muscle incubated in CON, ACM LF, ACM SFA, or ACM SFA/n-3 PUFA for 4 hours.

Values are means $\pm$ SEM, n=8.

* = significantly different from basal within the same treatment group, $p \leq 0.05$.

White bars = basal, Black bars = insulin-stimulated (0.5 mU/mL).

CON = control buffer; ACM LF = adipose conditioned medium from animals fed a low-fat diet; ACM SFA = adipose conditioned medium from animals fed a high saturated fatty acid diet; ACM SFA/n-3 PUFA = adipose conditioned medium from animals fed a high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

Appendix G

Changes in ATP and PCr content in soleus and EDL muscle incubated for 8 hours in M199.

	Soleus		EDL	
	Baseline	8 hrs	Baseline	8 hrs
ATP (nmol/g dry weight)	13.11	8.53	17.54	9.83
PCr (nmol/g dry weight)	30.89	21.79	41.12	21.44