Development of obesity-induced inflammation and insulin resistance: The role of adipose tissue, fatty acids, and toll-like receptors

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Development of obesity-induced inflammation and insulin resistance: The role of adipose tissue, fatty acids, and toll-like receptors

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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The innate immune receptors, toll-like receptor-2 (Tlr2) and toll-like receptor-4 (Tlr4), are implicated in the development of inflammation and insulin resistance in various cell populations. However, little is known about the relationship of these receptors in diet-induced obesity (DIO). We have shown that Tlr4 deficient C57BL/10ScN mice were protected against DIO, specifically when mice were fed a high saturated fatty acid (SFA) diet. Moreover, these mice exhibited a specific reduction in adipose tissue (AT) inflammation as evidenced by the reduction in nuclear factor kappa B (NFκB) activation, proinflammatory gene expression, and macrophage accumulation. There was also a marked increase in Thr2 expression in mice fed a high fat diet. 3T3-L1 adipocytes treated with the Tlr2 agonist, zymosan A (ZymA), exhibited increased NFκB and activator protein-1 (AP-1) activation, proinflammatory gene expression, reactive oxygen species (ROS) accumulation, and insulin resistance, similarly to palmitate or Tlr4 agonist lipopolysaccharide (LPS). These effects were attenuated in adipocytes treated with a specific c-jun N-terminal kinase (JNK) inhibitor (SP600125) indicating involvement of this intracellular kinase in SFA and Tlr agonist-mediated signaling. Finally, we demonstrated that Tlr2−/− mice were also protected from DIO. However, unlike Tlr4 deficient 10ScN mice there were no significant changes in body weight, energy intake, or lipid profile. Inflammatory and oxidative stress markers in serum (C-reactive protein (CRP), endotoxin, and monocyte chemoattractant protein-1 (MCP-1)) and AT (Tumor necrosis factor α (TNFα), interleukin-6 (IL-6), MCP-1, F4/80, and nitric oxide synthase-2 (NOS2) transcript abundance) were attenuated in Tlr2−/− and 10ScN mice, which was more apparent in mice fed high SFA diet. Insulin sensitivity was improved in 10ScN, but not Tlr2−/− mice. However, stromal vascular (SV) cells isolated from AT of Tlr2−/−
mice had a marked increase in insulin sensitivity, demonstrating the potential involvement of Tlr2 in obesity-induced insulin resistance in AT. Based on these studies, Tlr2 and Tlr4 may represent ideal targets for pharmaceutical, as well as dietary, interventions designed to attenuate obesity and related co-morbidities, including Type 2 diabetes mellitus (DM) and cardiovascular disease (CVD).
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Currently, approximately 60 million adults in the U.S. are classified as obese (BMI ≥ 30), and equally alarming, the prevalence of obesity has increased 30% during the past decade (1). The medical and economic implications of obesity are exacerbated by its co-morbidities, which include diabetes mellitus (DM) and atherosclerotic cardiovascular disease (CVD). Generally, a chronic inflammatory state develops in association with obesity, and likely contributes to the development of insulin resistance and its progression to DM (2,3). This chronic inflammation associated with obesity is evident by the increased concentrations of circulating proinflammatory cytokines, including tumor necrosis factorα (TNFα) and interleukin-6 (IL-6) (4,5). The exact mechanisms which regulate this inflammation are not yet clearly defined, but recent evidence has unequivocally implicated the adipocyte and its inflammatory pathways as pivotal factors in the development of insulin resistance (6,7).

Several genetic and diet-induced rodent models of obesity have revealed that obesity promotes increased expression of proinflammatory cytokines in adipose tissue (AT) (2,8), and that this may be mediated by activation of nuclear factor kappa B (NFκB) or activator protein-1 (AP-1) signaling pathways (9,10). Furthermore, obesity-linked inflammation parallels changes in adipose cell populations and phenotypes as AT is expanded through hyperplasia and hypertrophy. Excessive adipocyte hypertrophy initiates changes in the inflammatory characteristics of the adipocyte that include greater NFκB transcriptional activity and increased expression of proinflammatory cytokines (11). Excessive adipocyte hypertrophy has also been associated with increased cell death and recruitment of macrophages to AT (6,7). Macrophage infiltration represents a major contributor to obesity-
induced inflammation and is partially regulated by the chemokine, monocyte chemoattractant protein-1 (MCP-1) (12,13), which is known to be increased in the circulation of obese subjects (14).

Furthermore, innate immunity may be a primary mediator of obesity-induced inflammation in AT. Toll-like receptors (TLRs) represent key innate immune receptors that are expressed in adipocytes, preadipocytes, and macrophages (15-17). Interestingly, both Tlr2 and Tlr4 expression are increased with obesity and DM (16). Collectively, recent findings indicate lipopolysaccharide (LPS)-stimulated Tlr4 activation induces expression of TNFα, IL-6, and MCP-1 in adipocytes and preadipocytes through a NFκB dependent pathway (15,18,19). LPS also increased Tlr2 transcript abundance in adipocytes (20). The interaction between the LPS molecule and Tlr4 is mediated through the lipid A component of LPS, which consists of several fatty acids attached to a phosphate and a central glucosamine dimer (21). The fact that fatty acids can directly interact with Tlr receptors and stimulate downstream signaling pathways that lead to inflammation is therefore not surprising (Figure 1). Palmitate and other saturated fatty acids (SFA) clearly stimulate proinflammatory cytokine expression and NFκB activation in adipocytes (22).

Several recent investigations (23-25) have provided evidence linking obesity-induced inflammation to fatty acid signaling through Tlr4. Shi and colleagues (25) have shown that Tlr4−/− mice have improved insulin sensitivity and attenuated inflammatory markers in AT when stimulated with either lipid infusion or a high fat diet (25). These data are further supported by in vitro experiments in which free fatty acid (FFA)-induced inflammatory responses in adipocytes are attenuated when Tlr4 signaling is blunted by siRNA strategies. More recently, Lee et al. (26) observed that SFA, but not polyunsaturated fatty acids
(PUFA), activated Tlr2 dimerization and activated stress-linked kinases, such as NFκB and AP-1, in RAW264.7 macrophages. Thomas and colleagues (27) also demonstrated that the Tlr2 ligand, Zymosan-A (ZymA), when administered at low doses in vivo, stimulated chronic low grade inflammation contributing to the formation of new adipocytes. These studies underscore the important link between obesity and innate immunity, and how dietary SFA may exacerbate obesity and its related co-morbidities through direct interactions with Tlr2 and Tlr4 in AT. The significance of the current studies is that it will identify the involvement of Tlr2 and Tlr4 in obesity-induced inflammation. Moreover, elucidation of AT’s role in Tlr-mediated inflammation and insulin resistance.

Objectives and Specific Aims

Our goal is to elucidate the specific mechanisms by which SFA induce insulin resistance in adipocytes in relation to obesity and diabetes. Our central hypothesis is that SFA exacerbate obesity-induced inflammation and insulin resistance through interactions with not only Tlr4, but also through Tlr2, and that the SFA signaling through Tlr2 also culminates in activation of proinflammatory transcription factors NFκB and AP-1 in AT. The rationale for the work proposed herein is that understanding the relationship between innate immunity and obesity will allow recommendations for specific prevention and intervention strategies, and future recommendations directed at optimizing human nutrition and health and thereby reducing the risk of diseases associated with obesity. The Specific Aims are: (i) to determine whether Tlr2−/− and/or Tlr4−/− mice are protected against high fat diet-induced obesity, inflammation, and insulin resistance, (ii) to determine involvement of Tlr2 and/or
TLR4 signaling in adipocyte inflammation and insulin resistance, and (iii) to determine the ability of specific dietary fatty acids to induce inflammation through TLR2 and/or TLR4.

**Figure 1.** Fatty acid signaling through TLR2 and TLR4 in adipocytes. Our proposed mechanism of action of TLR2 and TLR4 mediated inflammation in adipocytes. Activation of TLRs requires stimulation by respective ligands, which include SFA. Following ligand binding an inflammatory signaling cascade is initiated resulting in activation of proinflammatory transcription factors, NF\(\kappa\)B and AP-1. The subsequent increase in transcription leads to the release of proinflammatory mediators, which can act in an autocrine (e.g. adipocyte), paracrine (e.g. adipocytes, macrophages, endothelial cells, and other AT-based cells), and endocrine (e.g. muscle and liver tissue) manner.
References


CHAPTER 2. REVIEW OF LITERATURE

Obesity and Diabetes: Role of Inflammation and Oxidative Stress

Obesity Prevalence and Implications

Over the past few decades the prevalence of obesity has risen dramatically worldwide. According to the Center for Disease Control, more than 20 states had obesity prevalence rates greater than 25% in 2006. Data from the 2003-2004 National Health and Nutrition Examination Survey (NHANES) reported the prevalence rates of obesity in adolescents and adults were 17.1% and 32.1%, respectively (28). These data indicate a 3% increase in obesity from 1999-2000.

Factors related to obesity include socioeconomic status (SES), education, ethnicity, and gender with interactions among these factors. For instance, African Americans and Mexican Americans show greater obesity prevalence with reduced SES, whereas this relationship is not evident in Asian Americans (29). Regardless of these distinctions among ethnic groups, there appears to be a significant rise in obesity independent of SES. Moreover, the trend is expected to continue with obesity prevalence possibly reaching 35%, 36%, 33%, and 55% by 2010 among white men, white women, black men, and black women, respectively (30).

Obesity is a major health concern in the U.S., as well as in the majority of developed countries. Individuals with a high BMI (>35) are estimated to have a significant reduction in life span, especially in younger adults (31). In white males, this reduction in life span approaches 22%. The increased mortality rates observed with obesity may be due to its associated co-morbidities, including DM, CVD, hypertension (HTN), and dyslipidemia.
Severely obese adults (BMI ≥40) exhibit 7.38 times greater risk of developing DM, 6.38 times greater risk of developing HTN, and 1.88 times greater risk of developing dyslipidemia as compared to healthy weight adults (BMI 18.5-24.9) (32). Furthermore, the prevalence rates of DM have closely paralleled the increase in obesity. According to the CDC, the number of individuals diagnosed with DM in the U.S. has increased from 5.6 million in 1980 to 15.8 million in 2005 independent of age, gender, and ethnicity. The majority of this increase is associated with the increased prevalence of obesity.

The impact of obesity and related co-morbidities on society includes not only the health consequences, but the financial burden. From 1987 to 2001, the health care costs in the U.S. have risen dramatically. Obese individuals account for 27% of this inflation, whereas 38% of health care spending is attributed to DM (33). In 2002, obesity-attributable medical costs approached 93 billion dollars, half of which was paid through Medicaid and Medicare (34). The financial burden of obesity and DM also affects private companies and businesses. For instance, although only 3% of an employee population is classified as obese, it accounts for 21% of total medical costs primarily through increased disability and absenteeism (35). Of all obesity related co-morbidities, DM represents the largest burden to the health care system. The American Diabetes Association (ADA) reported that in 2002, the direct (medical care and services) and indirect (disability and premature death) costs were over 132 billion dollars. However, it has been demonstrated that these costs can be greatly reduced with the advent of prevention vs. treatment programs targeted at addressing obesity before it manifests into DM or other co-morbidities (36).
**Metabolic Syndrome and Insulin Resistance**

Investigations regarding the relationship between obesity and DM have revealed a clustering of biological abnormalities known to precede the development of chronic DM. Referred to as the metabolic syndrome (MS), this cluster of abnormalities involves several obesity-related complications, including HTN, dyslipidemia, glucose intolerance, and central adiposity (Figure 2). The Third Report of the National Cholesterol Education Program...

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Figure 2. Development of Metabolic Syndrome (MS). The development of MS is preceded by central adiposity. The primary defects appear to be insulin resistance with concomitant hyperinsulinemia. When untreated these aberrations can mediate several risk factors (including glucose intolerance, dyslipidemia, and inflammation) that lead to chronic disease, such as Type 2 DM and CVD.

Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III) defines MS as three or more of the following criteria: abdominal obesity (waist...
circumference >102 cm in men and >88 cm in women); hypertriglyceridemia (1.69 mmol/L); low high-density lipoprotein (HDL) cholesterol (1.04 and 1.29 mmol/L in men and women, respectively); HTN (systolic blood pressure ≥130 and diastolic blood pressure >85 mm/Hg); impaired glucose tolerance (IGT) (6.1 mmol/L). Based on these criteria, estimate indicate that > 20% of U.S. adults have MS, which translates to more than 47 million Americans (37).

Substantial evidence now indicates that insulin resistance may be the primary defect contributing to the development of IGT, dyslipidemia, and HTN. Previous investigations (38) indicated that prevalence rates for insulin resistance in subjects with IGT, hypercholesterolemia, hypertriglyceridemia, and HTN ranged from 54 to 88%. Furthermore, the prevalence of insulin resistance in subjects exhibiting a combination of glucose intolerance, dyslipidemia, and hypertension was 95.2% (38). Insulin resistance, defined as reduced sensitivity to the action of insulin, is associated with impaired glucose uptake in peripheral tissues, particularly in skeletal muscle (39,40). The condition is also frequently accompanied by an increase in insulin secretion from pancreatic cells. This compensatory mechanism enables β-cells to maintain euglycemia through increasing circulating insulin concentration. When hyperinsulinemia can no longer compensate for increased insulin resistance, aberrations in glucose metabolism occur and eventually progress to DM (39,40). Abdominal obesity appears to be a major mediator of insulin resistance and hyperinsulinemia (41,42). Older, insulin resistant subjects exhibit greater accumulation of AT in the abdominal region. This centralized distribution of AT is associated with a significant reduction in insulin sensitivity (41). Additionally, obese postmenopausal women (body fat >45%) exhibiting preserved insulin sensitivity have nearly 50% lower visceral AT (VAT) compared to gender, age, and body fat-matched insulin resistant subjects (43). Accumulation of VAT is
also associated with the development of MS in normal weight, overweight, and obese elderly men and women (44). The role of subcutaneous AT (SAT) in the pathogenesis of insulin resistance and MS is less pronounced than that of VAT (45). In a review of 23 intervention strategies (46), VAT loss in response to diet, drug, and exercise intervention represented the highest percentage of weight loss than all other AT depots. This may be due to various genetic and physiological differences between depots. Vohl et al (47), reported that in severely obese subjects (BMI 45-81) nearly 350 genes were differentially regulated between VAT and SAT. Furthermore, VAT has been shown to be more lipolytic and catecholamine sensitive, as well as more insulin resistant, than SAT (48). These studies indicate that in addition to overall obesity, the distribution of AT is an important factor in the development of insulin resistance and MS.

**Inflammation and Insulin Resistance**

There is considerable evidence that inflammation is a primary mediator of obesity-related co-morbidities, including DM and CVD (49-56). Additionally, several studies have demonstrated that inflammation is an integral factor in the development of MS in several populations (52-54,56). Ishikawa, et al (52) reported that circulating C-reactive protein (CRP) concentrations, a common clinical indicator of systemic inflammation, was significantly higher in Japanese men and women diagnosed with MS than their non-affected counterparts. In this population, the odds ratio for having MS was also greatest in individuals categorized in the highest tertile of CRP (52). Data from the Insulin Resistance Atherosclerosis Study (IRAS) (49) confirmed that chronic subclinical inflammation, as indicated by increased CRP, is associated with dyslipidemia, central adiposity, HTN, and insulin resistance. These findings are consistent in African-American, Hispanic, and White
Non-Hispanic populations. Increased CRP levels are also associated with higher age-adjusted risks for developing DM, even after adjusting for presence of CVD, SES, physical activity, alcohol consumption, smoking, and family history of DM (54). In subjects with DM, the underlying aberration contributing to the development of insulin resistance appears to be a subclinical inflammatory state marked by increased concentrations of circulating leukocytes, fibrinogen, and CRP (55). Inflammation is also associated in the pathogenesis of CVD (50,51,57). Results from the Cardiovascular Health Study (57) indicated that in adults (≥65) with atherosclerosis in combination with increased CRP had a 72% greater risk for CVD-related death. This risk can be exacerbated in subjects diagnosed with DM, suggesting an even greater incidence of CVD-related mortality in patients with both inflammation and DM (58).

A novel approach for the treatment of DM and CVD is the use of anti-inflammatory medications. Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, have long been used for their ability to reduce blood glucose (59-61). The exact mechanism is not fully understood, but may be related to reduced FFA concentrations (59,62). One early investigation (63) demonstrated that ibuprofen reduced glucose concentration following a glucose challenge. This improvement in glycemic control was associated with a greater insulin response and reduced FFA (63). More recently, data from the Physicians' Health Study, a randomized, double-blind, placebo-controlled trial, indicated a 44% reduction in myocardial infarction with aspirin treatment compared to placebo (64). The development of atherosclerotic CVD and stroke is increased in individuals with DM. However, the ability of NSAIDs to reduce CVD-related morbidity and mortality in diabetic individuals remains unclear (65). Data from the DECLARE-DIABETES Trial (66) indicated that aspirin in
combination with two other anti-inflammatory medications was able to lower cardiovascular related complications in diabetic patients. Hundal and colleagues (67,68) observed that two weeks of aspirin treatment improved insulin sensitivity and the lipid profile in male and female diabetics. This improvement may be mediated in part by reduced IκB kinase β (IKKβ) activation (68), which is the upstream activator of the proinflammatory transcription factor, NFκB. Activation of IKKβ, either in obese mice or IKKβ agonist treated Fao hepatoma cells, resulted in reduced insulin signaling which was reversed by NSAID treatment (68). Overall, these studies indicate that treatment of inflammation improves obesity-related complications, including insulin resistance and dyslipidemia, which are major risk factors for CVD and DM.

**Cytokine and Chemokine-Induced Insulin Resistance**

**IL-6.** Proinflammatory cytokines, such as IL-6 and TNFα, are small proinflammatory proteins that are increased with obesity (4,5,69). The importance of IL-6 in obesity-induced inflammation is demonstrated by identification of single nucleotide polymorphisms (SNP) in the IL-6 promoter (70-73). Fernandez-Real et al. (71) identified a SNP in the 5’ flanking region of the IL-6 gene at position -174 in which a guanine was replaced by a cytosine. This SNP was associated with reduced plasma IL-6, as well as lower circulating insulin, TAG, and FFA in healthy subjects. This SNP was further associated with greater insulin sensitivity and glycemic control, as well as a reduced WBC (70). Alternatively, Kubaszek and colleagues (72), found this IL-6 SNP to be associated with reduced energy expenditure (EE), basal metabolic rate (BMR), whole body glucose uptake, and glucose oxidation in a healthy Finnish population (72). These findings were further supported by a recent study (73) that indicated this SNP is associated with higher risk of developing obesity-related metabolic disorders, including insulin resistance.
Despite the discrepancy in SNP associated phenotypes, it is evident that increased circulating IL-6 is associated with obesity related co-morbidities. Data from the Health, Aging and Body Composition Study (74) indicated the odds ratio of developing subclinical CVD was greatest in the tertile of patients with the highest concentration of serum IL-6. Additionally, Bahrami, et al (75) suggested that serum IL-6 was the strongest predictor of obesity-associated coronary heart disease. Recent data from the Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA)/Cooperative Research in the Region of Augsburg (KORA) study also indicated that both high IL-6 and CRP concentrations are associated with the development of DM (76). Furthermore, elevated IL-6 concentrations is associated with increased mortality in elderly adults (77). The impact of IL-6 on obesity-related co-morbidities may be dependent on the development of insulin resistance. Rush, et al (78) observed that IL-6 concentrations is positively associated with reduced β-cell function and insulin resistance in a population of Asian Indian immigrants. Furthermore, obese adolescents exhibited insulin resistance attributed to elevated IL-6 (79).

IL-6 involvement in insulin resistance may be mediated through impairment of insulin signaling (80-83). Chronic IL-6 administration has been shown to inhibit insulin signaling in adipocytes, muscle, and liver (82-84). Specifically, IL-6 was observed to inhibit autophosphorylation of the insulin receptor (IR) and downstream effectors including insulin receptor substrate-1/2 (IRS-1/2), phosphatidylinositol-3 kinase (PI3K), protein kinase-1 (Akt/PKB), and glucose transporter-4 (GLUT4) (80-84). These effects were mediated through activation of specific proinflammatory signaling intermediates, such as suppressors of cytokine signaling-3 (SOCS3) (81,82,84) and protein kinase C (PKC) (82). Activation of SOCS1/3 inhibited tyrosine phosphorylation of the insulin receptor (81,85). Furthermore,
PKC activation increased serine phosphorylation of IRS-1/2 (82) and thus contributed to the development of IL-6-mediated insulin resistance. These data suggest that IL-6 may induce insulin resistance through multiple mechanisms including direct impairment of insulin signaling.

**TNFα.** Hotamisligil et al. (4,86) originally proposed the involvement of the proinflammatory cytokine, TNFα, in obesity-associated insulin resistance. More recently, specific SNPs in the TNFα gene have been associated with DM (87-89). Moreover, certain SNPs predisposed diabetic patients to athertosclerotic-related complications (88). Fontaine-Bisson et al. (89) identified a SNP in the TNFα promoter (-238G>A) that is associated with reduced FFA clearance and insulin resistance in obese subjects with DM but not in lean controls. Additionally, the same SNP was observed at a higher frequency in patients with coronary artery disease, especially in subjects with DM or HTN. Analysis of the TNFα gene and promoter indicates the importance of this cytokine in the regulation of obesity induced insulin resistance.

Elevated TNFα levels are closely related to components of MS including, IGT, HTN, and dyslipidemia in obese adults (90) and adolescents (91). High serum TNFα levels are also associated with insulin resistance in elderly patients (92). Tam and colleagues (93) observed that neutralization of TNFα with NSAIDs contributed to improved lipid profile and insulin sensitivity in subjects with rheumatoid arthritis.

TNFα-mediated insulin resistance, as observed in obesity and DM, is suggested to involve impairment of insulin signaling. This may include reduced IR autophosphorylation, as well as decreased IRS-1/2 activation (94-96). The mechanism for reduced tyrosine phosphorylation is not fully characterized. However, it may involve activation of
phosphotyrosine phosphatase (PTPase) (96). Alternatively, TNFα is observed to increase serine phosphorylation of IR and IRS-1/2, thereby contributing to reduced insulin sensitivity (97-100). Serine phosphorylation of insulin signaling intermediates involves activation of downstream effectors, such as PKC or mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinases (ERK) (99), c-Jun N-terminal kinases (JNK) (100), and p38-MAPK (97). However, the exact pathway involved appears to be tissue-specific. These data implicate TNFα as a major contributor to insulin resistance and further supports the idea that proinflammatory cytokines are involved in obesity-related co-morbidities.

**MCP-1.** Chemokines are small proteins similar to cytokines, which also exhibit the ability to induce chemotaxis. Certain chemokines, such as MCP-1 and interleukin-8 (IL-8), promote migration of monocytes and neutrophils, respectively. Data from the Cooperative Health Research in the Region of Augsburg Survey identified serum chemokine concentrations as independent risk factors for DM (101). Moreover, MCP-1 concentration was associated with increased risk of CVD mortality in a 7-year longitudinal study (102). Recent work (103) has shown that a high concentration of circulating MCP-1 was associated with insulin resistance and DM. Several SNPs have been identified in the MCP-1 gene and promoter. Although there is still debate regarding the extent of the association, there is evidence to suggest that the A-2518G polymorphism in the distal regulatory region of MCP-1 is associated with heart disease and myocardial infarction (104,105). In addition, the G/G genotype at position -2518 in the MCP-1 promoter was associated with lower plasma MCP-1 and reduced prevalence of insulin resistance and DM (103).

Obesity is associated with increased circulating MCP-1 (13,69). Overexpression of this chemokine or its corresponding receptor (CCR2) stimulated insulin resistance and
hepatic steatosis in several rodent models of obesity (12,106). Furthermore, multiple obese mouse models, either transgenic (TG) or diet-induced (DIO), exhibited elevated MCP-1, which was associated with greater macrophage accumulation into peripheral tissues, including AT (12,106). More recently, it was observed that *in vivo* infusion of chemokine stimulated adipogenesis in mice (107). This effect may account for the increased adiposity and FFA observed in TG-MCP-1 mice (12).

There is limited evidence implicating the direct involvement of MCP-1 with insulin signaling. However, it appears that recruitment of macrophages into tissues important in glucose homeostasis, including the pancreas, liver, skeletal muscle, or AT, results in an inflammatory response that contributes to the development of insulin resistance. Additionally, MCP-1 involvement in oxidative stress and ROS formation represents another potential mechanism of chemokine-induced insulin resistance (108). Finally, increased MCP-1 expression in pancreatic islets and beta-cells could result in β-cell dysfunction and thus promote IGT. Regardless of the mechanism, it is clear that increased MCP-1 expression is involved in the pathogenesis of obesity-related complications.

**ADN.** Obesity is not only associated with increased proinflammatory cytokines and chemokines, but is also associated with reduced concentrations of anti-inflammatory proteins, such as adiponectin (ADN). Several genetic analyses (109,110) observed that a quantitative trait locus (QTL) on chromosome 3 (3q27), the site of the ADN gene, was strongly correlated with insulin resistance, MS, and DM. Data from the Metabolic Risk Complications of Obesity Genes Project (109) indicated that chromosome 3 also had the highest association with BMI, waist circumference, and insulin sensitivity. Additional genetic analysis observed the presence of multiple SNP in the ADN gene associated with
increased risk of DM and CVD (111-115). More specifically, the G/G genotype at position 276 in the ADN gene is associated with reduced ADN, as well as insulin resistance, DM, and CVD (111-113). Multiple investigations have since indicated that obesity is associated with dysregulation of ADN (116-118). Arita et al. (116) initially reported a negative correlation between BMI and plasma ADN in obese subjects. Furthermore, hypoadiponectinemia is associated with several components of MS, including insulin resistance, dyslipidemia, and inflammation (117,119,120). Xydakis and colleagues (120) demonstrated that obese individuals with MS had lower serum ADN compared to obese individuals without MS. Patients diagnosed with DM or CVD are also observed to have significantly lower plasma ADN (118,121,122). This is especially true for high risk populations, such as Pima Indians.

ADN possesses key anti-inflammatory properties, which likely contribute to its varied metabolic effects. Plasma ADN was negatively correlated with CRP, IL-6, and TNFα in obese subjects (123,124). Furthermore, ADN attenuated LPS-induced IL-6 and TNFα expression in both macrophages and adipocytes (19,125,126). LPS-stimulated NFκB activation was also prevented with ADN treatment (22), which may explain the observed reduction in proinflammatory cytokines. Conversely, administration of IL-6 or TNFα blunted ADN expression and secretion in 3T3-L1 adipocytes (127-129). Furthermore, monocyte proliferation and macrophage function, including phagocytic activity and cytokine release, were reduced by ADN (130,131). Foam cell formation, as determined by lower macrophage cholesteryl ester content and scavenger receptor expression, was also attenuated by ADN (132). These data indicate that ADN is a key anti-inflammatory mediator.

Several studies have examined the role ADN plays in vivo. Genetically engineered ADN knockout (KO) mice exhibited reduced insulin sensitivity in response to a high fat diet
(133-135). Maeda et al. (133) demonstrated that ADN deficient mice had impaired insulin signaling in skeletal muscle. Moreover, this effect was corrected by adenoviral produced ADN (133). Alternatively, Nawroki and colleagues (134) suggested that the reduction in insulin sensitivity in ADN KO mice was attributed to increased hepatic glucose production. Several additional studies (136,137) indicated the liver as the primary site of ADN action. These observed differences in the mechanism of action of ADN can be attributed to structural differences in the protein. ADN forms several distinct oligomeric complexes. These include a high molecular weight (HMW, >250 kDa), medium molecular weight (MMW or hexamer, ~180 kDa), and low molecular weight (LMW or trimer, ~90 kDa) species (138-141). Furthermore, a proteolytic cleavage product of ADN that includes the globular C-terminal of the protein (gADN) was also present in human plasma (142).

In humans, females have a greater proportion of HMW and hexamer complexes than males (138-140). Additionally, BMI is negatively correlated with the concentration of HMW species, but not the hexamer or trimer forms (139). Pajvani and colleagues (138) reported that lower HMW to total ADN was associated with lower insulin sensitivity in diabetic subjects. Furthermore, use of insulin sensitizing drugs, such as thiazolidinedione (TZD), increased HMW but not total ADN in these subjects (138). The HMW species is suggested to improve hepatic insulin sensitivity (138,141). Wang et al. (141) demonstrated the HMW species stimulated 5'AMP-activated protein kinase (AMPK) in liver, which lead to inhibition of gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase). Alternatively, gADN was associated with skeletal muscle insulin sensitivity in obese and diabetic humans (143,144) and rodents (142,145-147). Bruce and colleagues (144) observed that gADN in vitro stimulated glucose uptake and FA oxidation in
skeletal biopsies from lean and obese subjects. This effect was attributed to increased AMPK and acetyl-CoA carboxylase (ACC) activation. Administration of gADN also increased IR and IRS-1 activation, as well as glucose uptake in primary rat adipocytes (148).

**Oxidative Stress and Insulin Resistance**

ROS are small, highly reactive molecules possessing oxidative abilities primarily attributed to an unpaired electron. Examples include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH), and nitric oxide (NO•) (149). These molecules play important roles in cell signaling. However, accumulation of ROS can contribute to oxidation of amino acid side chains, protein-protein cross-links, and protein fragmentation (149).

Oxidative stress is implicated in the pathogenesis of multiple diseases (150). Several investigations have indicated a role of oxidative damage in atherosclerosis (151), HTN (152), and DM (153). Obesity has also been linked to increased oxidative stress (154,155). Data from the Framingham Heart Study (155) indicated that urinary 8-epi-prostaglandin F2 alpha (8-epi-PGF2 alpha), an F2-isoprostane, a marker of ROS, formed via a noncyclooxygenase pathway, was positively associated with BMI and waist circumference in male and female subjects. Moreover, for every 5 kg/m$^2$ of BMI there was a 9.9% increase in urinary 8-epi-PGF2 concentration, indicating very high levels of oxidative stress. Furukawa et al. (154) also observed a positive correlation between BMI and urinary 8-epi-PGF2 in healthy Japanese men and women. Additionally, a positive association has been shown (154) between obesity and plasma thiobarbiturate reactive substances (TBARS), an end product of lipid peroxidation.

Obesity-induced oxidative stress represents a potential link between obesity and its associated co-morbidities. Cardona and colleagues (156) reported that individuals with MS
exhibited increased markers of oxidative stress compared to patients without MS. This same group also demonstrated that antioxidant defense systems were impaired in healthy individuals following high fat feeding (157). The increased oxidative stress associated with obesity is specifically linked to the development of insulin resistance (158-160). Obese individuals diagnosed with DM exhibit increased oxidative stress and reduced antioxidative capacity compared to healthy controls (158). Mohora et al. (159) determined that obesity and DM were both associated with increased ROS, but obese subjects with DM exhibited a greater concentration of plasma malondialdehyde (MDA), a marker of oxidative stress. These findings are supported by additional groups (160) and clearly link oxidative stress as a bridge between obesity and DM.

The mechanisms involved in oxidative stress-induced insulin resistance have not been fully elucidated. However, multiple studies indicated involvement of ROS accumulation in hepatocytes (161), skeletal muscle (162-164), and adipocytes (165-167). Kumashiro and colleagues (161) demonstrated that overexpression of the antioxidative enzyme, superoxide dismutase 1 (SOD1) in the liver of diabetic db/db mice reduced hepatic ROS accretion and improved insulin sensitivity. Additionally, Dokken and colleagues (163) observed that isolated rat skeletal muscle incubated with H2O2 significantly inhibited insulin-stimulated glucose transport and glycogen synthesis. Furthermore, a mineralocorticoid receptor antagonist prescribed to control blood pressure, spironolactone, attenuated ROS induced insulin resistance through increased phosphorylation of IRS-1, Akt/PKB, and GLUT4 in soleus muscle of insulin resistant rats (162).

Recent evidence suggests that increased oxidative stress in skeletal muscle is secondary to changes in AT. Adipocytes themselves exhibited insulin resistance in response
to oxidative stress (165-167). Rudich et al. (165) showed that low grade oxidative stress results in impaired insulin-stimulated GLUT4 translocation and PI3K activation in 3T3-L1 adipocytes. Furthermore, basal, but not insulin-stimulated, glucose uptake was increased along with GLUT1 expression in response to increased H$_2$O$_2$ content (165). Several additional studies (164,166,168) confirmed that an increase in basal glucose transport occurs in response to oxidative stress in adipocytes. Moreover, it appears that increased basal glucose uptake may exacerbate ROS accumulation and inflammatory signaling in adipocytes (169-172) through activation of NFkB and AP-1 (173). These studies clearly link adipocyte-specific oxidative stress to the development of insulin resistance and therefore connect oxidative stress to the development of DM and related co-morbidities.

**Obesity-Induced Insulin Resistance: Role of Free Fatty Acids**

**Overview of Fatty Acid Induced Insulin Resistance**

Multiple investigations have demonstrated an increase in circulating FFA in obese humans (174-177) and animals (178,179). This elevation in FFA is largely attributed to increased basal AT lipolysis (180-182). However, the antilipolytic effect of insulin may also be impaired in obesity (183,184). Regardless of the exact mechanisms involved, increased FFA is associated with insulin resistance in obese individuals (185,186). Moreover, infusion of FFA in lean humans and rats stimulated insulin resistance (187,188). These data indicate that an increase in FFA represents a primary connection between obesity and insulin resistance.

Obesity-related co-morbidities, including DM and CVD, have also been directly associated with increased FFA (189,190). A cross-sectional analysis of offspring from parents diagnosed with DM (191), reported that non-diabetic offspring had increased FFA
that predisposed them to the development of DM. Pima Indians with the highest concentration of FFA had a 2.3 fold greater risk of DM than individuals with the lowest concentration of FFA (192). Impaired postprandial FFA clearance is also linked to CVD in diabetic individuals (193). Carlsson and colleagues (194) observed that higher FFA were associated with greater risk of myocardial infarction and stroke. Other markers of CVD, including endothelial dysfunction, vascular smooth muscle cell proliferation, and HTN were also associated with increased FFA (194). Additional studies (90,174) have indicated that elevated FFA is associated with the development of several other components of the MS, including HTN and dyslipidemia.

Insulin sensitivity can be improved by lowering FFA concentration. Studies using the hypolipidemic drug, acipimox, demonstrated that a reduction in FFA improved insulin sensitivity in obese and diabetic individuals (193,195,196). Santomauro et al. (196) reported that acipimox reduced plasma FFA by more than 60%, which improved insulin-stimulated glucose uptake by greater than 2-fold in obese subjects under euglycemic hyperinsulinemic clamp conditions. The peroxisome proliferator-activated receptor-alpha (PPARα) agonist, fenofibrate, is commonly used for treatment of dyslipidemia (197). Activation of PPARα is suggested to increase β-oxidation, improve lipoprotein profiles, and reduce inflammation (197). Data from the Bezafibrate Prevention Study showed a near 10% reduction in CVD-related mortality (198). Moreover, it has been observed that treatment with the PPARα agonist improved several additional complications of MS, including insulin resistance (198-200). Haluzik and colleagues (201) demonstrated that 14 d of fenofibrate treatment reduced blood glucose, FFA, TAG, and insulin resistance in lipogenic, simple carbohydrate diet fed mice. However, the insulin-sensitizing response of fibrates in humans is limited, which may
be due to lower PPARα expression in liver and skeletal muscle (202). The ability of lipid-lowering medication to effectively attenuate insulin resistance, as well as lower risk for developing DM and CVD, indicates that FFA are key mediators in obesity-related comorbidities.

**Substrate Competition and Non-Oxidative Fatty Acid Metabolism**

Accumulating evidence (185,186) supports the theory that disturbances in glucose metabolism are secondary to changes in FA metabolism. The exact mechanism of FFA-induced insulin resistance has not been fully elucidated. However, Randle et al. (203) originally proposed that the insulin resistant properties of FA were mediated through direct inhibition of glucose metabolism. More specifically, as intermediates of FA and TAG synthesis, such as citrate and acetyl-CoA, accumulate in the cytosol of muscle cells, enzymes involved in glucose oxidation, such as phosphofructokinase-1 and pyruvate dehydrogenase complex, are inhibited. This in turn reduces insulin-stimulated glucose uptake and metabolism (204-207). Overall, researchers have suggested that increased FFA induces peripheral insulin resistance and stimulates an initial hyperinsulinemic state, but as DM develops, β-cell dysfunction occurs and insulin secretion can no longer compensate for elevated FFA and thus hyperglycemia becomes apparent.

Elevated serum FFA concentration is toxic to cells. Increasing the concentration of lipid within non-adipose tissue, such as skeletal muscle, liver, and pancreatic β-cells, has a more profound effect on cellular metabolism than impairment of glucose metabolism. This is evident by the increased level of apoptosis in these tissues in response to lipid accumulation (208-210). Lipotoxicity, excess lipid accumulation in non-adipocytes, can contribute to obesity-mediated β-cell failure, non-alcoholic steatohepatitis (NASH), and skeletal muscle
insulin resistance (211). The consequences of lipotoxicity may be mediated through products of non-oxidative FA metabolism, such as diacylglycerol (DAG) and ceramide (209,211). The accumulation of long-chain fatty acyl-CoA (LCFA) in the cytoplasm of non-adipocytes has been associated with the synthesis of DAG and ceramide (208,209,212). This effect appeared to be more pronounced in response to SFA, such as palmitoyl-CoA, whereas PUFA appeared to be protective (213). The free radical, nitric oxide (NO) has also been correlated with ceramide concentration and may represent the link between the FA metabolites and β-cell apoptosis (209). In addition to the potential reduction of insulin synthesis, that DAG and ceramide have been shown directly to inhibit insulin signaling (208,214-216). This action may be mediated PKB and PI3K, which are key intermediates of insulin-mediated glucose uptake (208,214,215). More specifically, FA metabolites activate PKC (208) and protein phosphatase 2A (PP2A) (214), which are both shown to directly inhibit insulin-mediated GLUT4 translocation. Proinflammatory cytokines, such as IL-6 and TNFα, have also been shown to increase synthesis of ceramide (208), as well as directly stimulate PKC activation (4,86). Therefore, the overall impact of non-oxidative FA metabolites on insulin resistance may be secondary to inflammation.

**Dietary Fatty Acids Play a Role in Inflammation**

Dietary FA can be both pro- and anti-inflammatory. SFA may be responsible for the increase in proinflammatory cytokines and chemokines observed with obesity and MS (217-222). The consumption of a Westernized diet high in refined grains, red meat, SFA, and hydrogenated fats has been positively related to several markers of inflammation, including CRP, serum amyloid A (SAA), and IL-6 (223). Aeberli et al. (220) reported that total dietary fat is associated with CRP and IL-6 in obese children. Moreover, SFA intake is specifically
linked to the development of inflammation in humans. Fernandez-Real et al. (224) reported that palmitic and myristic acid consumption were negatively correlated to plasma ADN and insulin sensitivity. Alternatively, consumption of either omega 3 FA (n-3 FA) or monounsaturated FA (MUFA) have been associated with reduced inflammation and improved insulin sensitivity (225-229). More specifically, Pischon and colleagues (226) reported intake of the n-3 FAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were inversely associated with plasma concentrations of the soluble TNFα receptor (sTNF-R) and CRP. Data from the Nurses’ Health Study (225) demonstrated that EPA and DHA intake were also inversely related to CRP, IL-6, and several proinflammatory adhesion factors, including E-selectin, soluble intracellular adhesion molecule (sICAM-1), and soluble vascular adhesion molecule (sVCAM-1). Similar anti-inflammatory effects have been observed with consumption of a diet rich in MUFA (228,229). Fito et al. (228) observed that consumption of virgin olive oil reduced plasma IL-6 and CRP in patients diagnosed with coronary heart disease. Thus, substitution of dietary SFA or trans FA (TFA) with either n-3 FA or MUFA may attenuate obesity-induced inflammation and reduce the risk of DM and CVD.

The mechanism of SFA-induced inflammation and insulin resistance is not fully elucidated, but it is clear that certain SFAs stimulate a proinflammatory response in various cells and tissues. Palmitate and other medium chained SFA-induced proinflammatory gene expression in endothelial cells (230,231), hepatocytes (232), myotubes (233,234), macrophages (11), and adipocytes (22,100,235). Elevated circulating palmitate was also positively correlated with increased plasma IL-6 (231) and negatively correlated with plasma ADN (224). Moreover, human coronary artery endothelial cells exhibited increased IL-6
mRNA and protein expression in response to palmitate treatment (231). This vascular inflammation can in turn contribute to development of atherosclerosis (50). SFA-induced inflammation has also been associated with development of NASH (236). Joshi-Barve et al. (232) reported that palmitate-induced IL-8 expression stimulated lipid accumulation and insulin resistance in cultured hepatocytes (232). These effects have been dramatically reversed by blocking cytokine production with neutralizing antibody, improving hepatic insulin sensitivity (237).

Skeletal muscle accounts for over 80% of glucose uptake under insulin-stimulated conditions (238). Thus, skeletal muscle represents a major target of FA-induced inflammation and insulin resistance. SFAs, such as palmitate, increased IL-6 and TNFα expression in skeletal muscle cells and have been associated with reduced insulin sensitivity (233,234,239,240). Sinha and colleagues (234) demonstrated that palmitate treatment reduced insulin-stimulated glucose uptake, corresponding to a decrease in insulin-stimulated IRS-1, PI3K, and Akt/PKB phosphorylation in L6 myotubes. Furthermore, Jove et al. (240) reported that palmitate inhibited insulin-stimulated glucose uptake, as well as GLUT4 expression, in C2C12 muscle cells. These studies and others (233,239,241-243) indicated that SFA-induced insulin resistance was mediated through activation of distinct inflammatory pathways involving extracellular signal-regulated kinases (ERK), JNKs, and p38 mitogen-activated protein kinases (MAPK). These proinflammatory pathways lead to activation of transcription factors, such as NFκB and AP-1 (232,244,245). Blocking MAPK kinase activity or expression through use of specific inhibitors or genetic knockdown attenuates FA-induced insulin resistance in muscle cells. Furthermore, inhibition of NFκB activation appears to be of primary importance in blocking palmitate-induced insulin
resistance (233, 234, 239-241). Weigert and colleagues (233) reported that the proteosome inhibitor, MG132, completely blocked palmitate-induced NFκB DNA binding and corresponding IL-6 production in human myotubes. This relationship is consistent with other NFκB inhibitors, including pyrrolidine dithiocarbamate (PDTC), SN50, and parthenolide (234, 240, 241).

The ability of FFA to induce inflammation and insulin resistance in liver and skeletal muscle suggests that disturbances in these tissues are dependent on interactions with adipocytes. Dietze et al. (246) observed that co-culture of human muscle cells with adipocytes dramatically reduced insulin signaling in myotubes. In vivo studies observed an accumulation of FFA in skeletal muscle with obesity (247, 248). This ecotopic storage can then promote systemic insulin resistance (249, 250). Boden and colleagues (251) suggested that insulin resistance occurred in adipocytes prior to skeletal muscle. They provided evidence that reduced insulin sensitivity in adipocytes promoted greater lipolysis and reduced FFA uptake that consequently led to ecotopic fat storage. Moreover, increased FFA concentration directly contributed to insulin resistance in adipocytes. Several groups (11, 22, 100) demonstrated that FFA-induced proinflammatory cytokine and chemokine expression and impaired insulin signaling in adipocytes. Ajuwon et al. (22) reported that palmitate induced IL-6 expression and NFκB activation in 3T3-L1 adipocytes. FFA also increased TNFα and MCP-1 expression through activation of JNK- and NFκB-mediated pathways (11, 100). In addition to FFA, the proinflammatory cytokines/chemokines secreted from adipocytes (referred to as adipokines) also contributed to insulin resistance in non-AT (11, 100). The impact of adipocytes on inflammation and insulin resistance suggests that they represent a potential therapeutic target for treatment of obesity-related co-morbidities, such as DM.
Obesity-Induced Insulin Resistance: Role of Adipose Tissue

Adipose Tissue Cellularity and Changes with Obesity

Adipose tissue cellularity and changes with obesity

AT represents one of the most dynamic tissues in the human body. It can represent as little as 3% of total body weight in conditioned athletes to over 70% of total body weight in severely obese individuals (252). The cellular proliferation of AT is highly variable and depends greatly on depot location, as well as multiple genetic and environmental factors. However, despite substantial intra- and inter-individual differences there is a basic organization to AT, which consists of mature adipocytes, fibroblast-like preadipocytes, endothelial cells, lymph nodes, and nerves (252,253). Adipocytes represent the major cellular component of AT. There are two distinct classifications of adipocytes: the unilocular white adipocytes and multilocular brown adipocytes.

White adipocytes contain a large lipid droplet composed primarily of TAG and cholesteryl esters, ranging in size from 20 to 200 µM in diameter. Moreover, they exhibit distinct endocrine, paracrine, and autocrine properties via expression and secretion of multiple proteins, such as leptin, ADN, IL-6, TNFα, and MCP-1. Adipocytes are also targets for various signaling molecules released from β-cells, macrophages, neurons, hepatocytes, and adipocytes themselves. Some of the adipocyte-specific receptors mediating this signaling include insulin, cytokine (i.e. IL-6 and TNFα), adrenergic/cholinergic, and lipoprotein receptors (254). Therefore, it is clear that the physiological role of white adipocytes extends beyond simple energy storage and involves complex interactions with central and peripheral tissues in order to regulate energy metabolism and systemic homeostasis.

Brown adipocytes have a slightly different morphology than white adipocytes. For instance, these cells are smaller (20 to 40 µM), contain multiple lipid droplets, have
pronounced nuclei and cytoplasm, as well as increased capillary density (255). Brown adipocytes also exhibit a greater number of mitochondria contributing to the trademark brown color. The major functional difference between the two cell populations is the role of brown adipocytes in thermogenesis. Briefly, FAs stored within brown adipocytes are quickly accessed by mitochondria and subsequently oxidized generating a proton gradient. Normally, this gradient is used for ATP synthesis, but in brown adipocytes the uniquely expressed protonophore, uncoupling protein 1 (UCP1), mediates disruption of the gradient causing the protons generated in FA oxidation to be dissipated as heat (256). In rodents, this process plays an important role in thermal regulation. Newborn infants also possess high levels of brown adipocytes in several adipose depots (255). However, in adult humans the amount of these cells is dramatically reduced and are found dispersed among white adipocytes. In lean adults, researchers have suggested that, on average, there is one brown adipocyte for every 100 to 200 white adipocytes in VAT (257). Several studies (253,255) indicated that reduced sympathetic stimulation of these cells resulted in transdifferentiation of brown adipocytes into a phenotype similar to white adipocytes. Moreover, it appears that this process may be reversed by using β-adrenergic agonists (258). The ability of these two adipocyte populations to interchange phenotypes has important implications for health and disease.

Adipose expansion involves both adipocyte hypertrophy (increase in cell size) and hyperplasia (increase in cell number). Hypertrophy and hyperplasia occur naturally in normal growth phases, as well as with obesity (discussed below) (259,260). Adipocyte hypertrophy requires positive energy balance that results in accumulation of TAG. However, adipocytes are limited in the amount of lipid that can be stored. Hence, hyperplasia represents an important means by which AT can expand. Also referred to as adipogenesis, hyperplasia
involves proliferation of fibroblast-like preadipocytes and their subsequent differentiation into mature adipocytes (252,261). Proliferation of preadipocytes entails mitotic replication to increase cell number. Alternatively, differentiation of preadipocytes requires cell cycle arrest, as well as distinct molecular and cellular modifications that mediate conversion of preadipocytes to spherical, lipid-filled mature adipocytes (253,260,262-264). Generally, proliferation occurs prior to differentiation. However, researchers have suggested that partially differentiated cells may still be capable of replication (260). In normal human growth and development, differentiation is controlled by the expression of several adipogenic transcription factors, transcriptional coactivators, and cell-cycle proteins designed to increase the lipogenic capacity of the cell (262,263). Two transcription factors, CCAAT/enhancer binding protein α (C/EBPα) and PPARγ, are essential to adipocyte differentiation. Following initiation of growth arrest, preadipocytes undergo at least one more round of DNA replication and cell doubling, often referred to as clonal expansion (263,265). During this process, adipogenic transcription factors upregulate genes involved in adipogenesis. Complete (or terminal) differentiation of adipocytes is associated with complete exit from cell cycle and an increased expression of adipocyte-specific genes and proteins, including leptin, ADN, and TNFα (263).

Obesity is associated with significant increases in adipocyte hypertrophy and hyperplasia (252,263,266). In obesity, mature adipocytes reach their lipid storage capacity at an accelerated rate. This in turn contributes to increased hyperplasia. Regulation of adipogenesis is thought to be mediated through a paracrine mechanism in which hypertrophic adipocytes secrete growth factors in response to their expanding size (264). These growth factors, such as insulin-like growth factor (IGF), can then promote preadipocyte
differentiation and proliferation (267-269). Blocking of IGF-1 signaling with specific binding proteins attenuates obesity and implicates hyperplasia as a physiological mechanism contributing to obesity (270). Wang and colleagues (271) observed that mice lacking preadipocyte factor-1 (Pref-1), a known inhibitor of adipogenesis, exhibited increased adiposity, an effect that was reversed through transgenic overexpression of the preadipocyte marker. Moreover, use of synthetic inhibitors of adipogenesis is proposed as a potential treatment of obesity and related co-morbidities (272). Therefore, individuals with greater adipogenic potential may be predisposed to obesity (273).

**Obesity-Induced Changes in Adipose Tissue Fatty Acid Metabolism**

TAG stored in AT originates from either dietary fat that is packaged into chylomicrons in enterocytes of the small intestine (post-prandial) or from endogenously produced TAG that is packaged into very low density lipoproteins (VLDL) in the liver (post-absorptive) (274). Once in the circulation, these lipid rich lipoproteins deliver TAG and FA to peripheral tissues, including skeletal muscle and AT. Delivery of FFA to adipocytes requires hydrolysis of lipoprotein based TAG from chylomicrons and VLDL by lipoprotein lipase (LPL) (275) This process involves adipose-specific VLDL-receptor recognition of apoprotein E, located on VLDL and chylomicrons (276). LPL is initially synthesized in adipocytes and then exported to the luminal surface of capillary endothelium where it hydrolyses, with acylation stimulating protein (ASP), TAG to FFA and glycerol (277). Dysregulation of LPL, as observed in obesity has been associated with dyslipidemia and insulin resistance (275,278,279). Kitajima et al. (278) reported that systemically overexpressed LPL attenuated insulin resistance in obese rabbits. However, increased LPL activity in AT is associated with dyslipidemia and insulin resistance (279). The mechanism
underlying these differences is unclear, but may relate to the difference in the lipid oxidative activity of various tissues.

Following hydrolysis of TAG from lipoproteins in the endothelial lumen, FFAs are transported through the plasma membrane of adipocytes by specific FA transporters, such as CD36 and fatty acid transport protein (FATP). Expression of these transport proteins have been shown to be increased in obesity and DM (280,281). Gertow and Colleagues (281) observed that increased expression of FATP4 was positively correlated with obesity and insulin resistance in monozygotic twins. Based on these studies, it appears that regardless of the source of TAG (endogenously produced or dietary), increased storage of TAG in AT promotes dyslipidemia and insulin resistance in obese and diabetic individuals.

The majority of TAG is from dietary sources; however, de novo lipogenesis (DNL) significantly contributes to AT energy reserves. DNL is defined as the synthesis of FA from non-lipid sources, primarily glucose. In humans, DNL occurs extensively in the liver (282) and is increased substantially in individuals consuming a high carbohydrate (CHO) diet (283). FA generated by hepatic DNL are packaged into VLDL for transport to peripheral tissues (284). AT is also indicated as a site for DNL in humans. The contribution of DNL in AT compared to liver is lower on a per gram of tissue basis, but considering the total amount of AT, especially in obese individuals, the overall contribution of FA is similar between the two tissues (285). Furthermore, in humans, high a CHO diet appears to mediate little effect on DNL in AT (285). Alternatively, the reduction in DNL observed in liver with a high fat diet is not seen in AT (286), indicating that DNL in AT is less susceptible to nutrition or endocrine regulation. However, the mechanisms regulating DNL in AT and its implication in obesity are not fully understood.
AT is not only an important site of FA/TAG synthesis and storage but also represents a significant site of oxidative FA metabolism (287). Although the total amount of FA oxidized in WAT is insubstantial compared to skeletal muscle (288), the large concentration of mitochondria found within white adipocytes indicates these cells are involved in oxidation of FA (289). Furthermore, several studies (290) elucidated that while the amount of oxidation occurring in AT is small, it is inconsistent enough to reduce redistribution of lipid to other tissues such as liver. Masseen and colleagues (287,290) suggested that FA in cytosol of white adipocytes was cleared through mitochondrial oxidation, thus limiting release of FFA into circulation. Moreover, they proposed that with a greater increase in cytosolic FA concentration, mitochondrial uncoupling occurs, further reducing FFA released into circulation and thus preventing lipotoxicity (290). Mitochondrial uncoupling allows a greater amount of FA to be removed through the generation of heat rather than ATP production. In obesity an increase influx of FA into the cytosol of adipocytes may exceed the protective capacity of FA oxidation and mitochondrial uncoupling, thus resulting in greater amounts of FFA released into circulation (287,290). The subsequent increase in plasma FFA contributes to ectopic TAG accumulation in non-AT (i.e. pancreas, liver, and skeletal muscle), which promotes the development of insulin resistance (291).

Additional studies examining the oxidative capacity of white adipocytes suggested involvement of AMP-activated protein kinase (AMPK) and UCP1 in the regulation of adipose-specific FA oxidation (292,293). Rossmeisl et al. (293) reported that overexpression of UCP1 in AT of transgenic mice increased FA oxidation, as well as reduced FA synthesis and lipolysis in white adipocytes through an AMPK-mediated mechanism. Moreover, ADN was observed to activate AMPK signaling (294), suggesting that adipocytes regulate FA
oxidation in a variety of tissues, including adipocytes. Obesity is associated with a reduction in ADN and AMPK expression in adipocytes, indicating that downregulation of these genes may contribute to the reduced FA oxidation observed in obesity (295).

Lipolysis, which is the hydrolysis of TAG into FFA and glycerol in adipocytes, is catalyzed by hormone sensitive lipase (HSL) (284). Activation of HSL is mediated by catecholamines and additional endocrine signals, including adenosine, and prostaglandins through interactions with adrenergic receptors (296). Signaling through these receptors leads to activation of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) which in turn activates HSL (284). The cAMP/PKA also mediates phosphorylation of the phosphoprotein perilipin, which surrounds lipid droplets and serves as a docking protein for HSL (284). The antilipolytic effect of insulin is mediated through increasing intracellular phosphodiesterase that subsequently reduces PKA-mediated HSL activation (297). Obesity is characterized by high circulating FFA (185,251). This would suggest that obesity is associated with greater levels of lipolysis. However, several studies have demonstrated that adipocytes were resistant to catecholamine-induced lipolysis (298). Moreover, HSL activity has been shown to be dramatically reduced in obese individuals (284). This metabolic paradox may be partially explained by an increase in basal lipolysis. The exact mechanisms regulating basal lipolysis are not fully elucidated, but may be related to adipocyte size (299). Therefore, adipocyte hypertrophy leads to increased basal lipolysis that in turn leads to FFA-induced insulin resistance in skeletal muscle and liver.

**Obesity-Induced Cytokine Production in Adipose Tissue**

The primary connection between obesity and DM may be the development of AT inflammation. Hotamisligil and colleagues (4) originally demonstrated that obesity is
associated with increased TNFα release from AT. This group subsequently reported that adipose-specific TNFα mRNA expression was negatively correlated with insulin sensitivity in obese postmenopausal women (300). The source of this increased TNFα expression in AT was initially attributed to adipocytes, but several groups (6,301,302) recently reported that the stromal vascular fraction (SVF) of AT released greater levels of TNFα than neighboring adipocytes. Cells located in the SVF include preadipocytes, endothelial cells, smooth muscle cells, leukocytes, and macrophages. Weisberg and colleagues (7) suggested that adipose tissue macrophages (ATM) represented the major source of TNFα. Regardless of the source, TNFα contributes to the activation of several proinflammatory intermediates, such as NFκB and MAPK (including ERK1/2, p38 MAPK and JNK). Activation of these pathways in obesity mediates insulin resistance. Moreover, increased expression of TNFα in AT precedes the development of systemic insulin resistance, indicating that obesity-induced TNFα expression in AT is a mediator of the reduced insulin sensitivity observed in obesity.

The regulation of TNFα-induced insulin resistance in obesity may be mediated by direct or indirect mechanisms. Several groups (78,94,95,98) have demonstrated that TNFα mediates insulin resistance in skeletal muscle. Plomgaard et al. (95) reported that infusion of TNFα in healthy humans induced insulin resistance in skeletal muscle through disruption of insulin signaling. Specifically, TNFα inhibited tyrosine phosphorylation of IR and IRS, as well as increases serine (inhibitory) phosphorylation of IRS and Akt/PKB, leading to reduced insulin-stimulated glucose uptake (94,95,98). TNFα impairment of insulin signaling involves activation of proinflammatory intermediates, such as NFκB, ERK1/2, JNK, and PKC. Furthermore, obesity-induced activation of these pathways and subsequent serine phosphorylation of IR were attenuated in TNFα or TNFα receptor-deficient mice (303,304).
These data indicate the importance of this cytokine in obesity-induced insulin resistance. However, Ofei and colleagues (305) demonstrated that TNFα-neutralizing antibody failed to improve insulin sensitivity in obese individuals diagnosed with DM. This study suggested that \textit{in vivo} circulating TNFα may not be sufficient to induce obesity-induced insulin resistance.

One potential explanation is that TNFα released from AT functions in an autocrine or paracrine manner to regulate adipocyte metabolism. It is established that TNFα causes insulin resistance in adipocytes (306). This cytokine can block insulin-stimulated IR, IRS, and GLUT4 activation (307,308). Moreover, TNFα-induced insulin resistance in adipocytes involves signaling through JNK (100) and NFκB (307). This inflammatory signaling contributed to accumulation of ROS (309), which can further exacerbate insulin resistance. TNFα signaling also mediates several physiological changes in adipocyte metabolism, including reduced lipogenesis and increased lipolysis. The reduction in lipogenesis involves impaired insulin-stimulated glucose and FA uptake. Mechanisms mediating TNFα-induced inhibition of FA uptake are not well understood, but may involve downregulation of FATP, as well as decreased LPL activity (310,311). The induction of lipolysis and release of FFA contributes to the development of systemic insulin resistance, which is likely mediated through an inflammatory response initiated within skeletal muscle. Mechanistically, TNFα stimulates lipolysis through activation of kinases such as JNK and ERK1/2, which decreased the expression of antilipolytic genes (312). TNFα-induced lipolysis may also be exacerbated by inhibition of adipogenesis. Several groups (313,314) have reported that TNFα reduced PPARγ expression and activity. Therefore, TNFα-induced inflammation and insulin resistance in AT mediates systemic insulin resistance through release of FFA.
IL-6 is another important adipokine involved in obesity-induced insulin resistance. AT secretion represents 10 to 35% of circulating IL-6 (315). Adipocytes themselves express and secrete IL-6 (22,84); however, their overall contribution to sera IL-6 concentration may be limited as the SVF contributes a significant proportion of IL-6 released from AT (316). Adipocyte hypertrophy is coupled with increased IL-6 production (317). Moreover, Mohamed-Ali, et al. (318) reported that in obesity, adipocytes may contribute up to 30% of total circulating IL-6. There are two major signaling pathways activated by IL-6: the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and the MAPK cascade (319). Activation of these inflammatory pathways contributes to obesity-induced insulin resistance.

Like TNFα, the regulation of IL-6-induced insulin resistance in obesity may be due to direct and/or indirect effects on peripheral tissues, including skeletal muscle and liver. Several studies have suggested that IL-6 is responsible for hepatic insulin resistance (81,83,237). Klover and colleagues (237) demonstrated that reductions in plasma IL-6, mediated by neutralizing antibody specific for IL-6, were associated with improved hepatic insulin sensitivity. These effects can be attributed to improvements in insulin signaling, which include increased activation of IR, IRS, PI3K, and Akt/PKB (81,237). Moreover, IL-6 has been shown to induce STAT3 and suppressors of cytokine signaling (SOCS) (81,83,237). Blocking these inflammatory intermediates led to improvements in hepatic insulin sensitivity (237). Interestingly, the effect of circulating IL-6 on insulin sensitivity in skeletal muscle is less clear. In fact, minutes within starting exercise, the intracellular concentration of IL-6 increased 100-fold in muscle cells (237). Febbraio and colleagues (320) reported that this elevation in IL-6 during exercise was associated with an increase in whole body glucose...
uptake. Furthermore, IL-6 deficient mice show a reduced capacity for aerobic exercise (321). Therefore, these data indicate that in terms of skeletal muscle physiology, IL-6 has an important function in energy metabolism during exercise.

The paradoxical relationship between circulating IL-6 and systemic insulin resistance may be partially explained by the autocrine and paracrine effects of IL-6 on adipocytes. Several groups (80,84) reported that IL-6 blunted insulin-stimulated IR, IRS-1, Akt/PKB, and GLUT4 activation in adipocytes. Accompanying the development of insulin resistance is a reduction in adipose-specific LPL activity (322). Hence, the lipogenic activity of adipocytes is dramatically impaired by IL-6 through blocking the entry of substrates (glucose and FFA) necessary for TAG synthesis. Along with the reduction in lipogenesis is a corresponding increase in lipolysis (323,324). Peterson and colleagues (323) observed that acute infusion of IL-6 stimulated lipolysis in elderly subjects. The overall implication of IL-6 in adipocyte metabolism is a reduced capacity for lipid storage and increased flux of FFA into the circulation. Thus, increased adipocyte hypertrophy stimulates IL-6 production and release from adipocytes, which in turn exacerbates lipolysis and FFA-induced insulin resistance in skeletal muscle. This effect is further supported by a recent study that observed conditioned medium from mature lipolytic adipocytes that stimulate inflammation and insulin resistance in skeletal muscle (325).

**Obesity-Induced Macrophage Accumulation in Adipose Tissue**

Obesity is associated with increased cytokine expression and proinflammatory signaling in AT. Corresponding with this inflammation of AT is an increase in macrophage infiltration (3,6,7). Weisberg and colleagues (7) reported that adipocyte size was positively correlated with expression of the macrophage marker F4/80 in AT of obese mice. In humans,
the macrophage marker CD68 was correlated with BMI, as well as adipocyte size (7). The majority (85%) of adipose-specific cells are thought to be derived from bone marrow (7). However, the ability of preadipocytes to exhibit a macrophage-like phenotype, which includes expression of macrophage-specific cell surface markers (i.e. F4/80 and Mac-1) and phagocytic activity, also have implications in AT inflammation (326). In terms of ATM, it is known that these cells originate from progenitor cells in bone marrow. Furthermore, there are two major subpopulations of ATM, which includes a short-lived subset actively recruited to inflamed AT and a subset that is constitutively localized to AT (327). Lumeng and colleagues (328) also suggested that resident ATM undergo a phenotypic change in response to obesity that results in an enhanced proinflammatory response. Macrophage infiltration into AT (diapedesis) involves several steps that include: activation of capillary endothelium, increased expression of adhesion molecules and attachment of blood monocytes, transmigration of monocytes through endothelial cells, and differentiation of monocytes to ATM (329). Recruitment of macrophages is commonly observed in ischemic and hypoxic tissues (330). Within AT, macrophages localize primarily to sites of necrotic-like adipocyte cell death and aggregate in crown-like structures (CLS) around individual adipocytes (331). Cinti, et al. (331) demonstrated in HSL knockout mice that there was excessive adipocyte hypertrophy associated with necrosis and subsequent macrophage accumulation. Based on these data, it is evident that one of the ATM functions is to clear necrotic adipocytes.

Another proposed function of macrophages is the stimulation of AT remodeling as observed in obesity. The expansion of AT is associated with increased adipogenesis coupled with angiogenesis (332,333). Macrophages have been shown to mediate angiogenesis during tumor growth (334) and inflammation (335). More recently, Pang and colleagues (336)
reported that macrophages induced angiogenesis in AT of obese mice through increased expression of platelet-derived growth factor (PDGF). Hence, ATM may regulate neovascularization in expanding AT in response to reduced vascular density. Alternatively, macrophages were observed to impair adipogenesis in humans through inhibition of adipocyte differentiation (337-339). Kim and colleagues (339) also reported that macrophage inflammatory protein-related protein-2 (MRP-2) inhibited adipocyte differentiation but promoted preadipocyte migration. The ability of infiltrating macrophages to regulate adipocyte differentiation may in fact exacerbate obesity-induced inflammation and insulin resistance through several distinct mechanisms. Specifically, impairment of adipocyte differentiation contributed to reduced lipid storage capacity and subsequently increased circulating FFA (339), leading to insulin resistance in adipocytes or in peripheral tissues (i.e., skeletal muscle and liver). Furthermore, ATM may cause preadipocytes to exhibit a proinflammatory phenotype characterized by increased local and systemic cytokine concentrations (15,326). Regardless of the exact mechanism, the involvement of ATM in obesity-induced insulin resistance is apparent, as obese mice deficient in macrophage-specific inflammatory signaling were protected from obesity-induced insulin resistance (340).

The regulation of macrophage infiltration into AT is not fully elucidated but likely involves multiple mechanisms including the release of proinflammatory chemokines from hypertrophic adipocytes (7). Numerous chemoattractant proteins, such as MCP-1 and colony-stimulating factor (CSF), were suggested mediators of macrophage infiltration in AT (6,7). Leptin is also involved in endothelial cell activation and monocyte transmigration (329). However, leptin deficient ob/ob mice still exhibited macrophage accumulation in AT (6),
indicating involvement of additional chemokines. One potential candidate is MCP-1, which is thought to be one of the most potent chemokines regulating macrophage infiltration. Adipocytes, endothelial cells, and macrophages have all been observed to contribute to the release of this chemokine from AT (69,341,342). Christiansen et al. (343) reported that MCP-1 mRNA expression in AT was correlated with circulating MCP-1 concentration, indicating that AT is the primary source of the chemokine in obesity and was associated with insulin resistance. Westerbacka and colleagues (344) reported that obese and insulin resistant women exhibited a more pronounced increase in adipose-specific MCP-1 gene expression compared to lean subjects. Collectively, these studies suggest that adipocytes release chemokines in response to hypertrophy. This in turn stimulates recruitment of macrophages and increased production of cytokines/chemokines from adipocytes and macrophages, as well as increased lipolysis, thus promoting systemic inflammation and insulin resistance.

**Obesity-Induced Inflammation and Insulin Resistance: Toll-like Receptor 2 and 4**

**Overview of Toll-like Receptors**

Toll-like receptors (Tlrs) are germline-encoded receptors expressed on a variety of innate immune cells. In mammals, there are currently 13 identified isoforms of Tlrs (345). Each Tlr recognizes distinct structural motifs primarily expressed on foreign pathogens such as bacteria, viruses, fungi, and protozoa. These pathogen-associated molecular patterns (PAMPs) range from specific lipids to nucleic acid sequences. For instance, Tlr4 recognizes lipid based structures, such as LPS, whereas Tlr3 recognizes double stranded viral DNA (345,346). The cellular location of Tlrs is an important factor in determining their respective ligands. Tlrs located on the plasma membrane (i.e. Tlr1, 2, 4, 5, 6, 10, and 11) commonly recognize lipid or protein based PAMPs (347). Alternatively, intracellular based Tlrs (i.e.
TLR3, 7, 8, and 9) most commonly recognize bacterial and viral nucleic acids (347). The wide distribution in pathogen recognition of TLRs, as well as their extensive in vivo expression indicates their importance in host defense. TLRs are expressed on immune cells involved in innate and adaptive immunity, including macrophages, neutrophils, dendritic cells, and T and B lymphocytes (346). More recently, expression of these innate immune receptors were identified in cells not traditionally characterized as immune cells, such as intestinal epithelial cells (348), myotubes (349-351), and adipocytes (20).

The activation of TLRs through interactions with their respective ligand or PAMP leads to an inflammatory response mediated by a common signaling pathway (345,346). The initial event following ligand binding is the recruitment of adaptor proteins to the intracellular domain of TLRs. These adaptor proteins, include MyD88, TIR domain-containing adaptor protein (TIRAP), Trif, and TRAM (352). MyD88 is the most prominent adaptor protein involved in the majority of TLR signaling. In fact, only TLR3 and TLR4 are capable of signaling independently of MyD88 (352). The more prominent MyD88 dependent pathway involves several additional proteins including IL-1R-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6). Phosphorylation of IRAK activates TRAF6, which subsequently activates IKK and MAPK signaling pathways (347,352). Several groups (353,354) suggested that TRAF6 interacts with membrane-associated proteins, such as transforming growth factor-β-activated kinase 1 (TAK1) (transforming growth factor-(β-activated kinase 1) and transforming growth factor-(β-activated protein kinase-1-binding protein 1 (TAB1) leading to activation of IKK and MAPK pathways. However, the exact mechanisms are yet to be fully elucidated.
Activation of IKK mediates phosphorylation of IκB, marking the inhibitor protein for degradation. This is followed by nuclear translocation of NFκB, which induces expression of proinflammatory genes (355). There is evidence that MyD88-independent pathways may also induce NFκB activation through a delayed induction mediated through Tlr4 (356,357). Alternatively, activation of MAPK requires MyD88 for an inflammatory response to occur (352). Activation of the MAPK family, including JNK, ERK1/2, and p38, results in nuclear translocation of the transcription factor, AP-1 (358,359). Both NFκB and AP-1 are required for cytokine production, but it is unknown how activation of these transcription factors and the corresponding proinflammatory gene expression are affected by signaling through distinct Tlrs.

**Tlr4- and Tlr2-Induced Inflammation and Insulin Resistance**

**Tlr4.** LPS is a major component of the gram-negative bacterial envelope and a known inducer of inflammation in vivo (360). The proinflammatory component of LPS, endotoxin (or lipid A), is composed of FA and phosphate groups attached to a central glucosamine dimer (360). A specific serum binding protein transports endotoxin to cells expressing its corresponding receptor, which was recently identified as Tlr4 (361). The Tlr4 complex consists of the Tlr4 receptor itself and two co-receptors, MD-2 and CD14 (362). The MD-2 co-receptor is essential to Tlr4 function (363), whereas CD14 is a high affinity GPI-linked protein necessary for LPS binding (364). Following binding of LPS to CD14, Tlr4 is close enough to recognize endotoxin and initiate signal transduction through MyD88-dependent and independent pathways (360).

Several studies (365-370) have examined the impact of SNP in the Tlr4 gene on the development of CVD and DM. The findings of these studies are mixed but indicate that
certain polymorphisms exhibit protective effects. For instance, the Asp299Gly SNP was associated with changes in extracellular domain of Tlr4 causing reduced sensitivity to LPS (371). Data from the Bruneck Study (365) determined that 53 of 810 subjects were heterozygous for Asp299Gly SNP. Furthermore, carriers of this allele exhibited lower proinflammatory cytokines, ACP, and adhesion molecules, which was associated with lower atherosclerotic risk (365). Edfeldt and colleagues (370) also reported that this SNP was associated with reduced incidence of myocardial infarction in a Swedish population. Kolek et al. (369) reported a reduction in CVD and DM risk with individuals exhibiting the Asp299Gly SNP. More specifically, presence of the SNP has been associated with lower circulating CRP, with a simultaneous reduction in CVD and DM prevalence (369).

Alternatively, several studies have indicated that polymorphisms in Tlr4 have little impact on progression of chronic disease in humans (366,367). The disparities among these studies are difficult to explain and may be attributed to different populations.

Despite the conflicting genetic reports on Tlr4 polymorphisms in the development of CVD and DM, there is substantial evidence that the innate immune receptor is a major mediator of obesity-induced insulin resistance. Studies using the Tlr4 ligand, LPS, clearly showed the importance of the receptor in the development of insulin resistance in several cell types, including myotubes (372,373), hepatocytes (374), and adipocytes (23). Frost and colleagues (372) suggested that LPS-induced insulin resistance in C2C12 myotubes was mediated through Tlr4-induced activation of NFκB and JNK/AP-1, leading to production of TNFα and IL-6, respectively. Moreover, these effects were attenuated in skeletal muscle of Tlr4 deficient mice (372). Inflammation was also attributed to the LPS/Tlr4-mediated insulin
resistance in hepatocytes and adipocytes (23,374). Song et al. (23) observed that LPS stimulated TNFα and IL-6 expression, which in turn resulted in impaired insulin signaling.

There is also evidence that obesity is associated with increased circulating LPS, a major \textit{in vivo} agonist of Tlr4, and that this endoxemia is a primary mediator of insulin resistance (16,375). Cani and colleagues (375) demonstrated that plasma LPS was increased in obese mice. In humans, serum endotoxin levels are reported to be over 75% higher in diabetic subjects compared to non-DM controls (16). Furthermore, increased circulating LPS concentration was associated with insulin resistance in obesity, implicating a role of Tlr4 in obesity-induced inflammation. For instance, Tlr4 deficient mice were protected from obesity-induced insulin resistance (24,376,377). More specifically, Suganami and colleagues (24) observed that C3H/HeJ mice, with a missense mutation in the cytoplasmic domain of Tlr4, exhibited reduced TNFα expression in AT and improved insulin sensitivity following 16 weeks of a high fat diet (HFD) (24). These findings are supported by two recent studies (376,377). However, the impact of aberrant Tlr4 signaling on adiposity remains unclear. Tsukumo et al. (377) reported a reduction in epidydimal fat pad weight due to increased oxygen consumption and reduced respiratory exchange ratio, although the exact mechanisms are unknown. Alternatively, Poggi and colleagues (376) observed an increase in adiposity in HFD fed C3H/HeJ mice. The disparity between these studies may be due to distinct differences in diet composition, but this requires further investigation. Additional investigations demonstrated that Tlr4 may also be involved in the development of atherosclerosis (378-380). More specifically, Tlr4 is expressed in lipid-rich macrophage-infiltrated atherosclerotic lesions of mice and humans (380). Furthermore, Kim and colleagues (378) observed that Tlr4 knockout mice were protected from HFD-induced
vascular inflammation as determined by reduced NFκB activation and NO production. Overall, these studies indicate that Tlr4 may be a potential mediator of obesity-induced insulin resistance and related co-morbidities, including DM and CVD.

**Tlr2.** Of the various innate immune receptors, Tlr2 recognizes the most diverse number of microorganisms. PAMPs commonly recognized by Tlr2 include glycolipids (i.e. lipoteichoic acid and peptidoglycan), polysaccharides (i.e. zymosan), and whole bacteria (381,382). LPS was originally characterized as a Tlr2 agonist (383), but it was later determined that this effect was mediated by contaminating lipoproteins (384). However, two studies (385,386) reported that Tlr2 recognized atypical forms of LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*. The atypical forms of LPS differ in structure and function than the type recognized by Tlr4 by their different number of acyl groups in the lipid A component (387). Regardless of the exact agonist, Tlr2 activation requires dimerization with Tlr1 or Tlr6 (388,389). The specific heterodimer formed in the Tlr2 complex appears to be dependent on the size and number of fatty acyl chains. However, post-receptor signaling appears to be the same for both dimers.

There are currently 16 polymorphisms identified in the Tlr2 gene. Of these, half are non-synonymous mutations leading to amino acid exchanges and the remaining half are synonymous polymorphisms (390). There is limited research investigating the relationship between Tlr2 polymorphisms and obesity-related co-morbidities. However, two studies (390,391) demonstrated SNPs in the Tlr2 gene that were associated with reduced cytokine production. More specifically, Merx and Colleagues (390) reported a novel SNP in Tlr2 (arginine codon at 447 to stop codon) associated with reduced IL-6 and TNFα production in heterozygote Arg447 stop individuals. Additional genetic analyses have observed that
various SNP in Tlr2 were protective against several inflammatory conditions, including Type 1 DM (392) and coronary endothelial dysfunction (393).

Despite the limited genetic analysis, there is evidence to suggest involvement of Tlr2 in obesity-induced inflammation and insulin resistance. Creely, et al. (16) reported that expression of Tlr2 was increased in AT of obese diabetic individuals, whereas Tlr4 levels were unchanged. Furthermore, expression of this innate immune receptor was associated with increased inflammatory signaling, including activation of NFκB and induction of proinflammatory cytokine expression (16). Murakami and colleagues (394) utilized flow cytometry to analyze isolated adipocytes from obese mice and observed that these cells co-express Tlr2 and TNFα. Moreover, expression of these proteins was associated with insulin resistance (394). The exact mechanisms involved in regulation of Tlr2 expression are unclear but may be related to elevated endotoxin levels (16,20). As mentioned previously, certain forms of LPS may interact directly with Tlr2 (387). However, recent evidence indicates that LPS may also indirectly regulate Tlr2 through a Tlr4-specific pathway (20). These studies indicated a potential role of Tlr2 in the development of obesity-induced inflammation and insulin resistance.

**Fatty Acid-Induced Tlr2 and Tlr4 Signaling**

Investigations into the structure of endotoxin, the bioactive component of LPS, revealed the presence of acyl-linked SFAs (395). Furthermore, these acylated FAs were subject to hydrolysis, which subsequently reduced the endotoxic properties (396,397). Kitchens and colleagues (397) reported that deacylated LPS (dLPS) antagonized LPS-induced NFκB activation and TNFα expression in a human monocyte cell line. Moreover, deacylation of bacterial lipopeptides resulted in a reduction of Tlr2 to initiate a
proinflammatory response in human monocytes (398). These data suggest that FAs, specifically SFAs, act as endogenous ligands for Tlrs. Lee et al. (399) originally reported that SFA stimulated NFκB activation and cyclooxygenase-2 (COX2) activity through a Tlr4-dependent mechanism. Additional studies (25,400) confirmed the involvement of SFA in Tlr4 signaling that included activation of MyD88, IRAK-1, TRAF6, and PI3K-Akt pathways. However, Lee and colleagues (26) demonstrated that SFA increased COX2 promoter activity in bone marrow-derived macrophages (BMDM) independent of Tlr4. Moreover, this inflammatory response stimulated dimerization of Tlr2 with its co-receptors Tlr1 or Tlr6 (26). More recently, Nguyen et al. (401) demonstrated that FFAs mediated IL-6, TNFα, and MCP-1 gene expression in macrophages through Tlr2 and Tlr4 activation of JNK. Therefore, these data demonstrate the importance of both innate immune receptors in FA-induced inflammation.

Obesity is associated with an increase in circulating FFA (185,251). Subsequently this promotes Tlr signaling in insulin sensitive tissues, such as skeletal muscle and AT. For instance, Reyna and colleagues (402) showed FFA concentration was associated with an increase in Tlr4 gene and protein expression. Moreover, there was a strong correlation between insulin resistance and Tlr4 expression in obese and diabetic subjects (402). Alternatively, Senn (403) demonstrated that palmitate stimulated Tlr2 signaling in C2C12 myotubes, leading to activation of the proinflammatory mediators, JNK and NFκB, as well as reduced insulin-stimulated glucose uptake. Additionally, there is evidence that SFAs mediate inflammation and insulin resistance in AT through Tlr-dependent mechanisms. Shi et al. (25) observed that FFA-induced TNFα and IL-6 expression in adipocytes was attenuated in Tlr4 knockout mice. Furthermore, these mice were protected from FFA-induced NFκB activation
and impaired insulin signaling in AT (25). Additional studies (376-378) confirmed that Tlr4 deficient mice exhibited greater insulin sensitivity than their wild type counterparts, and that this effect may be a consequence of reduced inflammatory signaling in AT.

Macrophage infiltration is a primary contributor to AT inflammation. Furthermore, several studies (24,401,404) suggested that SFA signaling through Tlr4 is a potential mechanism involved in this process. Suganami and colleagues (404) utilized a co-culture system of adipocytes and macrophages to determine that FFA released from hypertrophied adipocytes serve as ligands for Tlr4 expressed on macrophages, as well as adipocytes themselves. Moreover, the Tlr4-NFκB pathway is critical in palmitate-induced TNFα expression in macrophages, and subsequent FFA release from adipocytes. These studies indicate that FFA released from hypertrophied adipocytes act in a paracrine and autocrine manner to stimulate Tlr receptors on macrophages and adipocytes located in AT and subsequently cause local inflammation and insulin resistance. Moreover, FFAs released from AT can then interact with Tlrs expressed in peripheral tissues, including skeletal muscle and liver, causing inflammation and insulin resistance in these tissues.

As discussed previously, certain MUFA and PUFA have been shown to exert anti-inflammatory properties both in vitro and in vivo (225-229). One potential explanation for these effects may be through antagonism of Tlr2 and Tlr4 signaling. Several investigations (26,399,400) reported that the PUFAs, DHA and EPA, antagonized SFA-induced Tlr signaling in RAW 264.7 macrophages. Moreover, Lee and colleagues (405) demonstrated that monocytes isolated from subjects fed 9 to 15 g fish oil (rich in omega-3 FA) per day for 8 weeks exhibited a reduction in Tlr2 and Tlr4 agonist-mediated COX2 expression and NFκB activation. Overall, these studies indicate that SFAs act as endogenous ligands for Tlr2
and Tlr4, whereas PUFAs antagonize Tlr signaling, likely through a direct interaction with these receptors.

Summary

The prevalence of obesity continues to rise dramatically in the U.S. and worldwide. Corresponding to this rise is an increase in obesity-related co-morbidities, such as DM and CVD. The underlying mechanism linking obesity to these diseases is the development of insulin resistance, which is further attributed to a state of chronic low-grade inflammation characterized by elevation in proinflammatory cytokines. Furthermore, FFA levels are increased in obesity and induce insulin resistance through a variety of mechanisms including inflammation and oxidative stress. However, obesity-induced changes in AT appear to precede the development of systemic inflammation and insulin resistance. Adipocyte hypertrophy represents the initial event contributing to AT inflammation. In response to increased lipid accumulation, adipocytes secrete greater amounts of FFA and proinflammatory cytokines into the circulation. These inflammatory mediators can then act in an autocrine or paracrine manner to activate surrounding cells, including macrophages, endothelial cells, and neighboring adipocytes. These events promote recruitment of immune cells into AT and further amplify the release of inflammatory mediators from AT, thus contributing to inflammation in other insulin-sensitive tissues (i.e., liver and skeletal muscle), resulting in subsequent insulin resistance. The ability of FFA, more specifically SFA, to induce inflammation and insulin resistance may be dependent on the presence of specific innate immune receptors. Tlrs recognize specific PAMPs on invading pathogens, initiating a proinflammatory response to aid in host defense. More recently, Tlr2 and Tlr4 were observed to mediate SFA-induced inflammation in AT and other insulin-sensitive tissues. Preliminary
investigations on these receptors demonstrate their potential involvement in DM and CVD. Furthermore, elucidating the role of Tlr2 and Tlr4 in AT inflammation will provide greater insight into the development of obesity-related co-morbidities and help identify novel nutritional and pharmaceutical therapies.

References


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CHAPTER 3. TLR4 DEFICIENCY SELECTIVELY PROTECTS AGAINST OBESITY INDUCED BY DIETS HIGH IN SATURATED FAT
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Abstract

Toll-like receptor-4 (Tlr4), a key pattern recognition receptor involved in innate immune response, is activated by saturated fatty acids (SFAs). To investigate the involvement of this receptor in obesity caused by consumption of diets high in fat, we utilized male Tlr4-deficient 10ScN mice and 10J controls. Mice were fed either low fat (low-fat control (LFC)), high unsaturated fat (high-fat control (HFC)), or high saturated fat + palmitate (HFP) diets ad libitum for 16 weeks. Relative to the LFC diet, the HFC diet resulted in greater epididymal fat pad weights and adipocyte hypertrophy in both Tlr4-deficient and normal mice. However, the 10ScN mice were completely protected against the obesigenic effects of the HFP diet. Moreover, macrophage infiltration and monocyte chemotactic protein-1 transcript abundance were lower in adipose tissue of 10ScN mice fed the HFP diet, and the hyperinsulinemic response was negated. Tlr4-deficient mice also had markedly lower circulating concentration of MCP-1 and much less nuclear factor-κB protein in nuclear extracts prepared from adipose tissue, irrespective of diet. In contrast, Tlr4 deficiency did not attenuate the induction of tumor necrosis factor-α or interleukin-6 expression in adipose tissue. These data indicate that Tlr4 deficiency selectively protects against the obesigenic effects of SFA by selectively altering obesity-related inflammatory responses in adipose tissue.

Introduction

Obesity is associated with chronic low-grade inflammation (1-4) that is characterized by increased circulating concentrations of proinflammatory cytokines (5-7) and acute phase
proteins (8,9), and decreased concentrations of the anti-inflammatory protein adiponectin (10). Although the mechanisms underlying the onset and progression of this inflammatory state remain somewhat ambiguous, two recent findings have strongly implicated the adipose tissue itself as a major contributor. First, genetic and diet-induced obesity models have not only shown increased expression of proinflammatory cytokines in the adipose tissue (1,3,11,12), but also a marked adipose-specific infiltration of macrophages that show gene expression profiles consistent with inflammation (13-15). Secondly, the adipocyte itself is capable of mounting a classical innate immune response initiated by ligand activation of Toll-like receptor-4 (Tlr4), followed by activation of nuclear factor-κB (NFκB), increases in proinflammatory gene expression and the release of these cytokines from cultured adipocytes (16-18). Furthermore, both Tlr2 and Tlr4 expression are increased in adipose tissue in association with obesity and Type 2 diabetes mellitus (DM) (19).

It is clear from multiple obesity models that adipocytes and macrophages, localized in adipose tissue, produce proinflammatory mediators, the mechanistic links among adipocyte hypertrophy, dietary fat, inflammation, and macrophage recruitment and activation have not been delineated. However, direct regulation of Tlr4 by certain saturated fatty acids (SFA) is a likely component of this process because palmitate and other SFA act directly to stimulate proinflammatory cytokine expression and NFκB activation in cultured adipocytes and macrophages (20,21). Furthermore, Suganami and colleagues (21) recently determined that free fatty acids (FFA) released from hypertrophic adipocytes can signal macrophages through Tlr4 and stimulate release of tumor necrosis factorα (TNFα). Additional studies by other groups (20,22-24) have shown that Tlr4 deficient mice are less susceptible to fat-induced
inflammation and insulin resistance, and that C3H/HeJ mice are protected against hyperglycemia and inflammation in adipose tissue when Tlr4 signaling is blunted (22).

Although the link between a functional Tlr4 receptor and obesity-induced inflammation in adipose tissue seems quite clear, whether this receptor contributes to the onset and progression of obesity is controversial. Elegant *in vivo* studies with a Tlr4 knockout mouse model have shown blunted inflammation, yet a greater adiposity in females (23). In contrast, Tsukomo et al. (25) recently reported evidence that a loss of function mutation in Tlr4 protects against high fat, diet-induced obesity. Albeit, there is currently a paucity of data that address the possibility that dietary SFA vs. unsaturated fatty acids (UFA) influence the ability of Tlr4 to promote adipose accretion. Consequently, we used the 10ScN mouse strain, which has a 74 kb deletion from chromosome 4 that precludes expression and production of Tlr4, to test the hypothesis that Tlr4 specifically mediates obesity and inflammation associated with a diet high in saturated fat.

**Materials and Methods**

*Animals and animal care.* Male Tlr4 deficient C57BL/10ScN mice (Cat No. 003752) derived from the C57BL/10 subline and corresponding control male C57BL/10J mice (Cat No. 000665), were obtained from Jackson Laboratories (Bar Harbor, ME) at 6-8 weeks of age. Subsequently, lack of Tlr4 mRNA was confirmed with real time PCR. Animals were housed individually in stainless steel wire-mesh cages at 21°C in a room with an automatically controlled 12-hour light:dark cycle. Mice were acclimated to the environment and provided unlimited access to food and water. Animals from each genotype were then randomly assigned to one of three experimental diets (n = 25 genotype/diet): 1) low fat control (LFC), high fat control (HFC), or high fat palmitate (HFP) for 16 weeks. Both high
fat diets were semi-purified, powdered diets based on American Institute of Nutrition recommendations (26) that were modified to induce obesity by providing 60% of calories from lipid (Table 1). The high fat diets were identical except the source of fat used was either soybean oil in the HFC (Harlan Teklad) or a mixture of lard (Harlan Teklad) and purified palmitate (Nu-Check Prep, Inc.) in the HFP. The LFC diet contained 12% of calories from lipid. Food intake was measured daily and used to calculate total energy intake. Mice were fasted overnight (6-8 h) and euthanized by CO2 asphyxiation for all blood and tissue collections. Blood was collected by heart puncture, placed on ice, and serum was collected by centrifugation and frozen at -80°C until analyzed. Animals were weighed and abdominal fat pads and liver were removed and weighed. All extracted tissues were immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction and immuno-histochemical analysis. All experimental protocols for animal care and use were approved by the Institutional Animal Care and Use Committee at Iowa State University, Ames, IA, USA.

**Analysis of Metabolic Parameters.** Serum concentration of glucose (Biovision) and insulin (Linco) were measured using commercial assay kits. Leptin, monocyte chemotactic protein-1 (MCP-1), adiponectin (ADN), and interleukin-6 (IL-6) serum concentrations were measured using ELISA kits (R&D Systems). Serum FFA was measured with NEFA-C (Wako Pure Chemical Industries, Inc.) kit.

**Quantitative real-time PCR.** Total RNA was extracted from frozen adipose tissue using a commercially available acid-phenol reagent (TRIzol, Invitrogen Corp.). Potential DNase contamination was removed with DNase-free™ (Ambion, Inc.). First-strand cDNA was synthesized using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen Corp.). Primer sequences for mouse sense and anti-sense primers are listed in
Table 2. Thermal cycling conditions for PCR reactions were 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Polymerase reaction products amplified by these primers were cloned into pGEMT vector (Promega) and sequenced for verification. Real-time reactions were carried out on an iCycler real time machine (BioRad) using the IQTM SYBR Green Supermix kit (BioRad). The abundance of each gene product was calculated by regressing against the standard curve generated in the same reaction with their respective plasmid. All genes of interest were normalized to beta actin and expressed as log starting quantity.

Immunohistochemistry. Frozen adipose tissue was fixed overnight at room temperature in 10% zinc-formalin solution and embedded in paraffin. Five micron sections were cut at 50 µm intervals and mounted on glass slides, deparaffinized in xylene, and stained for expression of F4/80 with monoclonal antibody (Serotec) as previously described (14). For each mouse, four different fields were selected and adipocyte area was determined using AxioVision v4.5.0.0 (Carl Zeiss, Germany). F4/80-positive macrophages were then counted at 40X magnification and divided by total cell number to obtain percent F4/80-positive cells.

Tissue and plasma fatty acid profile. Lipids from adipose and serum samples were extracted by the method of Lepage and Roy (27) with minor modifications. Briefly, 0.5 g of tissue was homogenized in 2.5 mL 4:1 methanol:hexane and 200 µL of 3.7 mmol heptadecanoic acid/L methanol was added to each sample as an internal standard. Fatty acid methyl esters were analyzed by gas chromatography on a Hewlett-Packard model 6890 (Hewlett-Packard, Palo Alto, CA) fitted with an Omegawax 320 (30 m x 0.32 mm ID, 0.25 µm) capillary column (Sigma-Aldrich, St Louis, MO USA). Hydrogen was the carrier gas.
The temperature program ranged from 80°C to 250°C with a temperature rise of 5°C/min. The injector and detector temperatures were 250°C and 1 µL of sample was injected and run splitless. Fatty acids were identified by their retention times on the column with respect to appropriate standards.

**EMSA and Activated NFκB p65 ELISA.** Adipose tissue nuclear extracts and mobility shift assay were prepared and validated as previously described (16). The consensus NF-κB oligonucleotides (Santa Cruz) were end-labeled with [-32P]ATP (PerkinElmer) using T4 polynucleotide kinase (Santa Cruz). Binding of nuclear proteins to the labeled probe was done by incubating 50 µg nuclear proteins with 50,000 cpm of labeled probe for 30 min at room temperature in a binding buffer [2 mM HEPES, 50 mM KCl, 2 mM EDTA, 10% glycerol, and 1% BSA (wt/vol)] in the presence of 2 µg poly(dI-dC) (Sigma) in a final reaction volume of 40 µL. For quantitative NFκB p65 DNA binding, 10µg of nuclear protein was assayed for the presence of activated p65 by ELISA using antibodies specific for activated p65 following binding to NFκB consensus sequence (TransAM Active Motif).

**Statistical analyses.** Data were tested for normality and analyzed using the mixed-model analysis with diet and genotype considered fixed effects, and the time dietary treatments were implemented for subgroups as a random effect. Significant interactions of main effects are indicated as Diet*Genotype effects. All indicated P-values were two-tailed and Bonferroni corrected. Differences were considered significant at P < 0.05 and a tendency at P < 0.10. Values are presented as least squares (LS) means ± SEM.

**Results**

*Tlr4 deficiency protects mice from increased adiposity caused by consumption of a diet high in saturated fat, without reducing caloric intake.* We examined whether the fatty
acid composition of the diet influenced the obesigenic effect of high fat diets in male Tlr4 mutant (10ScN) and normal (10J) mice. Regardless of diet, 10ScN mice weighed on average 3.8 g less than 10J mice after 16 weeks on the experimental diets (Genotype, $P<0.0001$, Figure 3A). Furthermore, the HFC diet increased body weight in both genotypes (Diet, $P<0.0001$), but average weekly energy intake (Figure 3B) was reduced by the HFC diet (Diet, $P<0.0001$). Additionally, epididymal fat pad weight was increased by the HFC diet in both genotypes. However, only the mice lacking Tlr4 were protected against the obesigenic effect of the HFP diet (Figure 3C, Diet*Genotype, $P=0.05$). Similar results were obtained for epididymal adipocyte size (Figure 3D) in that all mice fed the HFC diet had larger cells, but only the normal strain fed the HFP diet had enlarged cells vs. the LFC (Diet*Genotype, $P=0.05$).

To confirm diet-induced changes in fatty acid profiles of epididymal adipose tissue, we measured SFA, n-3, and n-6 fatty acid contents (Table 3). As expected, relative to the LFC and HFP diets, both palmitate and total SFA were reduced in mice fed the HFC diet (Diet, $P<0.002$), and n-3 fatty acids (docosahexaenoic acid and eicosapentaenoic acid) were lower in mice fed the HFP diet (Diet, $P<0.002$). Furthermore, the n-6 fatty acid content was higher in mutant mice (Genotype, $P<0.005$) and was also reduced by the HFP diet (Diet, $P<0.03$). However, the effects of diet and genotype on n-3 and n-6 fatty acid contents were not sufficient to alter the n-6 to n-3 ratio in this particular adipose depot.

Tlr4 deficiency improved markers of insulin sensitivity and inflammation. To gain insight into the effect of high fat intake on insulin sensitivity in Tlr4 deficient mice, we measured fasting serum glucose (Figure 4A) and insulin (Figure 4B) following 16 weeks on experimental diets. Glucose concentrations were lower in the mutant strain (Genotype,
P=0.0068), and were reduced in both genotypes fed either high fat diet (Diet, P=0.0014).

Insulin concentrations were also lower in Tlr4 mutant mice (Genotype, P<0.0001).

Alternatively, the HFC diet nearly doubled serum insulin in these mice, but this response only approached significance (Diet, P=0.111), and there was no effect on the normal strain (Diet*Genotype, P=0.15). However, these differences resulted in lower glucose to insulin ratios (GIR) in mutant mice fed the HFC diet as compared with the LFC or HFP diets, and the normal 10J mice had similar GIR, regardless of diet (Diet*Genotype, P=0.028).

We then measured several markers of inflammation that have been associated with obesity. The serum concentration of leptin was greater in all mice fed the HFC diet (Diet, P=0.0004, Figure 4D), and serum adiponectin was lower in the mutant strain (Genotype, P=0.043, Figure 4E). Additionally, the HFC diet increased serum adiponectin relative to the control and HFP diets, but only in the mutant strain (Genotype*Diet, P=0.031). Serum MCP-1 was seven-fold higher in 10J vs. mutant mice (Genotype, P<0.0001, Figure 4F), but was not influenced by diet in either genotype.

*Obesity-induced inflammation in adipose tissue was differentially regulated by dietary SFA vs. UFA, and was partly ameliorated by Tlr4 deficiency.* To determine if inflammatory markers were related to changes in adipose tissue, we quantified macrophage infiltration and measured several inflammatory markers in adipose tissue. The percentage of F4/80-positive cells (i.e., macrophages) in adipose tissue of 10J control mice was greater in mice fed either HF diet (Diet P<0.0001, Figure 5A). Alternatively, Tlr4 deficiency attenuated the SFA-induced, but not HFC-mediated, macrophage accumulation in 10ScN mice (Genotype*Diet interaction P=0.0002). Furthermore, the greater percentage of F4/80
positive cells was associated with the formation of distinct crown-like structures (CLS) (Figure 4B).

We then examined the mRNA abundance of selected proinflammatory chemokines and cytokines. MCP-1 expression was increased in the epididymal adipose tissue of 10J mice fed the high fat diets (Genotype*Diet, P=0.021, Figure 6A). There was also a trend for increased CCR2 transcript abundance in mice fed either HF diet (Diet, P=0.089, Figure 6B). Expression of the proinflammatory cytokines, TNFα (Figure 6C) and IL-6 (Figure 6D), were increased by over 2- and 3-fold in adipose tissue of mice fed the HFP diet (Diet, P=0.013 and P\leq0.0001, respectively). Furthermore, the magnitude of the increase in IL-6 was greater in mutant vs. normal mice fed the HFP diet (Genotype*Diet, P=0.074). Tlr2 expression was increased over 2-fold with diet-induced obesity, irrespective of genotype (Diet Effect, P=0.035, Figure 6E).

To determine if the proinflammatory transcription factor NFκB may be involved in adipose-specific inflammation, we utilized electrophoretic mobility shift assays as a qualitative assessment of NFκB translocation. There was a marked reduction in the nuclear localization of NFκB in Tlr4-deficient mice regardless of diet (Figure 7A). Similar quantitative results were obtained using a NFκB p65 DNA binding ELISA, which indicated adipose tissue nuclear NFκB concentration in mutant mice was 50% less than that in 10J mice (Genotype, P=0.0015, Figure 7B).

**Discussion**

The data presented herein establish a novel relationship between obesity and Tlr4. The absence of a functional Tlr4 due to the 74 kb deletion on chromosome 4 in the 10ScN strain selectively protects against the obesigenic effect of a diet high in SFA, but not that of
an isocaloric diet high in UFA in male 10ScN mice. Although we did not quantify total body fat mass in this study, adipocyte size mirrored epididymal fat pad weights, and thus reinforce our conclusion that the absence of Tlr4 protected against diet-induced obesity (DIO), but only when the diet was based on SFA. It is also important to note that this resistance to adipocyte hypertrophy occurred without a corresponding reduction in energy consumption, and is thus of metabolic origin, rather than a simple limitation in energy intake. These findings may implicate energy expenditure, and this possibility and potential underlying mechanisms are currently under investigation. Additionally, it is intriguing that recent findings indicated that the gut microbial population influences adipose accretion through effects on energy availability and regulation of the fasting-induced adipocyte factor (Fiaf). The Fiaf protein is a circulating lipoprotein lipase inhibitor expressed by the intestinal epithelium and adipocyte, and knockout models have shown that the absence of Fiaf protected against over accumulation of adipose tissue caused by introduction of a normal microbiota in germ-free mice (28). Consequently, it seems possible that the absence of Tlr4 precludes changes in the gut microbiota in response to SFA that would alter Fiaf expression and thereby influence triglyceride storage in adipocytes.

Recent reports (22-25) have indicated mixed results with regards to DIO in Tlr4 mutant or knockout mice. Our results are consistent with those of Tsukumo et al. (25), who also used males, but from the C3H/HeJ model. In contrast, Shi et al. (23) used females from a Tlr4 knockout model backcrossed with C57BL6J for six generations, and determined that these mice succumbed to DIO when fed a high-fat diet containing lard. Furthermore, Poggi et al. (24) reported heavier epididymal adipose weights and adipocyte hypertrophy in male C3H/HeJ mice consuming a high fat diet based on anhydrous milk fat, which likely varies
considerably in fatty acid composition. Although the mechanisms underlying these disparate results are not yet apparent, Tsukumo et al. (25) suggested that metabolic and physiologic differences relating to strain and background are likely to be important factors, and our data clearly indicate a strong linkage between Tlr4 function, dietary fat source, and DIO. Equally important, Cani et al. (29) provided convincing evidence that mice consuming a high fat diet containing both corn oil and lard had increased circulating concentration of bacterial lipopolysaccharide that triggered obesity and inflammation. Because mice lacking the CD14 co-receptor for Tlr4, which precludes formation of a functional Tlr4 complex, were resistant to this phenomenon, these researchers suggested that endotoxemia facilitated by dietary fat intake largely mediates the effects of a high fat diet on obesity and inflammation via a Tlr4-mediated signaling pathway. Collectively, the data substantiate the possibility that SFA promote obesity post-absorptively by direct activation of Tlr4, and (or) promote the uptake of gut-derived endotoxin, which also activates Tlr4 signaling. Consequently, it will be of utmost importance in the future to establish the effects of specific Tlr4 mutations and knockout models on obesity with respect to specific dietary fat sources and fatty acid profiles.

Chronic inflammation in adipose tissue is a co-morbidity of obesity that is strongly associated with the onset and progression of insulin resistance, and ultimately the transition to frank diabetes. We have shown previously that treating cultured adipocytes with palmitate results in activation of NFkB and proinflammatory cytokine expression and release into the culture media (30). Furthermore, using in vivo and in vitro experiments, Shi et al. (23) determined that a functional Tlr4 is a requisite component of the proinflammatory effects of palmitate or SFA on adipocytes. With respect to the present data, several points are notable.
First of all, the only inflammatory marker attenuated by the absence of Tlr4 was MCP-1. Whereas the high fat diets, irrespective of whether saturated or unsaturated in composition, resulted in increased MCP-1 expression in epididymal adipose tissue of normal mice, neither diet altered expression in mutant mice. Furthermore, the expression of both IL-6 and TNFα were markedly increased by the HFP diet, irrespective of genotype, and chemokine (C-C motif) receptor 2 (CCR2) expression was increased by the high fat diets in both genotypes. These results indicate not only that some inflammatory responses to high fat diets may be mediated independently of Tlr4, at least at the mRNA level, but also that some responses to high fat intake occur whether the fat is comprised predominantly of SFA or UFA. It should also be noted that even in the absence of epididymal adipose expansion and adipocyte hypertrophy in the mutant mice fed the HFP diet, both IL-6 and TNFα expression were increased. This possibly indicated a metabolic component to the proinflammatory effects of saturated fatty acids in adipocytes, as we have suggested previously, because inhibition of fatty acyl Co-A synthase attenuates the activation of NFκB by palmitate, but exacerbates IL-6 release into the culture media (30). Finally, the absence of a functional Tlr4 not only precluded the adipocyte hypertrophy in mice fed the HFP diet, but also blocked infiltration of adipose tissue with macrophages. This is consistent with the hypothesis that it is adipocyte hypertrophy that ultimately signals macrophage recruitment, and it is clear that this response does not depend on a functional Tlr4.

The serum MCP-1 and adipose NFκB data show a striking effect of genotype in that both were markedly depressed in the mutant mice, and were largely unresponsive to diet. Although serum MCP-1 concentration was barely measurable in mutant mice, MCP-1 adipose tissue expression differed little from that of normal mice. This perhaps indicated
that adipose tissue contributed little to circulating MCP-1 in these mice, but we cannot
discount the possibility that the adipose concentration was sufficient to be of physiological
significance. In regard to nuclear localization of adipose NFκB, both qualitative and
quantitative assessments indicated that the nuclear content of this important transcription
factor is significantly lower in mutant vs. normal mice with little effect of diet. We have
shown previously that the transcriptional activity of NFκB in adipocytes can be uncoupled
from proinflammatory cytokine responses (30), but it is perhaps important that Shi et al. (23)
noted a marked effect of dietary fat on NFκB activity in a knockout model.

In summary, our study indicated that the Tlr4 mutation in male 10ScN mice
selectively blocked SFA-induced obesity, and that this response was associated with an
absence of adipose infiltration with macrophages and improved markers of insulin sensitivity
as reflected in the lower ratios of glucose to insulin. These results point to a unique
relationship between Tlr4 and saturated fatty acids, and underscore the potential for
interactions between specific dietary fatty acids and Tlr4 mutations to impact the
development of obesity and the associated inflammation.

Acknowledgements

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Nutrition (CDFIN), and Nutrition and Wellness Research Center at Iowa State University.

References


33-35.


4 mutation are protected against the development of insulin resistance in white adipose tissue in response to a high-fat diet. Diabetologia.


Table 1. *Diet Composition*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Low-Fat Control (LFC)</th>
<th>High-Fat Control (HFC)</th>
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<td>g/kg</td>
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<td>100</td>
<td>100</td>
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1. Adapted from AIN-93M Diet
2. Harlan Teklad, Madison, WI
3. Nu-Check Prep, Inc., Elysian, MN
Table 2. Primer Sequences for Quantitative Real-Time Polymerase Chain Reaction

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<th>Target</th>
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Table 3. Adipose tissue fatty acid profiles in normal and Tlr4 mutant mice fed low fat control, high fat control, and high fat-palmitate diets for 16 weeks

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<tr>
<th>Diet</th>
<th>Genotype</th>
<th>n</th>
<th>Palmitate</th>
<th>SFA</th>
<th>n3 FA</th>
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<td>LFC*</td>
<td>10ScN</td>
<td>6</td>
<td>31.35 ± 3.47</td>
<td>33.68 ± 3.38</td>
<td>0.49 ± 1.00</td>
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<td>18.10 ± 3.22</td>
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<td>28.45 ± 3.18</td>
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<td>0.53 ± 0.99</td>
<td>15.18 ± 5.53</td>
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Main Effects

- **Genotype**: P = 0.48, P = 0.52, P = 0.18, P = 0.0055, P = 0.48
- **Diet**: P = 0.0026, P = 0.0014, P = 0.0025, P = 0.033, P = 0.21

Interaction

- **Diet*Genotype**: P = 0.32, P = 0.29, P = 0.22, P = 0.61, P = 0.67

*Low Fat Control (LFC), High Fat Control (HFC), and High Fat Palmitate (HFP) diets
Figure 3. *Tlr4* deficiency protects mice from increased adiposity due to consumption of a diet high in saturated fat.

All measures were taken after 16 weeks on experimental diets (n = 25 unless noted otherwise). (A) Body weight of 10ScN (white bars) and 10J (black bars) mice (Diet effect \(P<0.0001\), Genotype effect, \(P<0.0001\), Diet*Genotype interaction \(P=0.84\)). (B) Average weekly energy intake (kcal/week) of 10ScN and 10J mice (Diet effect \(P<0.0001\), Genotype effect \(P=0.34\), Diet*Genotype interaction \(P=0.94\)). (C) Epididymal fat pad weight of 10ScN and 10J mice (Diet effect \(P<0.0001\), Genotype effect \(P<0.0001\), Diet*Genotype interaction \(P=0.058\)). (D) Adipocyte size (\(\mu m^2\)) of 10ScN and 10J mice (n = 6, Diet effect \(P=0.0079\), Genotype effect \(P<0.0001\), Diet*Genotype interaction \(P=0.052\)). All displayed values represent LS means ± SEM for Diet*Genotype interaction. When interaction term was \(P<0.15\), Bonferroni correction was used to determine significant difference between means as indicated by different letters. Significant main effects (Genotype and Diet) were also indicated by * (Genotype effect) and # (Diet effect) over LS means.
Figure 4. Tlr4 deficiency improved markers of insulin sensitivity and inflammation in mice fed a diet high in saturated fat.

All measures were taken after 16 weeks on experimental diets (n = 10 unless noted otherwise). (A) Serum glucose (Diet effect P=0.0014, Genotype effect P=0.0068, Diet*Genotype interaction P=0.57), (B) serum insulin (Diet effect P=0.111, Genotype effect P=0.0001, Diet*Genotype interaction P=0.15), (C) glucose to insulin ratio (Diet effect P=0.038, Genotype effect P=0.0001, Diet*Genotype interaction P=0.028), (D) serum leptin (Diet effect P=0.0004, Genotype effect P=0.50, Diet*Genotype interaction P=0.15), (E) serum adiponectin (Diet effect P=0.25, Genotype effect P=0.043, Diet*Genotype interaction P=0.11), and (F) serum MCP-1 (Diet effect P=0.96, Genotype effect P=0.0001, Diet*Genotype interaction P=0.78) were measured in 10ScN (white bars) and 10J (black bars) mice following 16 weeks on diet. All displayed values represent LS means ± SEM for Diet*Genotype interaction. When interaction term was P≤0.15, Bonferroni correction was used to determine significant difference between means as indicated by different letters. Significant main effects (genotype and diet) were also indicated by * (genotype effect) and # (diet effect) over LS means.
Figure 5. Accumulation of F4/80-positive macrophages is attenuated in adipose tissue of high Tlr4 deficient (10ScN) mice fed the HFP diet.

Five micron sections were cut at 50µm intervals and mounted on glass slides, deparaffinized in xylene, and stained for expression of F4/80 with monoclonal antibody. Adipose sections were visualized under 40X magnification. (A) Percent F4/80 positive cells (n = 5, genotype effect P=0.65, diet effect P=0<0.0001, genotype*diet interaction P=0.0002), were measured in 10ScN (white bars) and 10J (black bars) mice following 16 weeks on diet. All displayed values represent LS means ± SEM for Diet*Genotype interaction. When interaction term was P<0.15, Bonferroni correction was used to determine significant difference between means as indicated by different letters. Significant main effects (genotype and diet) were also indicated by * (genotype effect) and # (diet effect) over LS means. (B) Representative images from 10ScN and 10J mice fed low fat (LFC), high fat (HFC), or HFP diet.
Figure 6. Adipose tissue inflammatory gene expression in Tlr4 deficient (10ScN) mice is differentially regulated in response to the HFP diet.

All measures were taken after 16 weeks on experimental diets (n = 6 unless noted otherwise). (A) MCP-1 (Diet effect P=0.70, Genotype effect P=0.31, Diet*Genotype interaction P=0.021), (B) CCR2 (Diet effect P=0.089, Genotype effect P=0.33, Diet*Genotype interaction P=0.35), (C) TNFα (Diet effect P=0.013, Genotype effect P=0.56, Diet*Genotype interaction P=0.93), (D) IL-6 (Diet effect P≤0.0001, Genotype effect P=0.98, Diet*Genotype interaction P=0.074), and (E) Tlr2 (Diet effect P=0.035, Genotype effect P=0.45, Diet*Genotype interaction P=0.65) were measured in 10ScN (white bars) and 10J (black bars) mice following 16 weeks on diet. All displayed values represent LS means ± SEM for Diet*Genotype interaction. When interaction term was P≤0.15, Bonferroni correction was used to determine significant difference between means as indicated by different letters. Significant main effects (genotype and diet) were also indicated by * (genotype effect) and # (diet effect) over LS means.
Figure 7. Tlr4 deficiency attenuates NFκB activation in adipose tissue of Tlr4 deficient (10ScN) mice.

Animals were fasted 6-8h and epididymal fat pad was collected. Nuclear extracts were obtained from adipose tissue and subsequently used for (A) NFκB EMSA, each lane represents an individual sample (n = 2). (B) NFκB p65 DNA binding ELISA. Nuclear extracts were used to quantify NFκB DNA binding; 10ScN (white bars) and 10J (black bars) (n = 4, Diet effect P=0.39, Genotype effect P=0.0015, Diet*Genotype interaction P=0.43). All displayed values represent LS means ± SEM for Diet*Genotype interaction. When interaction term was P<0.15, Bonferroni correction was used to determine significant difference between means as indicated by different letters. Significant main effects (genotype and diet) were also indicated by * (genotype effect) and # (diet effect) over LS means.
CHAPTER 4. THE C-JUN N-TERMINAL KINASE MEDIATES THE INDUCTION OF OXIDATIVE STRESS AND INSULIN RESISTANCE BY PALMITATE AND TOLL-LIKE RECEPTOR 2 AND 4 LIGANDS IN 3T3-L1 ADIPOCYTES
(Submitted to Hormone & Metabolic Research)

Abstract

Saturated fatty acids (SFAs) are known to induce inflammation and insulin resistance in adipocytes through toll-like receptor-4 (Tlr4) signaling, but the mechanisms are not well delineated. Furthermore, the potential roles of Tlr2 and the c-jun N-terminal kinase (JNK) in inflammation in adipocytes have not been investigated. We demonstrated that palmitate, lipopolysaccharide (LPS), and the toll-like receptor-2 (Tlr2) agonist, zymosan A (ZymA), induced insulin resistance in a time-and dose-dependent manner in 3T3-L1 adipocytes. Corresponding with the reduction of insulin sensitivity was an increased expression of IL-6, as well as activation of the proinflammatory transcription factors, nuclear factor kappa B (NFκB) and activator protein-1 (AP-1). Reactive oxygen species (ROS) accumulation was also observed in palmitate and Tlr agonist treated adipocytes. The JNK inhibitor, SP600125, attenuated insulin resistance mediated by SFA and Tlr agonists, which corresponded with a diminished proinflammatory response and reduced ROS accumulation. Collectively, these results demonstrated Tlr2 involvement in adipocyte inflammation and therefore implicated the receptor as a potential target for SFA. Moreover, activation of JNK also appeared to be essential to Tlr2-, as well as Tlr4-, induced insulin resistance and oxidative stress.

Introduction

Adipose tissue (AT) from obese individuals shows activation of several distinct inflammatory pathways, including those of NFκB and JNK (1,2). These proinflammatory signals stimulate production of cytokines and chemokines, including interleukin-6 (IL-6) and
monocyte chemotactic protein-1 (MCP-1), and thereby contribute to the systemic inflammation common in obesity (3,4). Importantly, obesity-induced inflammation in humans is associated with insulin resistance and an increased risk of diabetes mellitus (DM) (1,2,5). These strong associations between obesity, inflammation, and insulin resistance implicate the adipocyte as an important link in the development of DM.

Saturated fatty acids (SFA) activate inflammatory pathways and cause insulin resistance in myocytes (6-9), macrophages (10), and adipocytes (11). Furthermore, increased lipolytic rates in hypertrophied adipocytes result in greater release of non-esterified fatty acids (NEFAs), which can augment the inflammatory response of AT through the activation of neighboring macrophages and adipocytes (10). NEFAs are also implicated in the production of ROS and the ensuing oxidative stress (12). In the obese state, this increased flux of NEFA stimulates ROS production, which leads to impaired insulin signaling (13,14).

The molecular mechanisms involved in NEFA-induced inflammation, oxidative stress, and insulin resistance may involve several distinct pathways. In primary myocytes and hepatocytes, NFκB and AP-1 signaling are critical (9,15). Tlr4 activation was also implicated in SFA-induced inflammation and insulin resistance (3,16-20). LPS, a potent ligand for Tlr4, induces inflammation through JNK/AP-1 and NFκB signaling pathways in several cell types (21-25). Recently, Shi and colleagues (3) reported an attenuation of lipid-induced NFκB activation and insulin resistance in AT of Tlr4 knockout mice. Several additional studies (18,19,26,27) have confirmed that Tlr4 deficiency protects animals from obesity-induced inflammation and insulin resistance. Moreover, we recently demonstrated that Tlr4 deficient 10ScN mice, were specifically protected from SFA-induced adiposity and that they have reduced macrophage accumulation in AT (28). In contrast, the pro-
inflammatory cytokines IL-6 and TNFα were elevated in AT of 10ScN mice fed the same SFA-diet. These data suggest that whereas Tlr4 signaling plays a considerable role in SFA-induced inflammation and adiposity, pro-inflammatory pathways independent of Tlr4 must also exist.

Like Tlr4, the Tlr2 receptor is increased in AT of obese and diabetic individuals (22,29). Adipocytes of mice fed a high fat diet have greater expression of this pattern recognition receptor (30). It is known that SFA signal through Tlr2 and activate IKKβ/NFκB and JNK/AP-1 pathways in myotubes and epithelial cells, respectively (31-33). Additionally, Senn (31) reported that palmitate-induced insulin resistance in C2C12 myotubes is attenuated when Tlr2 signaling is blocked by either siRNA or a neutralizing antibody. ROS accumulation after vascular injury is also blunted in Tlr2 knockout mice (34). Thus, considering the recognized involvement of Tlr2 in oxidative stress and insulin resistance in multiple cell types, we hypothesized that Tlr2 agonists Zymosan A (ZymA) and palmitate would activate inflammatory signaling in the adipocyte and thereby promote inflammation, oxidative stress, and insulin resistance as observed in obesity.

The data presented herein indicated clearly that ZymA and palmitate activate JNK and cause NFκB and AP-1 nuclear translocation and proinflammatory cytokine production. Furthermore, inhibition of JNK blocked Tlr2 and Tlr4 agonist-induced ROS and insulin resistance in adipocytes. These data also provide novel evidence supporting a potential role for Tlr2 in dietary SFA-induced inflammation and insulin resistance.
Materials and Methods

Cells and Reagents. 3T3-L1 preadipocytes were obtained from American Type Culture Collection (Manassas, VA, USA). All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Cell Culture and Treatment. 3T3-L1 preadipocytes were grown in 5% CO₂ in low glucose (5.5 mM) DMEM containing 10% fetal bovine serum (FBS; Atlanta Biologicals, GA, USA) in the presence of a 1% penicillin/streptomycin mixture (growth media). At 2 d post-confluence (0 d), cells were induced to differentiate with medium containing 10% FBS, 1.7 µM insulin, 0.4 mM biotin, 1.0 µM dexamethasone, and 1.5 mM isobutylmethylxanthine for 48 h. Thereafter, fresh growth media supplemented with 1.7 µM insulin was added for 2-3 d followed by insulin-free growth media for the remaining culture period. At 10-12 d, cells were cultured in medium containing 10% FBS, 0.3 µM bovine serum albumin (BSA), and 0.1% methanol with one or a combination of the following treatments: 0.5 mM sodium palmitate, 10 µg/mL LPS from Escherichia coli 055:B5, 20 µg/mL ZymA from Saccharomyces cerevisiae, and 20 µM SP600125. At 24 h, medium was collected and fresh medium with 0.1% FBS was added with indicated treatments.

Quantitative real-time PCR. Total RNA was extracted from cells using a commercially available acid-phenol reagent (TRIzol, Invitrogen, Carlsbad, CA, USA). Potential DNase contamination was removed with DNase-free™ (Ambion, Foster City, CA, USA). First-strand cDNA was synthesized using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Primer sequences used to detect β-actin, Tlr4, Tlr2, MCP-1, and IL-6 are described elsewhere (28). Thermal cycling conditions for PCR reactions were 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 30
s, and 72°C for 30 s. Products amplified by these primers were cloned into pGEMT vector (Promega, Madison, WI, USA) and sequenced to confirm transcript identity. Real-time PCR reactions were carried out with an iCycler using the IQTM SYBR Green Super Mix kit (Bio-Rad, Hercules, CA, USA). The abundance of each transcript was calculated by regression against the standard curve generated on the same plate with the respective plasmid. Expression levels of genes of interest were normalized to β-actin and are reported as fold change based on log starting quantity.

**Analysis of Metabolic Parameters.** Total nitrite and IL-6 concentrations in medium were determined using commercially available ELISA kits (Biovision, Mountain View, CA, USA and R&D Systems, Minneapolis, MN, USA, respectively). Glycerol release was measured as previously described (35).

**Western Blot Analysis.** Cells were grown in 6 well tissue culture plates, and treated as described above. To harvest protein for analysis, media from cells was aspirated, and cells were rinsed once with ice cold PBS. Lysis buffer (10 mM Hapes pH 2.5, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovandate, 1 µg/mL leupetin, 1 µg/mL aprotinin, 1 µg/mL pepstatin, and 1 mM PMSF) was added to each well of cells, which were then scraped from the plate. Cell protein lysates were resolved by electrophoresis using 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane for western blot analyses. The membrane was blocked with 5% skim milk in tris buffered saline (TBS) for 1 h at room temperature, and then incubated with phospho-SAPK/JNK or SAPK/JNK monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After washing three times with TBST (0.05% Tween 20, 50 mM Tris–HCl,
pH 7.5, and 150 mM NaCl) for 15 min, the membrane was incubated for 1 h with anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase. The signal was detected using the Phototope®-HRP Western Blot Detection System (Cell Signaling Technology, Danvers, MA, USA).

NFκB and AP-1 Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts for mobility shift assay reagents were prepared and validated as previously described (36). Briefly, the consensus NF-κB and AP-1 oligonucleotides (Santa Cruz, CA, USA) were end-labeled with [-32P] ATP (PerkinElmer, Waltham, MA, USA) using T4 polynucleotide kinase (Santa Cruz, Santa Cruz, CA, USA). 10-30 µg of nuclear proteins were incubated with 50,000 cpm of labeled probe for 30 min at room temperature in binding buffer [2 mM HEPES, 50 mM KCl, 2 mM EDTA, 10% glycerol, and 1% BSA (wt/vol)] with 2 µg poly(dI-dC) in a total reaction volume of 40 µL. Probe-protein complexes were resolved by electrophoresis, and the gel was transferred to nitrocellulose and dried.

Intracellular ROS Accumulation Assay. 30 µM of carboxy-H₂DCFDA (DCF; Invitrogen, Carlsbad, CA, USA) was added to 1X Kreb’s Ringer Buffer (KRB; 12 mM HEPES, pH 7.4, 121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 0.33 mM CaCl₂). Cells were then incubated for 45 min at 37°C, after which medium was aspirated and cells washed three times with warm phosphate buffered saline (PBS). Culture plates were immediately read using a fluorescent plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA) with 435 nm excitation and 550 nm emission wavelengths, respectively. Fluorescence was normalized to total protein as determined with the bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA). Data are presented as percent of control.
2-Deoxyglucose (DG) Uptake. Cells were incubated with or without 5 µM cytochalasin B and with 100 nM insulin for 30 or 10 min at 37°C, respectively, in 1X Krebs Ringer buffer (KRB). Non-insulin stimulated control cells were treated identically. Cell media was then supplemented with 0.5 µCi/mL [3-H]-2-DG and 120 µM non-labeled 2-DG and cells were incubated for an additional 25 min. Glucose uptake was terminated by aspiration of media. Thereafter, the cells were washed with 1X KRB and lysed with 0.1 N NaOH. Cell lysates were used to determine 2-DG uptake via scintillation counting. Uptake was normalized to total lysate protein as determined by BCA assay. Data are presented as fold change of insulin-stimulated controls.

Statistical analyses. Data were tested for normality and analyzed using the mixed-model analysis. Treatment was considered the fixed effect and experimental repetition the random effect. The main effects (treatment and replicate) were tested against the treatment x replicate interaction term. When protected by a significant F-test, mean separation was accomplished using the least-squares mean separation (pdiff) procedure. Differences were considered significant at P < 0.05. Tendencies were defined at P < 0.10.

Results

Time dependent induction of insulin resistance by palmitate, ZymA, and LPS in 3T3-L1 adipocytes. Based on previous data, we investigated the ability of LPS and palmitate compounds (at previously studied doses) to mediate insulin resistance in a time dependent manner. Here we demonstrated that palmitate treatment reduced insulin-stimulated 2-DG uptake beginning at 12 h and continuing through 48 h (Fig. 8, P<0.05). Alternatively, ZymA and LPS only inhibited insulin-stimulated 2-DG uptake following 48 h of treatment (P=0.0022 and P=0.0048, respectively). Therefore, we treated adipocytes for 48 h with
palmitate, ZymA, or LPS in order to study mechanisms involved in the development of insulin resistance.

*ZymA induced inflammation and oxidative stress in adipocytes in a manner similar to that caused by palmitate and (or) LPS.* As shown in Table 4, the Tlr2 agonist, ZymA also caused a 2-fold increase in IL-6 gene expression (P=0.05) which corresponded with a greater than 10-fold increase in media IL-6 content (P≤0.0001, Table 4). Consistent with prior findings, palmitate resulted in an approximate 4-fold increase in IL-6 gene expression (P≤0.0001) and 30-fold increase in IL-6 media content (P≤0.0001). We further evaluated the inflammatory response in adipocytes by quantifying the expression of the pro-inflammatory chemokine, MCP-1. As with palmitate and LPS, ZymA increased MCP-1 expression vs. control cells (P≤0.0001, Table 4) with a near 2-fold greater transcript abundance. The expression of Tlr2 and Tlr4 were evaluated to assess potential feedback regulation of Tlr ligands on their respective receptors. Although Tlr2 expression was induced by all treatments (P≤0.0001, Table 4), Tlr4 was largely unresponsive to these pro-inflammatory mediators (Table 4), with the exception that LPS tended to reduce expression of Tlr4 (P=0.09). To investigate the roles that Tlr ligands play in the accumulation of ROS, we measured several markers of oxidative stress and ROS production. First, we quantified expression of the inducible nitric oxide synthase gene, and found that palmitate, LPS, and ZymA all caused a 5-fold increase in iNOS expression (P≤0.0001, Table 5). Next, we assessed ROS accumulation, and demonstrated that all three ligands caused a 50% increase in intracellular ROS (P<0.01, Table 5). However, an increase in media nitrite concentrations (25%, P=0.008, Table 5), indicative of extracellular oxidative stress, was apparent only in response to palmitate.
NFκB, AP-1, and JNK were activated by palmitate, ZymA and LPS in adipocytes. We next studied potential mechanisms underlying the pro-inflammatory effects of palmitate and the classic Tlr ligands. JNK has been implicated previously in proinflammatory signaling in adipocytes (37,38), and consequently, we quantified phosphorylation of JNK1 (Thr183, 46 kDa) and JNK2 (Tyr185, 54 kDa) as an indicator of activation in cells treated with palmitate, LPS, and ZymA. All three pro-inflammatory mediators significantly increased JNK phosphorylation (P<0.05, Fig. 9A-B). Activation of JNK is known to stimulate the activity of the pro-inflammatory transcription factors, NFκB and AP-1. After 48 h, there was increased activation of NFκB by palmitate, LPS, and ZymA (Fig. 9C). Consistent with previous results, the LPS treatment had the most pronounced effect on NFκB activation under the applied experimental conditions. Likewise, AP-1 activation was also increased by all of the pro-inflammatory mediators (Fig. 9D), and was greatest in cells exposed to ZymA.

Inhibition of JNK attenuated palmitate- and Tlr ligand-induced inflammation, oxidative stress, and insulin resistance in adipocytes. Our findings indicated that palmitate, LPS, and ZymA all induced JNK activation. Hence, we sought to determine whether inhibition of JNK would attenuate the inflammatory, oxidative, and insulin resistance responses in adipocytes. We first confirmed that there was a reduction of JNK phosphorylation by SP600125 in our adipocyte model. As compared with the non-inhibitor control cells, there was a 22% (P=0.045), 45% (P=0.0005), and 33% (P=0.0045) reduction in palmitate-, LPS- and ZymA-induced JNK activation, respectively, when cells were also incubated with the SP600125 inhibitor (Fig. 10A-B). Furthermore, as shown in Fig. 10C, inhibition of JNK completely abrogated the effect of palmitate on IL-6 expression (P≤0.0001), and attenuated the induction by LPS and ZymA. In contrast, inhibition of JNK
did not alter the effects of LPS or ZymA on MCP-1, Tlr2, or Tlr4 expression (Fig. 10D). However, there was a small (approximately 20%), but significant, decrease in palmitate-induced MCP-1 (P=0.023), Tlr2 (P≤0.0001, Fig. 10E), and Tlr4 (P=0.0014, Fig. 10F).

To establish whether the blunted inflammatory responses caused by inhibition of JNK were associated with changes in NFκB and AP-1 activation, adipocytes were treated with LPS, palmitate, or ZymA in the presence or absence of SP600125. The JNK inhibitor reduced the induction of NFκB by all three pro-inflammatory mediators (Fig. 10A), but this was qualitatively more pronounced for palmitate. Conversely, inhibition of JNK only reduced activation of AP-1 in cells stimulated with palmitate or ZymA (Fig. 10B).

*JNK mediated the insulin resistance caused by palmitate and Tlr ligands.* There was only a small (non-significant) reduction in LPS- and ZymA-induced iNOS expression in the presence of the JNK inhibitor (Fig. 10C). Conversely, palmitate-induced iNOS expression was dramatically reduced (over 60%, P≤0.0001) with inhibitor. Furthermore, ROS accumulation (Fig. 10D), as measured by DCF fluorescence, was reduced by 50-60% (P<0.0001) for cells stimulated with palmitate or Tlr agonist. To determine if the attenuated ROS accumulation impacted insulin sensitivity, we assessed glucose uptake using 2-DG (Fig. 10E). Insulin-stimulated glucose uptake was improved over 40% in cells stimulated with palmitate (P=0.0026), LPS (P=0.0018), or ZymA (P≤0.0001) when JNK was blocked by the inhibitor.

**Discussion**

The data presented herein indicated several important points regarding the regulation of inflammation and insulin sensitivity in adipocytes by palmitate and Tlr ligands. First of all, we showed that activation of Tlr2 resulted in a pro-inflammatory response and insulin
resistance that bore marked similarity to responses caused by palmitate or activation of Tlr4 by LPS. Palmitate and both Tlr agonists resulted in increased nuclear localization of the NFκB and AP1 transcription factors, and caused increased expression of MCP-1, Tlr2, and iNOS, as well as increased ROS accumulation. Furthermore, as with palmitate and LPS, activation of Tlr2 by ZymA activated JNK and attenuated the ability of insulin to stimulate glucose uptake (as determined with 2-DG).

The importance of Tlr2 activation as a contributor to inflammation in adipose tissue in vivo has not yet been established, but Nguyen et al. (39) recently demonstrated that NEFA activation of JNK involves both Tlr2 and Tlr4 in RAW 264.7 macrophages. The fact that Tlr2 is upregulated by palmitate, LPS, or ZymA, whereas Tlr4 is not, underscores the potential importance of signaling in adipose tissue via this receptor to obesity-linked inflammation. Furthermore, these receptors have a synergistic relationship in terms of activation of proinflammatory signaling. For instance, stimulation of Tlr4 leads to increased expression of Tlr2 and thus amplification of the inflammatory response (40). Moreover, the ability of these receptors to recognize similar ligands, including SFAs, suggests that Tlr2 and Tlr4 are major mediators of obesity-induced inflammation. In the present study, the more pronounced effect of palmitate may result from a combination of signaling through both of these innate immune receptors.

Our second major finding pertains to the importance of JNK activity to ROS accumulation and insulin-stimulated glucose uptake. Obesity-linked inflammation and oxidative stress contribute to the development of insulin resistance (12,37,41,42). Although the exact mechanism is not yet known, NEFA were associated with the generation of ROS in adipocytes (12). Lee and colleagues (43) recently demonstrated that a Tlr-JNK pathway
regulated ROS signaling in macrophages. Furthermore, Kaneto and colleagues (44) suggested that JNK mediates insulin resistance through generation of oxidative stress. Our data show that palmitate, LPS, and ZymA all induce JNK phosphorylation (activation), iNOS expression and ROS accumulation in adipocytes. Furthermore, the inhibitor used to disrupt activation of JNK not only blocked or attenuated the ability of palmitate, LPS and ZymA to stimulate phosphorylation of JNK, it also precluded the induction of ROS and the diminution in insulin-stimulated 2DG uptake by these pro-inflammatory factors.

We recognize the inherent limitations of kinase inhibitors, including their low specificity. However, it should be noted that SP600125 is shown to have >20-fold selectivity for JNK compared to a range of other kinases (45). Future experiments utilizing Tlr knockdown models will be necessary to further validate the specificity of SP6 to JNK. The redundancy of proinflammatory signals downstream of Tlr also suggest additional kinases may be involved in palmitate-, ZymA-, or LPS-induced inflammation. Regardless, our data strongly implicate JNK as a major determinant of ROS production in adipocytes, and confirm prior evidence (46) detailing the importance of ROS as determinants of insulin sensitivity in murine adipocytes.

Finally, we have identified the activation of AP-1 transcription factor as a component of the inflammatory response to palmitate, LPS, and ZymA, and presented initial evidence that activation of JNK may be required for activation of AP-1 by palmitate and ZymA, but not LPS. The fact that we showed a limited effect of JNK inhibition on LPS-induced AP-1 activation may relate to distinct differences between Tlr4 and Tlr2 signaling (47). For instance, LPS and other Tlr4 agonists are capable of signaling through both MyD88-dependent and independent pathways, whereas Tlr2 ligands require the MyD88
adaptor protein in order to generate an effective proinflammatory response (48,49). In this study, activation of NFκB was induced by palmitate and both Tlr agonists. Moreover, the Tlr4 agonist, LPS, had the greatest increase in NFκB activation (Fig. 3C), whereas the Tlr2 agonist, ZymA, had the greatest induction of AP-1 (Fig. 3D). Involvement of LPS in NFκB activation in adipocytes has been well documented (21,36). However, we have established herein that the Tlr2 agonist, ZymA, not only activates NFκB, but also induces AP-1 in adipocytes. The reduced ability of JNK inhibitor to fully inhibit LPS activation of AP-1 also suggested that JNK may be more involved in Tlr2 activation of AP-1.

The in vivo activation of AP1 and NFκB in AT is likely a concerted process involving macrophages, preadipocytes, and adipocytes. The contribution of mature adipocytes to this process is unclear, but may be paramount as activation of these proinflammatory transcription factors promotes lipolysis (41), leading to increased NEFA and activation of Tlr signaling within adipocytes and neighboring cells. However, elucidating the contribution of different AT-based cells to Tlr2 and Tlr4 mediated inflammation will require further investigation.

In conclusion, this study demonstrated involvement of Tlr2 agonists, ZymA, and palmitate, in adipocyte inflammation, oxidative stress, and insulin resistance. These data also indicated that JNK activation is essential to ZymA agonist induced NFκB and AP-1 activation. Based on these data, we believe that Tlr2, in addition to Tlr4, is an important mediator of obesity-induced inflammation. Further insight into this relationship may provide potential therapeutic strategies to inhibit Tlr agonists for alleviating obesity-associated health complications.
References


Table 4. Palmitate, ZymA, and LPS induced inflammation in 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-6 Protein</th>
<th>IL-6 Transcript</th>
<th>MCP-1 Transcript</th>
<th>Tlr2 Transcript</th>
<th>Tlr4 Transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.94(^a)</td>
<td>1.19 ± 0.27(^a)</td>
<td>1.12 ± 0.05(^a)</td>
<td>1.16 ± 0.05(^a)</td>
<td>1.29 ± 0.09(^ab)</td>
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<tr>
<td>Palmitate</td>
<td>29.5 ± 0.94(^c)</td>
<td>4.02 ± 0.18(^c)</td>
<td>1.42 ± 0.03(^b)</td>
<td>1.54 ± 0.03(^b)</td>
<td>1.48 ± 0.06(^b)</td>
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<td>LPS</td>
<td>2.10 ± 0.94(^a)</td>
<td>1.63 ± 0.21(^b)</td>
<td>1.59 ± 0.04(^c)</td>
<td>1.68 ± 0.04(^c)</td>
<td>1.04 ± 0.07(^a)</td>
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<tr>
<td>ZymA</td>
<td>13.1 ± 0.94(^b)</td>
<td>1.93 ± 0.18(^b)</td>
<td>1.80 ± 0.03(^d)</td>
<td>1.70 ± 0.03(^c)</td>
<td>1.31 ± 0.06(^b)</td>
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</table>

Treatment Effect: P ≤ 0.0001  P ≤ 0.0001  P ≤ 0.0001  P ≤ 0.0001  P = 0.0009

\(^a, b, c, d\) Significant difference between LS means indicated by different letters (P<0.05)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Nitrite</th>
<th>NOS2 Transcript</th>
<th>ROS Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.06a</td>
<td>0.82 ± 0.23a</td>
<td>1.16 ± 0.16a</td>
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<td>Palmitate</td>
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<td>3.15 ± 0.19b</td>
<td>1.89 ± 0.13b</td>
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<tr>
<td>LPS</td>
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<td>1.70 ± 0.16b</td>
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<tr>
<td>ZymA</td>
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<td>2.96 ± 0.16b</td>
<td>1.54 ± 0.15b</td>
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</tbody>
</table>

Treatment Effect: P = 0.0036 for Total Nitrite, P < 0.0001 for NOS2 Transcript, P = 0.0027 for ROS Accumulation.

*a,b,c* Significant difference between LS means indicated by different letters (P<0.05)
Figure 8. Palmitate, ZymA, and LPS induced insulin resistance in 3T3-L1 adipocytes.

10-12 d post-differentiated 3T3-L1 adipocytes were treated with 0.5 mM sodium palmitate, 10 µg/mL LPS, or 20 µg/mL ZymA for 3, 6, 8, 12, 24, and 48 h. Insulin-stimulated 2-DG uptake was then determined (n = 8, Treatment effect P<0.0001, Time effect P<0.0001, Treatment*Diet effect P<0.0001). A non-insulin stimulated control was included for reference. Results are expressed as fold change versus insulin stimulated control and represent LS means ± SEM. When Treatment effect was significant (P<0.05), post-hoc t-test was used to determine significant difference between LS means as indicated by different letters.
Figure 9. Palmitate, ZymA, and LPS induced JNK phosphorylation and NFκB and AP-1 nuclear translocation in 3T3-L1 adipocytes.

10-12 d post-differentiated 3T3-L1 adipocytes were treated with 0.5 mM sodium palmitate, 10 µg/mL LPS, or 20 µg/mL ZymA for 48 h (n= 4 unless noted otherwise). (A) Representative western blot showing phosphorylation of JNK (top panel) and total JNK levels (bottom panel). (B) Quantification of phosphorylated JNK by densitometry and expressed as fold change versus control (Treatment effect P=0.0089). Representative EMSA blot showing nuclear protein binding to labeled (C) NFκB and (D) AP-1 consensus sequences.
Figure 10. JNK inhibitor, SP600125, attenuated palmitate, ZymA, and LPS induced JNK phosphorylation and inflammation in 3T3-L1 adipocytes.

10-12 d post-differentiated 3T3-L1 adipocytes were treated with 0.5 mM sodium palmitate, 10 µg/mL LPS, or 20 µg/mL ZymA for 48 h (n=6-8 unless noted otherwise). (A) Representative western blot showing phosphorylation of JNK (top panel) and total JNK levels (bottom panel). (B) Quantification of phosphorylated JNK by densitometry (Treatment effect P<0.0001). (C) IL-6 gene expression (Treatment effect P<0.0001). (D) MCP-1 gene expression (Treatment effect P<0.0001). (E) Tlr2 gene expression (Treatment effect P<0.0001). (F) Tlr4 gene expression (Treatment effect P=0.0002). All displayed were expressed as fold change versus control and represent LS means ± SEM. When Treatment effect was significant (P<0.05), post-hoc t-test was used to determine significant difference between LS means as indicated by different letters.
Figure 11. JNK inhibitor, SP600125, attenuated palmitate, ZymA, and LPS induced NFκB and AP-1 nuclear translocation, oxidative stress, and insulin resistance in 3T3-L1 adipocytes.

10-12 d post-differentiated 3T3-L1 adipocytes were treated with 0.5 mM sodium palmitate, 10 µg/mL LPS, or 20 µg/mL ZymA for 48 h (n= 6-8 unless noted otherwise). Representative EMSA blot showing nuclear protein binding to labeled (A) NFκB and (B) AP-1 consensus sequences. (C) iNOS gene expression (Treatment effect P<0.0001). (D) DCF fluorescence (Treatment effect P<0.0001). (E) Insulin-stimulated 2-DG uptake (Treatment effect P<0.0001). All displayed values represent LS means ± SEM for Treatment effect. When Treatment effect was significant (P<0.05), post-hoc t-test was used to determine significant difference between LS means as indicated by different letters.
CHAPTER 5. ABSENCE OF TLR2 AND TLR4 HIGH FAT DIET-INDUCED OBESITY AND ADIPOSE-SPECIFIC INFLAMMATION AND INSULIN RESISTANCE
(To be submitted to *Obesity*)

Abstract

We previously demonstrated the involvement of toll-like receptor-4 (Tlr4) in saturated fatty acid (SFA)-induced inflammation and insulin resistance. However, less is known about toll-like receptor-2 (Tlr2), which also recognizes SFA. To investigate the involvement of this receptor in diet-induced obesity, we utilized male Tlr2−/− mice and C57BL6J controls. We also compared 10Scn mice lacking functional Tlr4 and their respective control 10J. Mice were fed a low fat (low-fat control (LFC)), high unsaturated fat (high-fat control (HFC)), or high saturated fat + palmitate (HFP) diet *ad libitum* for 16 weeks. Tlr2−/− mice were protected from DIO. However, unlike Tlr4 deficient 10ScN mice, there were no significant changes in body weight, energy intake, or lipid profile in Tlr2−/− mice fed any of the three diets as compared to their control. Serum markers of inflammation, including C-reactive protein, endotoxin, and monocyte chemotactic protein-1 (MCP-1) were lower in 10ScN mice fed HFP diet, whereas only MCP-1 levels were reduced in high fat fed Tlr2−/− mice. Transcript abundance of adipose markers of inflammation and oxidative stress, including tumor necrosis factor-α, interleukin-6, MCP-1, F4/80, and nitric oxide synthase-2, were attenuated by Tlr2 and Tlr4 deficiency. Furthermore, these effects were more striking in HFP-fed mice. Blood glucose and serum insulin were lower in 10ScN mice, but not in Tlr2−/− mice. However, stromal vascular (SV) cells isolated from AT of Tlr2−/− had a marked increase in insulin sensitivity. These data indicated that Tlr2, as well as Tlr4, play a significant role in the
development of obesity-induced inflammation and insulin resistance, specifically in mice fed a HFP diet.

**Introduction**

Obesity-induced inflammation is associated with insulin resistance and an increased risk for diabetes mellitus (DM) (1-3). Obese individuals exhibit activation of several distinct inflammatory pathways, including nuclear factor kappa B (NFκB) and c-jun N-terminal kinase (JNK) (1,2), which are associated with macrophage infiltration in AT and stimulation of proinflammatory cytokines, such as monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6). In addition, the incidence of DM is higher in obese versus healthy weight patients. The strong association between obesity, inflammation, and insulin resistance suggests that adipose tissue (AT) plays a prominent role in their development.

The underlying mechanism contributing to the proinflammatory response seen in AT of the obese is not fully elucidated, but may involve signaling through specific Tlrs. Shi and colleagues (4) reported attenuation of fatty acid (FA)-induced NFκB activation and insulin resistance in AT of Tlr4 null mice. Additional studies (5-8) have confirmed that Tlr4 deficiency improved insulin sensitivity and lowered inflammation in diet-induced obesity (DIO). We previously demonstrated that 10ScN mice, which have a 74 kb deletion from chromosome 4 that precludes expression and production of Tlr4, were specifically protected from saturated fatty acid (SFA)-induced adiposity and AT-specific inflammation (9). However, Tlr-4 deficiency did not fully attenuate high fat diet-induced inflammation in AT. These data suggested that pathways independent of Tlr4 may also mediate obesity-induced inflammation and insulin resistance.
One potential candidate pathway is that of Tlr2, which is increased in AT of obese and diabetic individuals (10,11). Since SFA are reported to contribute to activation of Tlr2, it is a likely mediator of obesity-induced inflammation (12). Thus, the goal of this study was to test the hypothesis that the absence of Tlr2 expression attenuates systemic inflammation, improves insulin sensitivity, and decreases SFA-induced AT inflammation in DIO mice.

**Materials and Methods**

_Animals and animal care._ Male and female B6.129-Tlr2tm1Kir/J (Cat No. 004650; Tlr2−/−) mice and C57BL/10ScN mice (Cat No. 003752; 10ScN) derived from the C57BL/6J and C57BL/10J sublines, respectively, were obtained from Jackson Laboratories (Bar Harbor, ME), along with corresponding control male and female C57BL/6J (Cat No. 000664) and C57BL/10J (Cat No. 000665) mice. Breeding colonies were established for each genotype. Aberrant Tlr2 and Tlr4 expression was confirmed by real time PCR. At 6-8 weeks of age, male offspring were housed individually in a room with an automatically controlled 12-hour light:dark cycle. Mice were acclimated to the cages and provided unlimited access to food and water.

Animals from each genotype were randomly assigned to one of three experimental diets (n = 15/genotype/diet): 1) low fat control (LFC), high fat control (HFC), or high fat palmitate (HFP) for 16 weeks. Both high fat diets were semi-purified, powdered diets based on American Institute of Nutrition recommendations (13) that were modified to induce obesity by providing 60% of calories from lipids (Table 6). The high fat diets were identical except the source of fat used was either soybean oil in the HFC (Harlan Teklad) or a mixture of lard (Harlan Teklad) and purified palmitate (Nu-Check Prep, Inc.) in the HFP diet. The LFC diet contained 12% of calories from lipid. Food intake was measured daily and used to
calculate total energy intake. Mice were fasted overnight (6-8 h) and euthanized by CO₂ asphyxiation for blood and tissue collections. Blood was collected by heart puncture, placed on ice, and serum was collected by centrifugation and frozen at -80°C until analyzed. Animals were weighed and abdominal fat pads were removed and weighed. Extracted tissues and carcasses were immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction and immuno-histochemical analysis. All experimental protocols for animal care and use were approved by the Institutional Animal Care and Use Committee at Iowa State University, Ames, IA, USA.

*Analysis of metabolic parameters.* Blood glucose, total cholesterol, and triacylglycerides (TAG) were measured using CardioChek P•A® system (Polymer Technology Systems, Inc.). Serum insulin (Linco), C-reactive protein (CRP) (Immunology Consultants Laboratory, Inc.), and MCP-1 (R&D Systems) were measured using commercial assay kits. Non-esterified fatty acids (NEFA) were measured with NEFA-C kit (Wako Pure Chemical Industries, Inc.). Serum endotoxin determinations were performed using a kit based upon a Limulus amaebocyte extract (LAL kit; Hycult Biotechnology). Samples were diluted 1:40. Internal control of recovery calculation was included in the assessment.

*Quantitative real-time PCR.* Total RNA was extracted and cDNA synthesized as described previously (9). Primer sequences are listed in Table 7. The abundance of each gene product was calculated by regressing against the standard curve generated in the same reaction with their respective plasmid. All genes of interest were normalized to beta actin and expressed as log starting quantity.

*Serum fatty acid profile.* Lipid from serum samples were extracted by the method of Lepage and Roy (15) with minor modifications as described previously (9).
**Stromal vascular cell (SVC) isolation and culture.** Male Tlr2−/− or 6J mice were euthanized by CO2 asphyxiation. Epidydimal fat pads were removed, trimmed, and diced before adding to sterile digestion cocktail (1X Krebs Ringer buffer (KRB) and 200 U/ml collagenase) for 1 h in 37°C shaking water bath. Inter-phase liquid, absent of floating adipocytes and debris, was centrifuged at 800 x g for 4 min. The resulting pellet was resuspended in red blood cell lysis buffer (pH 7.2; 155 mM HH4Cl, 5.7 mM K2HPO4, and 0.1 mM EDTA) for 5-6 min and then diluted with 1X PBS and centrifuged for 800 x g for 15 min. Following a second PBS wash, the pellet was resuspended in low glucose DMEM containing 10% FBS and 5% streptomycin/penicillin mixture. Growth media was changed after 4 h and then again at 24 h, after which media was replaced every 48 h. Cells were split twice before being frozen in media containing 15% DMSO. These stocks were subsequently used for all experiments.

When cells reached 70-80% confluence, adipocyte differentiation cocktail (growth media containing 1.5 mM IBMX, 1.0 µM dexamethasone, 1.7 µM insulin, and 0.4 mM biotin) was added for 48 h. Following an additional 48 h with differentiation cocktail, cells were cultured in growth media with insulin. At 14 d post-differentiation, approximately 40% of SVC exhibited adipocyte phenotype (e.g., lipid accumulation). Cells were then treated with 0.5 mM sodium palmitate, 10 µg/mL LPS from Escherichia coli 055:B5, or 20 µg/mL ZymA from Saccharomyces cerevisiae for 48 h and used thereafter for analysis of glucose uptake.

**2-Deoxyglucose (DG) Uptake.** SV cells were treated as described and glucose uptake was measured as previously described (16).
Statistical analyses. Data were tested for normality and analyzed using the mixed-model analysis with diet and genotype considered fixed effects, and the time dietary treatments were implemented for subgroups as a random effect. Significant interactions of main effects are indicated as Diet*Genotype effects. All indicated P-values were two-tailed and Bonferroni corrected. Differences were considered significant at $P < 0.05$ and a tendency at $P < 0.10$. Values are presented as least squares (LS) means ± SEM.

Results

Tlr2$^{-/-}$ and 10ScN mice are protected from diet-induced obesity independent of caloric intake. Unlike 10ScN mice, Tlr2$^{-/-}$ mice did not exhibit a reduction in body weight (Fig. 12A) or weight gain (Fig. 12B) compared to 6J controls (Genotype, $P=0.68$). In contrast, 10ScN mice had on average 2.9 g lower body weight relative to 10J mice (Genotype, $P=0.0018$). This relationship was especially evident in HFP fed 10ScN mice (Diet*Genotype, $P=0.0076$) and is consistent with our previous findings (9). To further evaluate the level of adiposity, we measured epidydimal fat pad weight (Fig. 12C) and observed that both Tlr2$^{-/-}$ and 10ScN mice had approximately 20% and 30% lower fat pad weight compared to their controls, respectively (Genotype, $P\leq0.0001$). Epidydimal fat pad weight was higher in high fat diet fed mice (Diet Effect, $P=0.032$) relative to LFC fed mice. These effects on fat pad weight were independent of energy intake as there were no intake differences between Tlr2$^{-/-}$ and 6J mice (Fig. 12D, Genotype, $P=0.58$). However, 10ScN mice actually exhibited greater energy intake than 10J mice (Genotype, $P=0.0097$), which was especially evident in HFC fed mice (Diet*Genotype, $P=0.0007$).

Tlr4 deficiency, but not Tlr2 deficiency, improved markers of insulin sensitivity, particularly in mice fed a high saturated fat diet. In the present study, we measured fasting
blood glucose (Fig. 13A) and serum insulin (Fig. 13B) to determine if Tlr2\(^{-/-}\) mice might also exhibit modification of insulin sensitivity. We have shown that blood glucose concentration was over 10% lower in 10ScN mice versus 10J controls (Genotype, \(P=0.019\)). However, we observed no significant difference in blood glucose or serum insulin between Tlr2\(^{-/-}\) and 6J controls (Genotype, \(P=0.12\) and \(P=0.66\), respectively). Dietary treatment also had no effect on blood glucose regardless of genotype (Diet, \(P=0.93\)). In contrast, serum insulin was approximately 30% higher in mice fed HFP compared to HFC (Diet, \(P=0.035\)) suggesting that a diet rich in SFA is more insulinemic compared to a diet rich in unsaturated FA. One caveat to this was that 10ScN mice had a tendency toward lower insulin concentration when fed the HFP diet compared to 10J controls (Diet*Genotype, \(P=0.10\)).

Absence of Tlr2 and Tlr4 expression differentially modified markers of systemic inflammation. We measured several proinflammatory markers in serum, including CRP (Fig. 13C), endotoxin (Fig. 13D), and MCP-1 (Fig. 13E). Circulating CRP concentration was greater in mice fed a saturated versus unsaturated fat diet (\(P=0.031\)), but this effect was blunted in Tlr4 deficient mice fed HFP diet (Diet*Genotype, \(P=0.0034\)). Furthermore, Tlr4 deficient mice showed a nearly 30% reduction in serum endotoxin regardless of diet (Genotype, \(P=0.048\)). In contrast, there was no detectable difference in serum CRP or endotoxin between Tlr2\(^{-/-}\) and 6J mice (\(P=0.46\) and \(P=0.79\), respectively). Serum concentration of the proinflammatory chemokine, MCP-1, was approximately 55% lower in 10ScN mice versus 10J controls (Genotype, \(P=0.0035\)). The reduction in MCP-1 concentration was more evident in Tlr4 deficient mice fed either high fat diet (Diet*Genotype, \(P=0.035\) (HFC) and \(P=0.05\) (HFP), respectively). Tlr2\(^{-/-}\) mice were also protected from the HFP diet-induced MCP-1 increase (Diet*Genotype, \(P=0.0038\), but not
from the HFC diet (Diet*Genotype, P=0.58). Therefore, it appeared that Tlr2, in addition to Tlr4, was involved in the stimulation of the SFA diet-induced serum MCP-1.

*Tlr2 and Tlr4 deficiencies differentially modified circulating lipid profiles in response to high fat diets.* We measured both the concentration and composition of serum lipids in order to determine their relationship with obesity-induced inflammation. We have shown that mice fed either high fat diet had a near 20% higher serum total cholesterol (Table 8, Diet, P=0.0075 (HFC) and P≤0.0001 (HFP), respectively), whereas serum TAG (Table 8) was elevated in LFC fed mice regardless of genotype (Diet, P≤0.0001). Furthermore, 10ScN mice exhibited approximately 20% greater serum TAG compared to 10J controls (Genotype, P=0.0049), which was more apparent in 10ScN mice fed LFC diet (Diet*Genotype, P≤0.0001). Similarly, circulating NEFA (Table 8) were higher in 10ScN mice fed the LFC diet (Diet*Genotype, P≤0.01). There were no observed differences in serum TAG or NEFA between Tlr2−/− mice and 6J controls (Genotype P=0.97 and P=0.85, respectively).

We next analyzed the composition of serum FA (Table 9). Total SFA, palmitate, and stearate content were all 21%, 14%, and 35% higher in serum of mice fed HFP and LFC compared to HFC diet, respectively (Diet, P≤0.0001). In contrast, total omega-6 and omega-3 FA content were approximately 18% and 25% greater in serum of mice fed HFC than HFP diet (Diet, P≤0.0001), which corresponds with higher serum linoleate, α-linolenate, and DHA (Diet, P≤0.0001). Polyunsaturated FA (PUFA) content was also different between LFC and HFP fed mice. Total omega-3 FA was greater in mice fed LFC (Diet, P≤0.0001), whereas total omega-6 FA was greater in mice fed HFP (Diet, P=0.0029), which is consistent with the observed DHA (Diet, P≤0.0001) and γ-linoleate concentrations (Diet, P≤0.01), respectively. Genotype had a minimal impact on FA composition. However, Tlr2−/− mice exhibited higher
circulating stearate (Genotype, $P=0.0003$) with a trend toward an increase in total SFA (Genotype, $P=0.083$) compared to 6J controls. Conversely, 10ScN mice had higher $\alpha$-linolenate (Genotype, $P=0.0067$) and linoleate (Genotype, $P=0.0960$) versus 10J controls. These data indicated that the circulating lipid profile was more closely related to dietary lipid content than genotype, thus indicating that the anti-inflammatory effects of Tlr2 or Tlr4 deficiency were not due to alterations in FA profile.

*Obesity-induced inflammation in adipose tissue was differentially regulated by high saturated and unsaturated fat diets, and was partly ameliorated by Tlr2 and Tlr4 deficiency.*

We first confirmed that Tlr4 (Fig. 14A) and Tlr2 (Fig. 14B) gene expression were deficient in AT of 10ScN and Tlr2$^{-/-}$ mice, respectively (Genotype, $P<0.0001$). Transcript abundance of Tlr4 was 2-fold greater in mice fed either high fat diet (Diet, $P=0.0068$ (HFP) and $P=0.020$ (HFC), respectively). SFA fed mice also exhibited a 3-fold increase in Tlr2 transcript levels compared to LFC diet fed mice (Diet, $P=0.0009$).

To determine whether Tlr2 or Tlr4 deficiency corresponded with changes in inflammation, we measured transcript abundance of TNF$\alpha$ (Fig. 14C), IL-6 (Fig. 14D), and MCP-1 (Fig. 14E) in AT of Tlr2$^{-/-}$, 10ScN, and the respective control mice. Tlr2$^{-/-}$ mice had 2-fold lower TNF$\alpha$ and MCP-1 transcript levels in AT compared to 6J mice (Genotype, $P\leq0.0001$ and $P=0.0087$, respectively). Similarly, TNF$\alpha$ transcript abundance was 2-fold lower in 10ScN mice versus 10J controls (Genotype, $P=0.0269$). Tlr2$^{-/-}$ mice were specifically protected from HFP diet-induced TNF$\alpha$, IL-6, and MCP-1 expression in AT (Diet*Genotype, $P=0.0010$, $P=0.051$, and $P\leq0.0001$, respectively). Tlr4 deficiency lowered IL-6 and MCP-1 transcript abundance by 2-fold in AT of HFP fed mice (Diet*Genotype, $P=0.045$ and $P=0.046$). These findings indicated that the ability of a high saturated fat diet to
induce expression of proinflammatory cytokines was significantly blunted without either functional Tlr2 or Tlr4.

We have shown that transcript abundance of macrophage marker, F4/80, was over 2-fold lower in Tlr2<sup>−/−</sup> and 10ScN mice relative to respective controls (Genotype, P=0.025 and P=0.032). Absence of Tlr2, as well as Tlr4, specifically attenuated F4/80 transcript abundance in HFP fed mice F4/80 (Diet*Genotype, P=0.0014 and P=0.0045, respectively).

In the present study, nitric oxide synthase-2 (NOS2) transcript abundance was 5.2- and 5.9-fold lower in both Tlr2<sup>−/−</sup> and 10ScN mice compared to 6J and 10J controls (Genotype, P<0.0001). This effect was more prominent with either high fat diet (Diet, P<0.0001), but the highest level of NOS2 transcript was in HFP fed mice (Diet, P=0.030). The ability of Tlr2 or Tlr4 deficiency to attenuate HFC-induced NOS2 was similar (Diet*Genotype, P≤0.0001). However, only 10ScN mice exhibited a protective effect against the HFP diet (Diet*Genotype, P≤0.0001). Overall, these results indicated that Tlr2 and Tlr4 were important mediators of macrophage accumulation and oxidative stress in AT of mice fed either high fat diet.

_Tlr2 deficient SV cells were protected from FFA-induced inflammation and insulin resistance._ We observed a limited effect of Tlr2 deficiency on blood glucose and serum insulin. These findings may reflect an increased susceptibility to DIO on the 6J background, which was clearly demonstrated by the greater body weight, adiposity, and energy intake in mice on 6J background (Tlr2<sup>−/−</sup> and 6J), relative to 10J background (10ScN and 10J) (Genotype, P≤0.0001). Thus, we investigated if insulin sensitivity in Tlr2<sup>−/−</sup> mice was improved at the cellular level. To do this we isolated and cultured primary SV cells from AT
of Tlr2−/− and 6J mice. Confluent cells were then treated with adipocyte differentiation media (~40% of cells exhibited lipid accumulation) and used for determination of 2-DG uptake.

We demonstrated that Tlr2−/− SV cells exhibited greater insulin sensitivity than 6J cells, regardless of treatment (Fig. 15, Genotype, P≤0.0001). Furthermore, Tlr2 deficient SV cells treated with palmitate, LPS, or ZymA for 48 hr had improved insulin sensitivity compared to 6J cells (Treatment, P<0.0001). However, only ZymA-induced insulin resistance was completely attenuated in Tlr2−/− SV cells compared to insulin-stimulated controls (Treatment*Genotype, P<0.0001). The inability of Tlr2 deficiency to fully block palmitate mediated insulin resistance indicated an alternative pathway. Tlr4 represents a potential candidate, as LPS-induced insulin resistance was unaffected by Tlr2 deficiency (Treatment*Genotype, P≤0.0001). These data indicated that absence of functional Tlr2 improved insulin sensitivity in SV cells isolated from AT, but failed to completely block SFA-induced insulin resistance due to Tlr2-independent pathways, such as Tlr4.

Discussion

Obesity is associated with chronic low grade inflammation, which contributes to the development of insulin resistance (17). The molecular mechanisms involved are not fully elucidated but may involve activation of Tlr2 and Tlr4 signaling pathways in AT. Several recent studies (4,6,7,20) have identified a role of Tlr4 in obesity and FA-induced inflammation. Herein, we demonstrate that Tlr2−/− mice were protected from DIO without significant changes in energy intake or lipid profile. These mice also exhibited a dramatic attenuation of systemic and AT inflammation. Moreover, SV cells isolated from AT of Tlr2−/− mice had a marked increase in insulin sensitivity. These data indicated that Tlr2 is involved in obesity-induced inflammation and insulin resistance.
Diets high in SFA are associated with increased inflammation (21). We have previously reported that Tlr4 deficiency specifically protected against a high SFA diet-induced adiposity and inflammation (9). In this study, we confirmed this effect in 10ScN mice, but also demonstrated that absence of functional Tlr2 blunted the high SFA diet-induced inflammation. Moreover, the attenuated inflammatory response in Tlr2−/− and 10ScN mice suggests that the absence of these receptors protects against the elevated serum SFA observed in HFP-fed control mice. The ability of SFA to induce inflammation through Tlr4 is well documented in vitro (5,22). However, only a few studies (12,23) have implicated Tlr2 as a potential target for SFA, including Senn (23), who reported that Tlr2 siRNA reversed palmitate-induced activation of IKKβ/NFκB in C2C12 myotubes. These data herein confirm the idea that Tlr2 is an endogenous target for SFA, and thus promotes obesity-induced inflammation.

AT inflammation, as evidenced by macrophage infiltration and proinflammatory gene expression, contributes to the development of insulin resistance (17,18). Impaired insulin signaling in AT often precedes the onset of systemic insulin resistance (24). We previously reported that Tlr4 deficient mice exhibited lower macrophage infiltration in AT (9). In the present study, we observed that transcript abundance of the macrophage specific marker, F4/80 was markedly reduced in Tlr2 and Tlr4 deficient mice. This reduction was more apparent in HFP-fed mice, and corresponded with lower expression levels of multiple proinflammatory genes (e.g. TNFα, IL-6, and MCP-1). Additionally, the high fat diet-induced NOS2 transcript was reduced in Tlr2−/− and 10ScN mice, which supports our previous findings that Tlr2 and Tlr4 ligands induce ROS in 3T3-L1 adipocytes (16). Thus,
Tlr2 and Tlr4 deficiency protected against AT inflammation and oxidative stress, especially when fed a high SFA diet. 

Several investigations (4,6-8) reported Tlr4 involvement in obesity and FA-induced insulin resistance. Alternatively, Caricilli et al. (25) showed that injection of Tlr2 antisense oligonucleotide attenuated insulin resistance as measured by an insulin tolerance test. Furthermore, inhibition of Tlr2 also improved insulin sensitivity in AT and muscle of DIO mice. Our data showed that only 10ScN mice exhibited improved serum markers of insulin sensitivity. However, to determine if insulin sensitivity was improved in AT, we studied insulin sensitivity in primary SV cells from Tlr2−/− mice. Although we observed a marked improvement in insulin sensitivity in the Tlr2−/− mice, the absence of Tlr2 failed to fully protect against palmitate-induced insulin resistance. These findings indicate that Tlr2 does mediated AT specific inflammation, and may be as involved in obesity-induced insulin resistance as is Tlr4. In summary, our study demonstrates a requisite role of Tlr2 and Tlr4 in obesity-induced inflammation and insulin resistance. The absence of these receptors protects against systemic and AT specific inflammation, especially in mice fed a high SFA diet. We therefore assert that both Tlr2 and Tlr4 represent potential dietary and pharmaceutical targets for reducing inflammation and insulin resistance associated with obesity.

References


### Table 6. Diet Composition

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<th>Ingredient</th>
<th>Low-Fat Control (LFC) $^1$</th>
<th>High-Fat Control (HFC) $^1$</th>
<th>High-Fat Palmitate (HFP) $^1$</th>
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<tr>
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<td>g/kg</td>
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1. Adapted from AIN-93M Diet
2. Harlan Teklad, Madison, WI
3. Nu-Check Prep, Inc., Elysian, MN
**Table 7. Primer sequences for quantitative real-time polymerase chain reaction**

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Beta-Actin</td>
<td>TGAGAGGGAAATCGTGGTGACAT</td>
<td>ACCGCTCGTTGCCAATAGTGATGA</td>
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<tr>
<td>Tlr2</td>
<td>TCCCTTGACATCAGCAGGAACACT</td>
<td>GCAGCGCGAGCAAGAAGAAAGAAA</td>
</tr>
<tr>
<td>Tlr4</td>
<td>CGGCTCTGGCATCATCCATTGTGGGT</td>
<td>TCCTCCATTTCCAGGTAGGTGTTT</td>
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<tr>
<td>MCP-1</td>
<td>CCAAGAAAGGAATGGGTCCAGACAT</td>
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<tr>
<td>TNF-α</td>
<td>CCAACGGCATGGATCTCAAAGACA</td>
<td>AGATAGCAAAATCGGCTGACGGGTGT</td>
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<tr>
<td>IL-6</td>
<td>TCCAGTTGCTTTCTTGGGACTGAT</td>
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<tr>
<td>NOS2</td>
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<td>F4/80</td>
<td>TGCCACAAACACTCTCGGAAGCTAT</td>
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Table 8. Lipid profiles in Tlr2 and Tlr4 deficient mice and wild type controls fed LFC, HFC, or HFP diet for 16 weeks

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>Serum Cholesterol (mmol/L)</th>
<th>Serum Triacylglyceride (mmol/L)</th>
<th>Serum NEFA (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ScN</td>
<td>LFC</td>
<td>5.00 ± 0.59&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.53 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.48 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10ScN</td>
<td>HFC</td>
<td>6.39 ± 0.53&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.73 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.51 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>10ScN</td>
<td>HFP</td>
<td>6.17 ± 0.51&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.75 ± 0.07&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.48 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10J</td>
<td>LFC</td>
<td>5.41 ± 0.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.96 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.65 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10J</td>
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<td>5.18 ± 0.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.71 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.38 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>HFP</td>
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<td>1.30 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Tlr2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>LFC</td>
<td>6.69 ± 0.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.92 ± 0.08&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.84 ± 0.27&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tlr2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>HFC</td>
<td>9.20 ± 0.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.76 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.02 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Tlr2&lt;sup&gt;-/-&lt;/sup&gt;</td>
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<td>0.92 ± 0.09&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.33 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>2.08 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>2.10 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
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Main Effects

<table>
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<tr>
<th></th>
<th>Genotype</th>
<th>Diet</th>
<th>Interaction</th>
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<tr>
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<td>P ≤ 0.0001</td>
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<td>P = 0.019</td>
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<td>Diet</td>
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<td>P = 0.052</td>
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<sup>a, b, c, d</sup> Significant difference between LS means indicated by different letters (P<0.05)
Table 9. Serum fatty acid profiles in Tlr2 and Tlr4 deficient mice and wild type controls fed LFC, HFC, and HFP diet for 16 weeks

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<thead>
<tr>
<th>Genotype</th>
<th>Diet</th>
<th>FA</th>
<th>C20:4n6</th>
<th>C20:5n3</th>
<th>C20:6n3</th>
<th>C20:5n3</th>
<th>C20:6n3</th>
<th>C20:5n3</th>
<th>C20:6n3</th>
<th>C20:5n3</th>
<th>C20:6n3</th>
<th>C20:5n3</th>
<th>C20:6n3</th>
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</thead>
<tbody>
<tr>
<td>10SxN</td>
<td>LFC</td>
<td>15.0 ± 1.3</td>
<td>18.5 ± 1.3</td>
<td>29.5 ± 1.8</td>
<td>13.0 ± 2.4</td>
<td>1.00 ± 0.7</td>
<td>7.50 ± 1.1</td>
<td>45.0 ± 1.8</td>
<td>43.5 ± 2.1</td>
<td>7.50 ± 1.3</td>
<td>45.0 ± 1.8</td>
<td>43.5 ± 2.1</td>
<td>7.50 ± 1.3</td>
</tr>
<tr>
<td>10SxN</td>
<td>HFC</td>
<td>21.0 ± 1.4</td>
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<td>46.3 ± 1.5</td>
<td>8.3 ± 1.9</td>
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<td>6.33 ± 0.9</td>
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<td>6.33 ± 1.0</td>
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<tr>
<td>10SxN</td>
<td>HFP</td>
<td>24.0 ± 1.7</td>
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<td>26.0 ± 1.8</td>
<td>20.5 ± 2.4</td>
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<td>5.50 ± 1.1</td>
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<td>49.5 ± 2.1</td>
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<td>17.6 ± 0.8</td>
<td>25.4 ± 1.1</td>
<td>11.3 ± 1.5</td>
<td>1.20 ± 0.4</td>
<td>7.40 ± 0.7</td>
<td>45.6 ± 1.1</td>
<td>42.9 ± 1.3</td>
<td>7.40 ± 0.8</td>
<td>45.6 ± 1.1</td>
<td>42.9 ± 1.3</td>
<td>7.40 ± 0.8</td>
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<tr>
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<td>43.7 ± 1.5</td>
<td>46.0 ± 1.7</td>
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<td>4.00 ± 1.0</td>
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<tr>
<td>Thr2-/+</td>
<td>LFC</td>
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<td>9.62 ± 0.6</td>
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<td>9.62 ± 0.6</td>
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<td>HFC</td>
<td>18.8 ± 1.2</td>
<td>11.7 ± 0.9</td>
<td>37.5 ± 1.3</td>
<td>16.0 ± 1.7</td>
<td>4.25 ± 0.5</td>
<td>6.25 ± 0.8</td>
<td>30.8 ± 1.3</td>
<td>57.5 ± 1.5</td>
<td>7.25 ± 0.9</td>
<td>30.8 ± 1.3</td>
<td>57.5 ± 1.5</td>
<td>7.25 ± 0.9</td>
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<tr>
<td>Thr2-/+</td>
<td>HFP</td>
<td>23.5 ± 1.0</td>
<td>16.8 ± 0.8</td>
<td>22.2 ± 1.0</td>
<td>21.4 ± 1.5</td>
<td>2.17 ± 0.4</td>
<td>4.00 ± 0.6</td>
<td>41.8 ± 1.0</td>
<td>45.7 ± 1.2</td>
<td>4.17 ± 0.7</td>
<td>41.8 ± 1.0</td>
<td>45.7 ± 1.2</td>
<td>4.17 ± 0.7</td>
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<tr>
<td>6F</td>
<td>LFC</td>
<td>21.8 ± 1.2</td>
<td>12.5 ± 0.9</td>
<td>22.5 ± 1.3</td>
<td>18.8 ± 1.7</td>
<td>2.25 ± 0.5</td>
<td>7.00 ± 0.8</td>
<td>37.0 ± 1.3</td>
<td>43.3 ± 1.3</td>
<td>9.00 ± 0.9</td>
<td>37.0 ± 1.3</td>
<td>43.3 ± 1.3</td>
<td>9.00 ± 0.9</td>
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<tr>
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<td>HFC</td>
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<td>38.3 ± 1.3</td>
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<td>3.67 ± 0.5</td>
<td>6.67 ± 0.9</td>
<td>31.3 ± 1.3</td>
<td>56.7 ± 1.7</td>
<td>6.57 ± 1.0</td>
<td>31.3 ± 1.3</td>
<td>56.7 ± 1.7</td>
<td>6.57 ± 1.0</td>
</tr>
<tr>
<td>6F</td>
<td>HFP</td>
<td>24.2 ± 1.6</td>
<td>15.2 ± 0.8</td>
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<td>21.4 ± 1.5</td>
<td>1.80 ± 0.4</td>
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<td>41.8 ± 1.3</td>
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<td>42.4 ± 1.1</td>
<td>41.8 ± 1.3</td>
<td>5.40 ± 0.8</td>
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</table>

Main Effects:
- **Genotype**
- **Diet**

Interaction:
- **Diet*Genotype**

Significant difference between LS means indicated by different letters (P ≤ 0.05)
Figure 12. Tlr2⁻/⁻ and 10ScN mice were protected against DIO.

All measures were taken after 16 weeks of LFC (white bars), HFC (black bars), or HFP (grey bars) diet (n = 15 unless noted otherwise). (A) Body weight of 6J, Tlr2⁻/⁻, 10ScN, and 10J mice (Diet effect P= 0.58, Genotype effect, P<0.0001, Diet*Genotype interaction P=0.0085). (B) Total weight gain of 6J, Tlr2⁻/⁻, 10ScN, and 10J mice (Diet effect P= 0.58, Genotype effect, P<0.0001, Diet*Genotype interaction P=0.0085). (C) Epididymal fat pad weight of 6J, Tlr2⁻/⁻, 10ScN, and 10J mice (Diet effect P=0.032, Genotype effect P<0.0001, Diet*Genotype interaction P=0.040). (D) Total energy intake (kcal) of 6J, Tlr2⁻/⁻, 10ScN, and 10J mice (Diet effect P=0.82, Genotype effect P<0.0001, Diet*Genotype interaction P=0.013). All displayed values were adjusted for initial body weight and represent LS means ± SEM for Diet*Genotype interaction. When main effects (diet and genotype) or interaction term were P≤0.15, Bonferroni correction was used to determine significant difference between means as indicated by different letters.
Figure 13. Absence of Tlr2 and Tlr4 expression differentially modified markers of systemic insulin sensitivity and inflammation.

All measures were taken after 16 weeks of LFC (white bars), HFC (black bars), or HFP (grey bars) diet. (A) Blood glucose from 6-8 h fasted 6J, Tlr2−/−, 10ScN, and 10J mice (n = 15, Diet effect P= 0.81, Genotype effect, P=0.066, Diet*Genotype interaction P=0.65). (B) Serum insulin (n = 5, Diet effect P=0.096, Genotype effect P=0.0002, Diet*Genotype interaction P=0.15). (C) Serum CRP from 6J, Tlr2−/−, 10ScN, and 10J mice (n = 5, Diet effect P=0.095, Genotype effect, P<0.0001, Diet*Genotype interaction P=0.066). (D) Serum endotoxin (n = 5, Diet effect P=0.57, Genotype effect P<0.0001, Diet*Genotype interaction P=0.34). E) Serum MCP-1 (n = 5, Diet effect P=0.42, Genotype effect P=0.0002, Diet*Genotype interaction P=0.090). All displayed values were adjusted for initial body weight and represent LS means ± SEM for Diet*Genotype interaction. When main effects (diet and genotype) or interaction term were P≤0.15, Bonferroni correction was used to determine significant difference between means as indicated by different letters.
Figure 14. Absence of Tlr2 and Tlr4 expression protected against SFA-induced inflammation in AT.

All measures were taken after 16 weeks of LFC (white bars), HFC (black bars), or HFP (grey bars) diet. (A) Tlr4 transcript abundance in AT of 6J, Tlr2−/−, 10ScN, and 10J mice (n = 6, Diet effect P=0.014, Genotype effect, P≤0.0001, Diet*Genotype interaction P=0.24). (B) Tlr2 transcript abundance in AT of 6J, Tlr2−/−, 10ScN, and 10J mice (n = 6, Diet effect P=0.0031, Genotype effect P<0.0001, Diet*Genotype interaction P=0.073). (C) TNFα transcript abundance in AT of 6J, Tlr2−/−, 10ScN, and 10J mice (n = 6, Diet effect P=0.66, Genotype effect P≤0.0001, Diet*Genotype interaction P=0.040). (D) IL-6 transcript abundance in AT of 6J, Tlr2−/−, 10ScN, and 10J mice (n = 6, Diet effect P=0.34, Genotype effect P=0.0005, Diet*Genotype interaction P=0.015). (E) MCP-1 transcript abundance in AT of 6J, Tlr2−/−, 10ScN, and 10J mice (n = 6, Diet effect P=0.13, Genotype effect P<0.0001, Diet*Genotype interaction P=0.14). (F) F4/80 transcript abundance in AT of 6J, Tlr2−/−, 10ScN, and 10J mice (n = 6, Diet effect P=0.19, Genotype effect P<0.0001, Diet*Genotype interaction P=0.084). (G) NOS2 transcript abundance in AT of 6J, Tlr2−/−, 10ScN, and 10J mice (n = 6, Diet effect P≤0.0001, Genotype effect P<0.0001, Diet*Genotype interaction P<0.0001). All displayed values were adjusted for initial body weight and beta actin transcript level. Values represent LS means ± SEM for Diet*Genotype interaction. When main effects (diet and genotype) or interaction term were P<0.15, Bonferroni correction was used to determine significant difference between means as indicated by different letters.
Figure 15. SV cells isolated from Tlr2−/− exhibit improved insulin sensitivity, but were not fully protected against FA-induced insulin resistance.

14 d post-differentiated SV cells, in which approximately 40% exhibited an adipocyte phenotype (e.g. lipid accumulation), were treated with 0.5 mM sodium palmitate, 10 µg/mL LPS, or 20 µg/mL ZymA for 48 h. Insulin-stimulated 2-DG uptake was then determined (n = 6-8, Treatment effect P<0.0001, Genotype effect P<0.0001, Treatment*Diet effect P=0.0007). A non-insulin stimulated control was included for reference. Results are expressed as fold change versus insulin stimulated 6J control and represent LS means ± SEM. When Treatment effect was significant (P<0.05), post-hoc t-test was used to determine significant difference between LS means as indicated by different letters.
CHAPTER 6. SUMMARY

The rise in obesity corresponds to the development of DM and CVD (1,2). The molecular mechanism linking obesity to these inflammatory diseases is not fully elucidated but may involve AT-specific inflammation (3). Adipocyte hypertrophy is associated increased release of proinflammatory cytokines, as well as accumulation of macrophages in AT (4,5). Corresponding to obesity-induced inflammation is an increased release of FFA from adipocytes, which signals neighboring or downstream cells (6). The ability of FFA, especially SFA, to mediate inflammation may be dependent on the presence of specific innate immune receptors, Tlr2 and Tlr4. Several studies (7-9) have implicated the involvement of these receptors in the development of inflammation and insulin resistance in various cell populations. However, little is known about the relationship of these receptors in DIO, more specifically, the role of AT inflammation in the development of systemic inflammation and insulin resistance.

We demonstrated that 10ScN mice, with a 74 kb deletion from chromosome 4 that precludes expression of Tlr4, were protected against DIO. This effect was more prominent in mice fed a high SFA diet. Moreover, these mice exhibited a specific reduction in AT inflammation as evidenced by the reduction in proinflammatory transcription factor activation and subsequent gene expression. There was also a decrease in macrophage accumulation in AT of mice fed the HFP diet. These data indicated that Tlr4 was involved in high fat diet-induced adiposity and inflammation in AT. However, Tlr4 deficiency did not fully attenuate TNFα and IL-6 transcript abundance in AT of high saturated diet fed mice. Corresponding to these findings was an increase in Tlr2 expression, which because of its
ability to recognize SFA, represented an additional mechanism mediating obesity-induced inflammation.

To determine involvement of Tlr2 in AT inflammation, we then utilized 3T3-L1 adipocytes to investigate the ability of the Tlr2 agonist, ZymA, to mediate inflammation and insulin resistance. Our data showed that ZymA induced proinflammatory gene expression similarly to palmitate and LPS treatment. Furthermore, the Tlr2 agonist stimulated NFκB nuclear translocation, although not to the same extent as the Tlr4 agonist, LPS. We also demonstrated for the first time in adipocytes that ZymA caused nuclear translocation of the proinflammatory transcription factor, AP-1. Thus, Tlr2 involvement in adipocyte inflammation was apparent. Along with the proinflammatory response observed with ZymA, LPS, and palmitate treated cells was an increased accumulation of ROS, which closely paralleled expression of NOS2 transcript abundance. The ability of inflammation and oxidative stress to mediate insulin resistance is well documented (6,10,11). Therefore, we evaluated insulin-stimulated glucose uptake in adipocytes treated with SFA or Tlr agonist and showed a significant reduction in insulin sensitivity with these treatments.

Activation of the intracellular kinase, JNK, is associated with Tlr signaling (7,12) thus, we investigated the ability of a highly specific JNK inhibitor to reduce inflammation and oxidative stress, as well as improve insulin sensitivity. There was a marked attenuation of insulin resistance in ZymA, LPS, and palmitate treated adipocytes, which corresponded to a reduction in NFκB and AP-1 activation, proinflammatory gene expression, and ROS accumulation. From these data we concluded that Tlr2 is likely involved in obesity-induced inflammation and oxidative stress in AT, as well as insulin resistance in adipocytes.
Additionally, it appeared that JNK activation was essential to the SFA and Tlr agonist mediated effects.

Based on our studies with Tlr4 deficient mice and cultured adipocytes, we then investigated the role of Tlr2 in obesity-induced inflammation and insulin resistance. Moreover, we wanted to determine whether the dietary source of FA (saturated vs. unsaturated) differentially modified inflammation and insulin resistance. Our data showed that Tlr2−/− mice were protected from DIO. However, unlike Tlr4 deficient 10ScN mice, there were no significant changes in body weight, energy intake, or lipid profile. These findings indicated that Tlr2 deficiency significantly modified body composition, which may reflect metabolic changes in energy metabolism. For instance, reduced adiposity in Tlr2−/− mice associated with energy uncoupling could mediate lower AT accumulation.

We also demonstrated that serum markers of inflammation, including CRP, endotoxin, and MCP-1 were lower in 10ScN mice fed a HFP diet, whereas only MCP-1 was reduced in the Tlr2−/− mice fed a high fat diet. The disparity in these data may be due to distinct genetic differences in background strain. The 6J background is more susceptible to DIO, which in our studies was indicated by higher body weight and adiposity compared to mice on 10J background. Therefore, the higher propensity toward obesity in Tlr2−/− mice may mask potential changes in markers of systemic inflammation. Despite differences in background, both Tlr2 and Tlr4 deficiency improved AT markers of inflammation and oxidative stress including TNFα, IL-6, MCP-1, F4/80, and NOS2 transcript abundance. Similar to our previous findings, the protective effect of Tlr deficiency was more apparent in mice fed the high SFA diet. In Tlr2−/− mice, attenuation of AT inflammation did not correlate with improved circulating glucose or insulin concentration. However, SV cells isolated from
AT of Tlr2^{-/-} mice had a marked increase in insulin sensitivity, demonstrating the potential involvement of Tlr2 in obesity-induced inflammation and insulin resistance.

The ability of both Tlr2 and Tlr4 to recognize FA and initiate proinflammatory signaling in adipocytes, and other cell types, demonstrated the tremendous overlap of inflammatory pathways. In order to completely understand the importance of these receptors in obesity and FA-induced inflammation, it will be necessary to target each receptor simultaneously either with siRNA or generation of double knockout mice. Furthermore, to fully elucidate the impact of Tlr2 and Tlr4 on adiposity and insulin resistance, energy expenditure will need to be evaluated, as well as more sensitive measures of insulin sensitivity, such as the euglycemic-hyperinsulinemic clamp. Our studies also demonstrated that Tlr2^{-/-} and 10ScN mice had a greater protective effect when fed diet high SFA. Therefore, future studies need to address the ability of various FA to induce an inflammatory response through Tlr2 and Tlr4.

Overall, we have presented evidence that Tlr2 and Tlr4 were involved in obesity-induced inflammation, as well as insulin resistance. Furthermore, the absence of these receptors in AT attenuated inflammation, oxidative stress, and insulin resistance at the cellular level (i.e., adipocytes), which in turn reduced systemic inflammation and insulin resistance. The underlying mechanism involved recognition of specific FA by Tlr2 and Tlr4, and more specifically interaction with SFA. Based on these studies, Tlr2 and Tlr4 may represent ideal targets for pharmaceutical, as well as dietary, intervention designed to attenuate obesity and related co-morbidities, including DM and CVD.
References


