The Effect of Butter, Naturally Enriched with Trans-10, Cis-12 Conjugated Linoleic Acid, on Insulin Resistance and Inflammation in High Fat-Fed Rodents

by

Amanda Stefanson

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Human Health & Nutritional Sciences

Guelph, Ontario, Canada

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ABSTRACT

THE EFFECT OF BUTTER, NATURALLY ENRICHED WITH TRANS-10, CIS-12 CONJUGATED LINOLEIC ACID, ON INSULIN RESISTANCE AND INFLAMMATION IN HIGH FAT-FED RODENTS

Amanda Stefanson                      Advisor:  
University of Guelph, 2013            Professor D C Wright

This study was conducted to evaluate dietary CLA\textsubscript{t10,c12}, given the studies showing that supplemental doses has a negative impact on insulin resistance. To that end, we produced a 60% high fat diet composed of butter produced from milk collected from dairy cows suffering from subacute ruminal acidosis (SARA) – a condition that can sometimes result from the dairy industry production practice of feeding a high grain, low forage diet to milk-producing cows. This butter represents the most CLA\textsubscript{t10,c12}-enriched source of naturally occurring CLA\textsubscript{t10,c12} for humans. We compared the effects of the high CLA\textsubscript{t10,c12} butter against a commercially available butter in a high fat diet-induced rodent model of insulin resistance. After 8 weeks, whole body glucose homeostasis was evaluated along with markers of inflammation, indicated by mitogen-activated protein kinase (MAPK) activation.
Acknowledgments

I would like to thank Dr. David Wright for his support, guidance, and good humor throughout this project. His positive attitude and advising style ensured that even the difficult times were a pleasure. I would also like to extend my thanks Dr. David Dyck and Dr. Brian McBride for their important contributions to this project. Tara MacDonald deserves special thanks for all her patient help in the lab, but to all my wonderful lab mates – it has been a joy to work with you all!
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<tbody>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>AS160</td>
<td>Akt substrate of 160 kDa</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BiP</td>
<td>binding immunoglobulin protein</td>
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<tr>
<td>CLA</td>
<td>conjugated linoleic acid</td>
</tr>
<tr>
<td>CLA&lt;sub&gt;c9,t11&lt;/sub&gt;</td>
<td>cis-9, trans-11 conjugated linoleic acid</td>
</tr>
<tr>
<td>CLA&lt;sub&gt;mix&lt;/sub&gt;</td>
<td>commercially available ~50:50 blend of CLA&lt;sub&gt;t10,c12&lt;/sub&gt; and CLA&lt;sub&gt;c9,t11&lt;/sub&gt;</td>
</tr>
<tr>
<td>CLA&lt;sub&gt;t10,c12&lt;/sub&gt;</td>
<td>trans-10, cis-12 conjugated linoleic acid</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglyceride</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter type 4</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of nuclear factor kappa B kinase</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin 8</td>
</tr>
<tr>
<td>IRE-1</td>
<td>inositol-requiring enzyme 1</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>IκBα</td>
<td>inhibitor of nuclear factor kappa B</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MFD</td>
<td>milk fat depression</td>
</tr>
<tr>
<td>MUFA</td>
<td>mono-unsaturated fatty acid</td>
</tr>
<tr>
<td>Nfr2</td>
<td>nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>p38</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR-like eukaryotic initiation factor 2 α kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>SARA</td>
<td>subacute rumenal acidosis</td>
</tr>
<tr>
<td>SAT</td>
<td>subcutaneous adipose tissue</td>
</tr>
<tr>
<td>SCD</td>
<td>stearoyl CoA desaturase</td>
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<tr>
<td>SFA</td>
<td>saturated fatty acid</td>
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T2D        type 2 diabetes
TLR4      toll-like receptor 4
TNFR      tumor necrosis factor-α receptor
TNFα      tumor necrosis factor-α
UCP       uncoupling protein
UPR       unfolded protein response
VAT       visceral adipose tissue
WAT       white adipose tissue
WT        wild type
Chapter One: Literature Review

DIABETES, OBESITY AND INSULIN RESISTANCE

Obesity rates have nearly doubled in the last 3 decades; according to the World Health Organization, more than 10% of the world’s adult population was obese as of 2008\textsuperscript{1}. Obesity is the greatest risk factor for the development of type 2 diabetes\textsuperscript{2,3}. Diabetes is projected to be the 7\textsuperscript{th} most common cause of death by 2030\textsuperscript{4}. Ninety percent of diabetes cases are type 2 diabetes (T2D)\textsuperscript{5}, which is considered mainly a lifestyle disease\textsuperscript{6}. Insulin resistance is an intermediary condition of varied etiology, characterized by a reduced responsiveness to the effects of insulin; insulin resistance can often be reversed with diet and lifestyle interventions before the development of the pancreatic dysfunction and insulin insufficiency characteristic of T2D.

CELL SIGNALING PATHWAYS INVOLVED IN INSULIN RESISTANCE

Overview of insulin signaling

In response to postprandial increases in plasma glucose, insulin is secreted into circulation from pancreatic beta cells. Circulating insulin binds the transmembrane insulin receptors embedded in the cell membranes of metabolic tissues (skeletal muscle, adipose tissue and liver primarily). Upon insulin binding to the insulin receptor the tyrosine kinase domain is activated, which in turn phosphorylates tyrosine residues on other target proteins\textsuperscript{7}. The tyrosine phosphorylation of cytosolic insulin receptor
substrate (IRS) proteins activates the phosphotidylinositide-3-kinase (PI3K) pathway responsible for most of the metabolic actions of insulin\textsuperscript{7-9}. IRS-1 activates PI3K which then adds a third phosphate group to membrane-bound phosphatidylinositol bisphosphate (PIP2) to form phosphatidylinositol triphosphate (PIP3); this reaction recruits protein kinase B (PKB) and 3-phosphoinositide dependent protein kinase-1 (PDK1) from the cytosol to separate membrane PIP3. PIP3-bound PKB can then be activated by phosphorylation carried out by a nearby PIP3-bound PDK1. Activated PKB phosphorylates AS160, resulting in glucose transporter type 4 (GLUT4) translocation from cytosolic vesicles (under low plasma insulin conditions) to the plasma membrane (under higher plasma insulin conditions). GLUT4 is a membrane transport protein responsible for facilitating insulin-stimulated glucose uptake from the blood into metabolic tissues for storage and reducing circulating glucose levels. Interference at any point in insulin signaling can reduce GLUT4 translocation, ultimately reducing the capacity for glucose clearance and contributing to insulin resistance. The activity of IRS-1 can be reduced by serine phosphorylation (classically Ser308) by various agents\textsuperscript{10,11}; in fact, it has many tyrosine and serine phosphorylation sites that act together to finely modify IRS signaling\textsuperscript{12}. 
Figure 1: Simplified insulin signaling cascade

Overview of mitogen-activated protein kinase-mediated pathways

Mitogen-activated protein kinases (MAPK) are a family of cytosolic serine/threonine protein kinases that are involved in transducing intracellular signals, and respond to widely varying signals including growth factors and stress mediators such as cytokines, reactive species, radiation, heat shock and electrophilic lipids\textsuperscript{13,14}. This enzyme family is characterized by a three-tiered signal transduction cascade (generalized as MAP3K, MAP2K and MAPK) (Figure 2). MAP3 kinases are often localized at the intracellular side of the plasma membrane and are involved in receiving extracellular signals mediated by various membrane receptor complexes\textsuperscript{15}. The canonical signal transduction cascade involves the activation of MAP3 kinases by membrane receptor complexes, which then pass the signal on to MAP2 kinases, which ultimately activate MAP kinases resulting in the activation of transcription factors and subsequent changes in gene transcription\textsuperscript{15,13}. 
Although activated MAPKs are also central to intracellular signaling, involved in innumerable cell signaling events, each one having hundreds of potential substrates\textsuperscript{16}. The most well-characterized MAPK pathways are those mediated by extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38). The ERK pathway is primarily activated by growth factors, the p38 pathway is primarily activated by stress mediators, and the JNK pathway is intermediate – activated both by growth factors and stress mediators\textsuperscript{13,9}.

**Interference with insulin signaling by mitogen-activated protein kinases**

MAPKs can interfere with insulin signaling either directly or indirectly. In their role as mediators of transcription factor activation, MAPKs can propagate wider stress and inflammation processes that can indirectly affect insulin signaling. ERK is somewhat unique among the MAPK pathways as it is insulin responsive, involved in growth and development, and responsible for much of the effect of insulin on gene transcription\textsuperscript{13,9}. JNK is involved in the activation of several transcription factors, most notably activator protein-1 (AP-1). Once c-Jun is activated by JNK, it can form half of the heterodimer that makes up AP-1\textsuperscript{17}. The other half of AP-1 is c-Fos\textsuperscript{18}, which can be activated in various ways by any of ERK, JNK or p38, therefore a confluence of intracellular signals converge to mediate AP-1 activation\textsuperscript{19}. P-38 is involved in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF\kappa B) – a transcription factor sequestered in the cytosol when bound to inhibitor of nuclear factor kappa B (I\kappa B) under normal conditions. P-38 is involved in the activation of inhibitor of nuclear factor kappa B kinase (IKK), which phosphorylates I\kappa B, releasing NF\kappa B for nuclear translocation\textsuperscript{20}. 
AP-1 and NFκB are generally proinflammatory transcription factors that regulate, but do not initiate, apoptosis\textsuperscript{21,17,22}.

In their role as intracellular signal transducers, activated MAPKs can directly interfere with insulin signaling. ERK itself can be activated by insulin as the MAP3K in this pathway is downstream of IRS-1 activation\textsuperscript{8}. Activated IKK can directly interfere with insulin signaling by serine phosphorylation of insulin receptor or IRS-1; phosphorylation by p-38 is one method of IKK activation\textsuperscript{23}. Of the MAPKs, JNK is most often implicated in insulin resistance because activated JNK can directly reduce IRS-1 activity by serine phosphorylation\textsuperscript{16}. JNK activity is increased in obese mice and JNK1 knock out mice are protected from obesity induced insulin resistance\textsuperscript{24}. 
Figure 2: MAPK signaling cascades

**HIGH FAT DIET INDUCED INSULIN RESISTANCE AND INFLAMMATION**

High fat diet-induced obesity is correlated with the development of insulin resistance in metabolic tissues (adipose tissue, skeletal muscle, liver) and chronic low-grade inflammation. Indices that measure whole body insulin sensitivity, such as the hyperinsulinemic-euglycemic clamp method, show that whole body insulin resistance develops rapidly in response to a high lipid intake and progressively deteriorates over time as the high fat diet continues. However, insulin resistance is a very broad term describing reduced responsiveness to the effects of insulin. Many different factors
arising from different etiologies contribute to insulin resistance, and originate at different time points in the development of high fat diet-related insulin resistance.

**Early development of insulin resistance and over-nutrition**

The early effects of a high lipid intake results in a decrease in insulin sensitivity at the whole body level and can be detected within hours or days of starting a high fat diet. Skeletal muscle is a major metabolic tissue accounting for ~80% of glucose disposal under normal conditions, and reduced skeletal muscle insulin sensitivity is clearly apparent in established obesity, but does not contribute significantly to the early stages of insulin resistance development. Under normal conditions, insulin suppresses endogenous glucose synthesis pathways in the liver. Among the earliest responses to high lipid intake is reduced hepatic suppression of gluconeogenesis. A large influx of blood lipids is metabolically challenging for adipocytes, as excess lipids will be stored in adipose tissue using energy-dependent processes. Adipocytes do not have large glucose stores, and the inability to suppress hepatic gluconeogenesis may be an adaptive response to ensure adequate energy availability to adipocytes in the face of this short-term challenge. The endoplasmic reticulum (ER) is a key organelle involved in cellular metabolism. When oversupply of lipids persists, the metabolic demands may outstrip the capacity of the ER, resulting in ER stress.

**Endoplasmic reticulum stress**

The endoplasmic reticulum (ER) is a key regulator of protein, lipid and cholesterol metabolism. It is the major site of protein synthesis, triglyceride and lipid droplet
formation, calcium storage and is also a major cellular sensor of nutrient status\textsuperscript{29}. When metabolic demands exceed the capacity of the ER, such as with nutrient excess, unfolded and misfolded proteins accumulate in the lumen; the signaling pathways initiated by the ER stress response is termed the unfolded protein response (UPR)\textsuperscript{30,31}. One factor contributing to ER stress is insufficient availability of the chaperone proteins involved in protein folding, such as binding immunoglobulin proteins (BiP), resulting in a backlog of unfolded proteins\textsuperscript{29}.

The ER contains three stress-sensing trans-membrane protein-mediated pathways: 1) PKR-like eukaryotic initiation factor 2α kinase (PERK), 2) inositol-requiring enzyme-1 (IRE-1) and 3) activating transcription factor 6 (ATF-6)\textsuperscript{29}. Both de novo proteins awaiting post-translational folding and the intraluminal domain of these three trans-membrane proteins are bound to a BiP\textsuperscript{29}. Excess protein synthesis reduces the availability of BiP for stress-sensing proteins, which results in their release from the ER membrane to initiate downstream signaling, endeavoring to restore homeostasis\textsuperscript{29}. The UPR mediates cell intrinsic stress responses by 1) PERK-mediated activation of antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and pro-inflammatory transcription factor NFκB, 2) IRE-1-mediated JNK activation, and 3) create electrophilic stress by producing excess reactive oxygen species (ROS)\textsuperscript{29,32}.

MAPKs participate in propagating stress signaling from the ER in various ways. When ER stress extends past the capacity to restore homeostasis, IRE-1 forms a complex with tumor necrosis factor receptor-associated factor (TRAF2) and apoptosis signal-regulating
kinase-1 (ASK1); it is this protein complex that activates JNK in the ER\textsuperscript{32}. JNK activation subsequently can directly suppress insulin signaling by serine phosphorylation of IRS-1\textsuperscript{33} and JNK activation in the ER is linked to the development of insulin resistance and T2D\textsuperscript{34}. Activation of PERK and IRE-1 pathways also activate NFκB by IKK activation mediated by p-38 or IκB degradation\textsuperscript{35}.

**Intermediate development of insulin resistance and increasing adiposity**

When a high fat diet continues beyond the short term, excess lipids are stored in enlarging adipocytes and fat mass in general begins to increase. Signs of adipose tissue inflammation develop rapidly in response to high lipid intake and precedes the development of inflammation in other metabolic tissues (skeletal muscle and liver)\textsuperscript{28,27,36}. The earliest signs of adipose tissue inflammation appear as an upregulation of proinflammatory genes (TNFα, IL-6, IL-8, MCP-1) apparent in both mature adipocytes and the stromal fraction of adipose tissue, containing preadipocytes, dendritic cells (resident macrophages) and other non-adipocyte components of adipose tissue\textsuperscript{28,27}. This is in contrast to obesity-linked inflammation, where proinflammatory gene expression is almost exclusively the domain of the stromal fraction\textsuperscript{37}.

Insulin resistance progressively worsens as high fat feeding continues and adiposity increases. Adipose tissue insulin resistance emerges prior to the development of reduced glucose uptake in skeletal muscle and liver, and concomitantly with the first appearance of increased reactive lipids\textsuperscript{26}. Under normal conditions, insulin stimulates glucose uptake and suppresses lipolysis in adipose tissue; insulin resistance in adipocytes is characterized
by reduced uptake of circulating fatty acids and increased lipolysis, resulting in increased serum free fatty acids.

**Free fatty acids, insulin resistance and toll-like receptors**

Elevated serum free fatty acids are characteristic of obesity, and a high fat diet alone increases adipose tissue lipolysis. Increased secretion of FFA into circulation from adipose tissue is a key event in the development of skeletal muscle insulin resistance, which accelerates the decline in whole body glucose homeostasis, and increasing local cell-to-cell pro-inflammatory signaling in adipose tissue.

It is well established that lipid infusion rapidly causes reduced insulin sensitivity in skeletal muscle, causing inflammatory stress and increased circulating TNFα. JNK and p-38 activation are increased in skeletal muscle in response to lipid infusion. Extracellular free fatty acid signaling through toll-like receptor (TLR) on the cell surface contributes to the initiation of inflammatory responses, in part by the activation of MAPKs and IKK. Lipid-induced insulin resistance in soleus muscle, mediated through TLR4, requires saturated fatty acid (SFA)-induced ceramide production.

The role of TLR is perhaps best understood as a pattern recognition receptor that propagates the innate immune response to bacterial lipopolysaccharide (LPS), however TLR also has a role in lipid-induced insulin resistance. TLR4 signaling is potently activated by circulating SFAs resulting in upregulation of IL-6 and TNFα; unsaturated fatty acids do not activate TLR4. In fact, n3 PUFA can completely abrogate TLR4
signaling. **TLR4** interference in mice protects against acute lipotoxicity and high fat diet-induced insulin resistance. **TLR4** knockout mice maintained greater insulin sensitivity and skeletal muscle insulin signaling than wild type mice when challenged with an 8-hour lipid infusion. They also demonstrated reduced JNK phosphorylation and IκBα degradation. Despite developing greater obesity than wild type mice, **TLR4** knockout mice on a HFD were more insulin sensitive and had reduced proinflammatory cytokine production in the liver, but especially in adipose tissue (IL-6, TNFα, MCP-1). Similarly, mice with a mutation in **TLR4** were more insulin sensitive and had more robust insulin signaling in liver, skeletal muscle and adipose tissue than did wild type mice fed a high fat diet, and did not experience high fat diet-induced increases in JNK or IκBα activation. Therefore, not all high fat diets will be expected to have the same potency to induce insulin resistance, depending on the fatty acid composition.

**Later development of insulin resistance and established adiposity**

In obesity, resident macrophages are induced to express a pro-inflammatory phenotype that causes the recruitment of infiltrating macrophages. A positive feedback loop mediated by FFA and TLR4 (which is overexpressed in obesity) increase adipose tissue inflammation: increased FFA release from adipocytes triggers TLR4 signaling both in adipocytes, as well as resident and infiltrating macrophages, which upregulates TNFα production by macrophages via MAPK activation, further exacerbating inflammation and insulin resistance in adipocytes. TNFα causes increased lipolysis in adipocytes, and interferes with the phosphorylation of insulin receptor and IRS-1. in a p-38 and IKK-
dependent manner. The macrophage population of adipose tissue can rise from 10% of total cells in lean conditions to 50% in obesity.

**Inflammation, insulin resistance and tumor necrosis factor-α**

Early studies showed that TNFα interference increased insulin sensitivity in obese rats. Obese women had 2.5 times more TNFα expression in abdominal subcutaneous adipose tissue despite very low or non-detectable plasma amounts; weight loss resulted in increased insulin sensitivity concomitant with reduced adipose TNFα expression. Wellen and Hotamisligil (2003) postulate that the source of serum TNFα is stressed adipocytes, whereas increasing tissue concentrations are due to secretions of TNFα from local activated macrophages that have a paracrine effect on surrounding cells. In ob/ob mice, obesity alone reduced insulin-stimulated tyrosine phosphorylation of insulin receptor by 70% in adipose tissue, 35% in skeletal muscle and 25% in liver. Knocking out TNFα restored insulin receptor activation to levels approaching lean controls in adipose tissue and skeletal muscle, but not liver, and protects against high fat diet-induced inflammation (systemic and adipose), elevated plasma free fatty acids and insulin resistance. In cultured murine adipocytes, TNFα increased the serine phosphorylation of IRS-1 and prolonged exposure reduced mRNA and protein levels of insulin receptor, IRS-1 and GLUT4. TNFα rapidly induces NFκB in adipocytes, along with a proinflammatory or macrophage-like set of genes associated with preadipocytes; TNFα also suppresses genes involved in normal metabolic function of mature adipocytes (GLUT4, HSL, LC-FACoA, PPARγ, C/EBPα) in an NFκB dependent manner. Long-term exposure reduces protein levels of GLUT4, insulin receptor, IRS-1 and PKB. The
main intracellular transducers of TNFR are IKK, JNK, p38 and ERK. Activated MAPKs can interfere with insulin signaling by the serine phosphorylation of IRS proteins and insulin receptor autophosphorylation directly, as well as contribute to the initiation of pro-inflammatory gene expression.

CONJUGATED LINOLEIC ACIDS

Origins of conjugated linoleic acids

Conjugated linoleic acid (CLA) is a group of geometric and positional isomers of linoleic acid (LA; 18:2 Δ9,12) where the two unsaturated carbon bonds are separated by a single carbon-carbon single bond. CLA cannot be produced by mammalian enzymes and so the only source of CLA for humans is dietary consumption of the meat, milk and derived products of ruminant animals. CLA is produced by bacteria in the rumen of herbivorous mammals when PUFAs are converted to saturated fatty acids by a process termed biohydrogenation. Some of the FA products of this process are absorbed by the animal before complete saturation and make up part of the fatty acid profile of meat and milk produced by these animals.

The three most abundant CLA isomers in milk and dairy products are the cis-9,trans-11, trans-7,cis-9, and trans-10,cis-12 isomers that make up approximately 76.5, 6.7 and 1.1% of total CLA, with other isomers present in trace amounts. The synthesis of CLA is mainly endogenous in ruminants with a small amount synthesized in the rumen, the trans-7,cis-9 isomer is exclusively endogenous, and CLA is exclusively synthesized by bacteria in the rumen. The total CLA content of milk increases with the
proportion of forage (high fiber content) in the diet of milk-producing ruminants. A high grain (high starch content) diet reduces rumen pH and may cause a condition known as milk fat depression (MFD) or if prolonged, subacute ruminal acidosis (SARA). Low rumen pH alters the fatty acid profile of milk and favors the production of CLA\text{t10,c12}. As CLA\text{t10,c12} makes up a higher proportion of the fatty acid profile, it is known to inhibit stearoyl-CoA desaturase (SCD), which depresses the endogenous synthesis of CLA\text{c9,t11} and CLA\text{t7,c9}, further increasing the proportion of CLA\text{t10,c12} in milk.

CLA can also be industrially produced by the alkali isomerization of linoleic acid resulting in an approximately 50:50 ratio of CLA\text{c9,t11} and CLA\text{t10,c12} (CLA\text{mix}). Due to their ease of production, CLA\text{c9,t11} and CLA\text{t10,c12} are the most widely studied CLA isomers, however CLA\text{mix} does not accurately represent naturally occurring ratios of CLA. The health effects of CLA are isomer specific. CLA\text{c9,t11} and CLA\text{t10,c12} often have opposing health effects in humans, and so studies using CLA\text{mix} without taking this into account tend to overemphasize the effects of CLA\text{t10,c12}.

**HEALTH EFFECTS OF CLA**

CLA (CLA\text{mix}) has successfully been marketed as a weight loss supplement due to the desirable changes in body composition seen in some animal studies that showed dramatic reductions of adipose tissue, sometimes concomitant with an increase in lean body mass. However, insulin resistance has also been observed as an outcome of CLA supplementation. Both these effects are linked to the CLA\text{t10,c12} isomer.
Effects of CLA on body composition and insulin resistance in at the whole body level in rodent models

A 1997 study was the first to demonstrate the beneficial effects of CLA$_{\text{mix}}$ on body composition; CLA-supplemented mice showed up to 60% less fat mass and up to 14% greater lean body mass$^{78}$. However, the dramatic reduction in adipose tissue seen in rodent studies appears to be lipodystrophic and occurs concurrently with an increase in insulin resistance. Additionally, the magnitude of the effect depends on the proportion of total dietary fat intake made up by CLA$_{10,\text{c12}}$.

In mice, high dose CLA-induced reduction in adiposity is due to lipodystrophy, resulting in the complete ablation of some adipose depots. Female mice consuming a diet composed of 10% kcal from fat and containing 0.1 g CLA/100 g diet for 5 months had 40% less WAT than controls, with no apparent effect on insulin sensitivity$^{79}$. Higher doses (1 g CLA/100 g diet) resulted in even greater loss of fat mass (76% retroperitoneal AT and 79% subcutaneous AT) and a dramatic reduction in insulin sensitivity after 9 weeks$^{79}$. When continued for 5 months, there was a complete ablation of BAT, and near ablation of WAT; only parametrial WAT was conserved despite a 27% reduction$^{80}$. This was due both to apoptosis – signs of which appeared as early as day 4 – as well as a reduction in the size of surviving adipocytes, which were 41% smaller after 5 months$^{80}$. This dose resulted in a nearly complete inhibition of GLUT4 mRNA (91%), along with a substantial upregulation of TNF$\alpha$ and UCP-2 in WAT, and an apparent compensatory upregulation of GLUT4 mRNA in skeletal muscle (1.5 fold upregulation in gastrocnemius)$^{80,79}$. Reduced GLUT4 expression in WAT and BAT was detectable by
day 4\textsuperscript{80}. These effects were accompanied by a modest deterioration in glucose tolerance, dramatic reduction in insulin sensitivity and elevated plasma insulin\textsuperscript{80}. In addition to the absolute dose of CLA, the proportion of CLA to total fat intake can mediate its effects. In a 10% fat diet, this dose of CLA (1 g CLA/100 g diet) makes up 25% of total fat, whereas in a 60% fat diet it represents only 3% of total fat and the effects are moderated\textsuperscript{79}. High-fat diet alone reduced GLUT4 expression in WAT by 62% as compared to mice fed a low fat diet, and high-dose CLA only reduced it a further 24%; TNF\(\alpha\) and UCP-2 expression were upregulated, but not as dramatically as when the diet contained 10% kcal from fat\textsuperscript{79}.  

**Molecular mechanisms of CLA action**

CLA\(_{t10,c12}\) may exacerbate the effects of a high fat diet on inflammation and insulin sensitivity via MAPK stress kinase signaling. In vitro, CLA can effect cell signaling in the short term and inflammation in the medium and long term.

CLA\(_{t10,c12}\) causes an increase in phospholipase C activity (PLC) which cleaves membrane phospholipids releasing a molecule of inositol 1,4,5-trisphosphate (IP\(3\)) and a diacylglyceride (DAG)\textsuperscript{81,82}. IP\(3\) binds to its receptor on the ER, causing calcium release\textsuperscript{81}; DAG is converted by diacylglyceride kinase (DAGK) into phosphatidic acid which also stimulates calcium release from the ER\textsuperscript{82}. Increased intracellular calcium occurs within 3 minutes of CLA\(_{t10,c12}\) treatment in primary human adipocytes\textsuperscript{81}. This effect is followed by increased expression of chaperone proteins (heat shock proteins) which suggests the UPR has been initiated by ER stress\textsuperscript{81}. ER stress ultimately activates MAPKs (ERK,
JNK, c-Jun) and increases expression of inflammatory genes, while decreasing the expression of genes involved in energy metabolism (PPARγ, GLUT4, ACC, SCD1)\textsuperscript{81,82}. This is followed by a reduction in insulin-stimulated glucose uptake\textsuperscript{81}. PLC inhibitors only partially attenuate these effects, which suggests CLA-induced inflammation is only partially mediated by PLC\textsuperscript{81}. DAGK inhibitors also attenuate intracellular calcium accumulation and changes in gene expression, reducing inflammatory markers and increasing insulin sensitivity\textsuperscript{82}. Additionally, the inhibition of intracellular calcium accumulation initiated by CLA\textsubscript{t10,c12} results in a blunted increase of ROS, inflammatory gene expression, and ERK and JNK activation\textsuperscript{83}. Calcium inhibitors block the binding of NFκB to the promoter regions of IL-8 and COX-2 that is upregulated by CLA\textsubscript{t10,c12} due to the release of cytosol-bound NFκB\textsuperscript{83}.

Microarray analysis of the effect of CLA\textsubscript{t10,c12}-treated adipocytes demonstrated that among the earliest effect of CLA\textsubscript{t10,c12} is to cause ER stress, which precedes other changes in inflammatory gene expression\textsuperscript{84}. Oversupply of nutrients in high fat feeding and obesity causes ER stress in metabolic tissues resulting in cellular stress due to MAPK activation and increasing reactive species\textsuperscript{34,29}. The combination of MAPK signaling and increasing electrophilic stress causes an upregulation first of antioxidant enzymes, and if electrophilic stress continues, of proinflammatory NFκB and AP-1 that initiate a broader proinflammatory response\textsuperscript{29}.

CLA\textsubscript{t10,c12} has other effects that are more directly linked to insulin sensitivity. PPARγ is a key transcription factor in the adipocyte phenotype regulating fatty acid and glucose
metabolism, which causes increased fatty acid uptake and adipogenesis. It is the molecular target of insulin sensitizing thiazolidinedione drugs used in the treatment of T2D. \(\text{CLA}_{10,12}\) indirectly inhibits PPAR\(\gamma\) expression in adipocytes and reduces adipogenesis via the activation of NF\(\kappa\)B, which increases IL-6 production responsible for PPAR\(\gamma\) suppression\(^8^5\). Additionally, \(\text{CLA}_{10,12}\) increased the serine phosphorylation of IRS-1 in the liver of rats fed a high fat diet for 8 weeks\(^8^6\).

\(\text{CLA}_{10,12}\) increases lipolysis and reduces fatty acid uptake into adipocytes. Reduced glucose and fatty acid uptake in adipocytes is dependent on ERK and NF\(\kappa\)B activation, as well as pro-inflammatory cytokines (IL-6, IL-8 TNF-\(\alpha\))\(^8^7\). In human adipocytes, \(\text{CLA}_{10,12}\) caused a strong and sustained activation of ERK, concomitant with increased IL-6 and IL-8 secretion, which preceded \(\text{CLA}_{10,12}\)-induced delipidation, decreased insulin-stimulated glucose uptake, fatty acid metabolism, and downregulation of PPAR\(\gamma\)\(^8^7\). \(\text{CLA}_{\text{mix}}\) reduces body fat by increasing lipolysis, and reduces lipid uptake by inhibiting lipoprotein lipase in adipocytes\(^8^8,8^9\).

**Health effects of CLA in human clinical trials**

In human clinical trials, \(\text{CLA}_{10,12}\) seems to result in a very small improvement of body composition in people with pre-existing obesity, usually limited to a small reduction in fat mass and often accompanied by a worsening of insulin resistance\(^9^0-9^4\). These effects seem to occur at relatively low doses, with no additional beneficial effects on body composition with increased amounts\(^9^0\). In fact, CLA is also associated with increased inflammation and so higher doses may not be advisable\(^9^5\). When normalized to body
weight, the doses used in human studies are far lower than those used in animal studies which produced dramatic results.

**Human dietary intake of CLA**

Food frequency questionnaires and food diaries can be used to estimate dietary intake of nutrients by calculating intake based on nutritional databases. Ritzenthaler et al. (2001) created actual food duplicates of CLA-containing items reported in food frequency questionnaires and food diaries from 93 adults in order to directly measure CLA content of food items to get a more accurate estimate of dietary CLA intake. They concluded that these types of estimates slightly underreport dietary intake of CLA and suggest that typical dietary intake is on average 212 mg/day for men, and 151 mg/day for women. This amounts to approximately 19 mg/day of CLA_{t10,c12} and 193 mg/day of CLA_{c9,t11} for men, and 11 mg/day CLA_{t10,c12} and 140 mg/day of CLA_{c9,t11} for women. The effective daily dose of CLA_{t10,c12} works out to 0.235 mg/kg for men and 0.177 mg/kg for women based on the average body weight reported in the study. This is far lower than amounts consumed by CLA supplementation and lower by orders of magnitude than the doses used in most animal studies.

**Effect of CLA_{t10,c12} in healthy adults**

The effects of CLA_{t10,c12} tend to be more pronounced in those who are already overweight or obese. Healthy postmenopausal women consuming either 2.2 or 0.4 g CLA_{t10,c12} per day had increased markers of inflammation and lipid peroxidation in the higher dose group. A study of healthy men receiving either CLA_{t10,c12} or CLA_{c9,t11} in
doses ranging from 0.5 to 2.5 g/day found no effect of either isomer on insulin sensitivity or resistance\textsuperscript{98}. Plasma triglycerides, cholesterol and low density lipoprotein were slightly elevated among men receiving CLA\textsubscript{t10,c12}, but the differences were very small, despite being statistically different\textsuperscript{98}. In healthy subjects, the effects of CLA were negligible, however the doses used were lower than those typically used in studies of pre-existing obesity.

\textit{Effect of CLA\textsubscript{t10,c12} in obese adults}

The effects of CLA are more pronounced in overweight and obese subjects than in healthy ones. Obese men presenting with metabolic syndrome receiving 3.4 g/day of CLA\textsubscript{t10,c12} experienced a 578\% increase in lipid peroxidation and 110\% increase in circulating C-reactive protein\textsuperscript{95}. Subjects of both genders who were overweight or obese receiving the same dose for 12 weeks had a small reduction in body fat mass, but no difference in lean body mass or plasma lipids\textsuperscript{90}. This study showed no added benefit on body composition of doses exceeding 3.4 g/day\textsuperscript{90}. Similar results were seen in obese men receiving 4.2 g/day CLA\textsubscript{mix}\textsuperscript{91}. These men experienced a small (~1\%) but statistically significant reduction in abdominal fat, with no other changes in body composition\textsuperscript{91}. CLA may also interfere with insulin sensitivity. Obese men were administered 3.4 g/day of either CLA\textsubscript{t10,c12} or CLA\textsubscript{mix}; hyperinsulemic-euglycemic clamp results showed that CLA\textsubscript{t10,c12} caused a 15\% reduction in insulin sensitivity, while the lower CLA\textsubscript{t10,c12} dose contained in CLA\textsubscript{mix} had no effect\textsuperscript{92}. CLA\textsubscript{t10,c12} treatment resulted in increased fasting glucose and circulating C-reactive protein, while CLA\textsubscript{mix} had no effect\textsuperscript{92}. CLA\textsubscript{t10,c12} also resulted in increased proinsulin – an indication of T2D and CVD risk – while the effect of
CLA\textsubscript{mix} was not significant\textsuperscript{92}. Thrush et al. (2007) found no change in body composition of overweight, non-diabetic subjects after 12 weeks consuming 4 g/day of CLA\textsubscript{mix}, however whole body glucose tolerance was decreased and ceramides accumulated in skeletal muscle (vastus lateralis)\textsuperscript{99}. Overall, a meta-analysis of 18 clinical trials concluded that 3.2 g CLA\textsubscript{mix} /day resulted in a 0.2 lb loss of body weight per week without any accompanying dietary changes\textsuperscript{93}. A separate meta-analysis similarly concluded that CLA supplementation also resulted in a small increase in lean body mass\textsuperscript{94}. 
Chapter Two: Introduction

Conjugated linoleic acid (CLA) is a group of geometric and positional isomers of linoleic acid (LA; 18:2 Δ9,12) where the two unsaturated carbon bonds are separated by a single carbon-carbon single bond. CLA cannot be produced by mammalian enzymes and so the only source of CLA for humans is dietary consumption of the meat, milk and derived products of ruminant animals. The most abundant, and well studied, CLA isomers are CLA\textsubscript{c9,t11} and CLA\textsubscript{t10,c12} which make up approximately 76.5% and 1.1% of total naturally occurring CLA, respectively. These isomers have different metabolic effects and CLA\textsubscript{t10,c12} has been shown to reduce fat mass in rodents and humans, as well as increase insulin resistance and inflammation.

In order to increase productivity, Canadian dairy cows are fed a high grain, low forage ration, that can sometimes produce a condition known as subacute ruminal acidosis (SARA). SARA changes the rumen pH resulting in increased production of the CLA\textsubscript{t10,c12} isomer. In light of the evidence that high CLA\textsubscript{t10,c12} intake can cause insulin resistance and inflammation, we endeavored to discover whether there was a health risk to consumers relating to changes in insulin sensitivity or inflammation associated with elevated CLA\textsubscript{t10,c12} in dairy products. To that end, a 60% fat rodent diet was produced, composed entirely of butter manufactured from milk collected from dairy cows experiencing SARA. This butter represents the most CLA\textsubscript{t10,c12}-enriched source of naturally occurring dietary fat for humans.
Using an established model of high fat diet-induced insulin resistance, female Sprague-Dawley rats were fed either a low (10%) or high (60%) fat diet for 8 weeks. SARA butter was compared against a commercially available butter and a custom non-SARA butter was included to account for any variability arising from the production of the custom SARA butter. The 5 treatment groups in this study included the 10% low fat diet (LFD; negative control) and 60% high fat diets (HFD): lard-based diet (LARD; positive control), CLA\textsubscript{t10,c12}–enriched butter (SARA), non-SARA butter (NonSARA), and commercial butter (COM). The effects of the diet on whole body glucose homeostasis and inflammation, indicated by MAPK activation, were evaluated after 8 weeks.

We hypothesized that rodents consuming a high fat diet for 8 weeks would gain fat mass. Subsequently, we expected that all high fat diets would result in increased insulin resistance as compared to low fat-fed controls, and that of the butter-based diets, the SARA group would show the greatest deviation from normal whole body glucose homeostasis. We also expected to see indication of cellular or tissue inflammation in the high fat-fed groups; assuming that adipose tissue would be the initiator of inflammation, we expected to see the greatest degree of MAPK activation in adipose tissue, followed by skeletal muscle and liver depending on how far the high fat diet-induced pathology had developed\textsuperscript{105}. 
Chapter Three: Methods

DIET PREPARATION

In order to evaluate the potential risk of high dietary levels of $\text{CLA}_{10c,12c}$, a custom butter was produced at the University of Guelph containing the maximum possible natural amount of this isomer. SARA was induced in dairy cows with a high grain, low forage diet, and the milk harvested was used to produce a high $\text{CLA}_{10c,12c}$ butter (SARA) as well as a control butter produced from the milk of healthy cows (NonSARA). The NonSARA group was included in the study to account for any potential differences between custom and commercially produced butters. A popular local consumer brand of butter was purchased commercially (COM; Gaylea Brand unsalted, Ontario, Canada).

Five rodent diets were used in the study (Table 1). A negative control low fat diet (LFD) containing 10% kcal from fat (5.5% soybean oil and 4.5% lard) and a positive control high fat diet (LARD) containing 60% kcal from fat (5.5% soybean oil and 54.5% lard) were obtained from Research Diets, New Jersey, USA (D12450B, D12492). A premix identical in composition to the LARD excluding lard (D12492px) was used to blend custom butter-based diets with 60% kcal from fat (5.5% soybean oil and 54.5% butter). Due to the higher water content of butter as compared to lard, the amount of butter added to make up 60% kcal from fat was based on the analysis of custom butters (fat content 83.2% ± 0.45). Butter diets were prepared by blending 295 g of butter with 528.8 g of diet premix in a food mixer (Hobart Canada, Ontario, Canada).
Table 1: Diet compositions

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<th>LFD kcal</th>
<th>Butter g</th>
<th>Butter kcal</th>
<th>HFD g</th>
<th>HFD kcal</th>
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kcal/g

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% kcal from fat

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ANIMAL, HOUSING & EXPERIMENTAL GROUPS

Twenty five female Sprague Dawley rats (mean body weight 137.6 g ± 1.337) were obtained from Charles River (Quebec, Canada) and individually housed in a 12-hour reversed light/dark cycle with free access to water and a standard chow diet for a 1 week acclimation period. Female Sprague-Dawley rats were selected to stay consistent with previous work on the effects of SARA butter (unpublished). Rats were randomly assigned to one of five dietary treatment groups (LFD, COM, NonSARA, SARA or LARD) and received food and water ad libitum for 8 weeks (Figure 3). Body weight and food intake were recorded several times per week for the experimental period. All ethical protocols as outlined by the Animal Care Committee in compliance with the guidelines outlined by the Canadian Council on Animal Care at the University of Guelph were followed.
Figure 3: Experimental design of the current study

WHOLE BODY INSULIN SENSITIVITY TESTS

During the final week, whole body glucose homeostasis was evaluated with three types of tolerance tests to evaluate systemic glucose tolerance, insulin sensitivity and liver-specific insulin sensitivity. Glucose, insulin and pyruvate tolerance tests were performed in the same week with a day of rest between each test.

Glucose tolerance test

Glucose tolerance testing (GTT) measures insulin resistance by estimating the rate of systemic glucose disposal. Equalized glucose doses are administered by injection into the intraperitoneal body cavity and blood glucose levels are subsequently measured at
established time points. Under normal conditions, the glucose bolus results in a brief
spike in blood glucose levels that are quickly returned to normal. Glucose intolerance is
evidenced by a reduced capacity to clear the glucose bolus, which demonstrates a degree
of insulin resistance or alternatively insulin insufficiency.

A 20% D-glucose solution was prepared in pure H2O. Rats were fasted for 6 hours and
basal blood glucose was determined with blood from the tail tip using standard glucose
strips and glucometer (OneTouch Ultra 2, LifeScan Inc., Milpitas, CA, USA). A glucose
bolus (2 g glucose/kg body weight) was administered by intraperitoneal injection and
blood glucose levels were evaluated at 15, 30, 45, 60, 90 and 120 minutes post injection.

**Insulin tolerance test**

Insulin tolerance testing (ITT) measures insulin sensitivity by evaluating the magnitude
of response to an equalized insulin bolus administered as described above. Under normal
conditions of insulin responsiveness, blood glucose drops quickly to a negative peak and
is slowly normalized as metabolic glucose homeostasis is restored. When sensitivity to
insulin is reduced, the drop in blood glucose is lessened and the return to basal glucose
levels more rapid, and suggests insulin resistance.

Stock insulin solution was diluted in saline to obtain a 0.025U/100μl solution. Basal
blood glucose was determined in non-fasted rats with blood from the tail tip as described
above. Blood glucose levels were determined at 10, 20, 30, 45, 60, 90 and 120 minutes
after an intraperitoneal insulin injection (0.75 U insulin/kg body weight).
**Pyruvate tolerance test**

Pyruvate tolerance testing (PTT) evaluates the efficiency of hepatic glucose output. Equalized pyruvate doses are administered and blood glucose evaluated as described above. Under normal conditions of insulin sensitivity, pyruvate – a substrate for hepatic gluconeogenesis – stimulates a rise in blood glucose corresponding to an increase in hepatic gluconeogenesis. The rise in blood glucose would be expected to stimulate insulin secretion to restore glucose homeostasis.

A 40% pyruvate solution in saline was prepared and the pH was adjusted to 7.35. Rats were fasted for 6 hours and basal blood glucose was determined as described above. A pyruvate bolus (2 g pyruvate/kg body weight) was administered by intraperitoneal injection and blood glucose levels were evaluated at 15, 30, 45, 60, 90 and 120 minutes post injection.

Group means were compared using the area under the curve (AUC) taken from baseline blood glucose (mmol/L) for each animal; the ITT used the AUC below baseline blood glucose. Additionally, each time point was compared within each tolerance test to clarify at which point the group means diverged, and in order to more clearly display the variability and significance.

**TISSUE HARVESTING**

Prior to surgeries, animals were fasted for 6 hours and anesthetized with sodium pentobarbital (1 mg/kg). Two skeletal muscles were extracted to represent potential
differences in metabolism between fiber types (type I slow twitch soleus muscle and type II fast twitch tricep muscle); two types of adipose tissue were harvested to evaluate potential differences between depots (gonadal adipose tissue is more highly correlated with insulin resistance than subcutaneous adipose tissue) and frozen immediately in liquid nitrogen and stored at -80°C awaiting subsequent analysis. The liver was also removed and frozen immediately in liquid nitrogen; samples from the left lobe were used in subsequent Western blot analysis.

**WESTERN BLOT ANALYSIS**

All tissues were prepared for Western blotting from whole cell lysates. Protein was extracted from tissue subsamples by submerging in an ice-cold cell lysis buffer supplemented with protease inhibitors to stabilize proteins and their phosphorylation states. Samples were homogenized for 2 1-minute cycles using a Fast Prep® tissue homogenizer (MP Biomedicals, LLP, Quebec, Canada). Samples were centrifuged at 15,000 rpm for 10 minutes and the supernatant removed by pipette, avoiding the surface lipid layer. This was repeated twice to minimize lipid contamination of the samples. The protein concentration of the samples was determined spectrophotometrically on a 96-well plate using a BSA assay. Protein content of samples were equalized in the preparation of samples for Western blotting by diluting samples to the same concentration with cell lysis buffer; final samples were prepared with a 1:1 of sample + cell lysis buffer to LPS solution. Proteins were separated by electrophoresis on 1.5 mm 10% SDS-PAGE acrylamide gels (1.5 hours at 100-140 mV). Protein bands were transferred onto nitrocellulose membranes in a methanol-containing transfer buffer at 4°C (1.5 hours at
0.2 amps). Equal loading was verified with nonspecific protein staining with Ponceau-S stain (Sigma Aldrich, Missouri, USA). Membranes were incubated for 1 hour in a 5 % skim milk powder solution in a Tris-buffered solution with Tween (TBST) to block non-specific protein binding, and then incubated with primary antibodies (~1:1000 dilution in a 5% BSA TBST solution) overnight at 4°C (SAPK/JNK, P-SAPK/JNK, ERK1/2, P-ERK1/2, p38 MAPK, P-p38 MAPK; Cell Signaling, Massachusetts, USA). Membranes were washed in TBST to remove excess primary antibody, incubated 1 hour with a horseradish-peroxidase-linked secondary anti-rabbit antibody at room temperature, and subsequently rewashed in TBST followed by TBS in preparation for imaging. Membranes were briefly incubated with an electrochemiluminescence solution (Western Lightning with ECL, Perkin Elmer, Massachusetts, USA) and detected via chemiluminescence and quantified via densitometry with an imaging system.

**STATISTICAL AND OTHER ANALYSIS**

Statistical analysis used in this study compared means with one-way ANOVA to determine group effects of diet treatments\textsuperscript{106}. The Dunnett post-hoc test was used compare treatment groups against control group (LFD)\textsuperscript{106}. All statistical analysis was calculated using Prism 5.0 software (GraphPad Software Inc. 2008, San Diego, USA). Two-tailed, unpaired Student’s t-test statistical analysis was used to compare blood glucose values between COM and NonSARA tolerance tests\textsuperscript{106}. Statistical significance was accepted at \( p \leq 0.05 \).

The equation used to calculate percent change is as follows:
\[
\frac{\text{final value} - \text{initial value}}{\text{initial value}} \times 100
\]

The equation used to normalize food intake to body size (Figure 7) is as follows:

\[
\frac{\text{mean daily food intake (g)} \times \text{kcal of the diet (kcal/g)}}{\text{mean body weight (g)}}
\]
Chapter 4: Results

RODENT DIETS

The fatty acid profile of butters can be seen in Table 2. There were no substantial differences in the fatty acid profile between COM and NonSARA butters. Notably, COM butter contained twice the amount of $\text{CLA}_{10,12c}$ than butter produced from verifiably healthy cows (NonSARA), which may be due to the current incidence rate of SARA in the commercial dairy industry (0.008 g/100 g NonSARA and 0.016 g/100 g COM). SARA butter exhibited the characteristic fatty acid type increases; 12-fold more $\text{CLA}_{10,12c}$ and 14-fold more trans-10 18:1 from NonSARA butter. This shift in the fatty acid profile is consistent with the literature, but interestingly, SARA butter also contained more than twice the amount of $\text{CLA}_{e9,t11}$ than NonSARA butter. Commercial butter contained twice as much $\text{CLA}_{10,12c}$ as NonSARA butter (0.016 g/100 g and 0.008 g/100g, respectively), and SARA butter contained nearly 10 times more (0.097 g/100 g $\text{CLA}_{10,12c}$). SARA butter also contained more total CLA than both COM and NonSARA butters (1.44, 0.62 and 0.55 g/100 g, respectively).
Table 2: Fatty acid profile comparison of butters (g/100 g)

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<tr>
<th>Fatty acid</th>
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<th></th>
<th>SARA</th>
<th></th>
<th>COM</th>
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<td>0.0120</td>
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<td></td>
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<td></td>
<td>COM</td>
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BODY WEIGHT AND FOOD CONSUMPTION

Following 8 weeks of high-fat feeding, the growth curves were similar between groups (Figure 4); there was no statistical difference between the group means of final body weight ($p = 0.1938$), although the LFD group was the lowest (Figure 4 & 5). The LFD group had the highest mean daily food intake by weight (Figure 6), but when the total caloric value of mean daily food intake was normalized to body weight, all groups consumed nearly identical calories relative to their body size (Figure 7).
Figure 4: Growth curve of rats fed either low or high fat diets for 8 weeks
Figure 5: Final body weight of rats fed either low or high fat diets for 8 weeks
Figure 6: Mean daily food intake of rodents fed either a low or high fat diet for 8 weeks
Figure 7: Mean daily food intake in kcal of rats fed either low or high fat diets for 8 weeks.
WHOLE BODY GLUCOSE TOLERANCE

There was no significant difference in the area under the curve (AUC) between groups for any of the tolerance tests, therefore tables have been included for each time point of each tolerance test in order to more clearly express the variability and significance of the differences between groups. Despite the lack of statistical significance, there was a consistent trend across all the tolerance tests. The lard-based diet resulted in the greatest deviation of whole body insulin sensitivity relative to the low fat diet; of the butter-fed groups, the SARA group demonstrated the greatest deviation in whole body insulin sensitivity relative to the low fat diet (Figure 8, 10 &12).

Glucose tolerance test results

ANOVA statistical analysis did not uncover a statistical difference between groups either by comparing AUCs ($p = 0.5041$) or at any individual time point of the GTT (Figure 9, Table 3). The total AUC was 1.9%, 24.4%, 62.2% and 69.9% greater than LFD for COM, NonSARA, SARA and LARD, respectively (Figure 9). Peak blood glucose measured 15 minutes after glucose injection was 12.8%, 16.9%, 28.6% and 47.9% greater than LFD for COM, NonSARA, SARA and LARD, respectively (see Table 3 for values).
Figure 8: Glucose tolerance test results for rats fed either a low or high fat diet for 8 weeks.
Figure 9: Total area under the curve for glucose tolerance test
Table 3: Blood glucose values (mmol/L) at each time point of a glucose tolerance test

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</tbody>
</table>

Statistically significant results are indicated with a * (p-value < 0.05)
**Insulin tolerance test results**

ANOVA statistical analysis did not uncover a statistical difference between groups mean AUC ($p = 0.3682$) (Figure 10 & 11). The total AUC was 21.7%, 31.9%, 35.2% and 45.3% lower than LFD for COM, NonSARA, SARA and LARD, respectively (Figure 11). However when evaluated at individual time points, the LARD group was significantly different from LFD at the 10, 20, 30, 45 and 120-minute time points; SARA differed significantly from LFD only at the 10-minute time point and NonSARA differed significantly from LFD only at the 20-minute time point (Table 4). Peak drop in blood glucose measured 20 minutes after glucose injection was 21.6%, 41.0%, 29.8% and 52.3% greater than LFD for COM, NonSARA, SARA and LARD, respectively (see Table 4 for values).
Figure 10: Insulin tolerance test results for rats fed either a low or high fat diet for 8 weeks
Figure 11: Total area under the curve for insulin tolerance test
Table 4: Blood glucose values (mmol/L) of each time point of an insulin tolerance test

<table>
<thead>
<tr>
<th>Time</th>
<th>LFD</th>
<th>COM</th>
<th>NonSARA</th>
<th>SARA</th>
<th>LARD</th>
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</thead>
<tbody>
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<td>Mean</td>
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</table>

Statistically significant results are indicated with a ‘*’ (p-value < 0.05)
**Pyruvate tolerance test results**

ANOVA statistical analysis of the group mean AUC for the PTT uncovered a statistical difference \( (p = 0.0466) \) only between LFD and LARD groups using the Dunnett post-hoc test (Figure 12 & 13), however this effect was due to large responses of two individuals in the LARD group (AUC for LARD group were 54.8, 69.8, 136.1, 514.5 and 515.0).

The PTT response curves were consistent across all subjects with the exception of these two subjects from the LARD group (see Appendix B). The total AUC was 0.9%, 16.5%, 25.7% and 302.7% greater than LFD for COM, NonSARA, SARA and LARD, respectively (Figure 13). Peak blood glucose measured 15 minutes after glucose injection was 0.3% lower than LFD for COM, and 1.8%, 4.6% and 25.8% greater than LFD for NonSARA, SARA and LARD, respectively (see Table 5 for values).

Statistically significant differences appeared only between LFD and LARD groups at the 30 and 45-minute time points (Table 5).
Figure 12: Pyruvate tolerance test for rats fed either a low or high fat diet for 8 weeks
Figure 13: Total area under the curve for pyruvate tolerance test
Table 5: Blood glucose values (mmol/L) of each time point of a pyruvate tolerance test

<table>
<thead>
<tr>
<th>Time</th>
<th>LFD Mean</th>
<th>LFD SE</th>
<th>COM Mean</th>
<th>COM SE</th>
<th>NonSARA Mean</th>
<th>NonSARA SE</th>
<th>SARA Mean</th>
<th>SARA SE</th>
<th>LARD Mean</th>
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<td>0.8171</td>
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Statistically significant results are indicated with a '*' (p-value < 0.05)

Table 6: Results of unpaired Student's t-test comparison of blood glucose values between commercial butter and NonSARA butter-fed rodents

<table>
<thead>
<tr>
<th>Tolerance test</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>GTT</td>
<td>0.6610</td>
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<tr>
<td>ITT</td>
<td>0.2881</td>
</tr>
<tr>
<td>PTT</td>
<td>0.8380</td>
</tr>
</tbody>
</table>
MOLECULAR MARKERS OF CELL STRESS AND INFLAMMATION

There was no substantial activation of any of the MAP kinase pathways that were evaluated (ERK, JNK, p38) by any of the high fat diets in this study (Figures 14-18). In gonadal adipose tissue, there was a statistically significant increase in the phosphorylation of ERK (2.8 fold relative to LFD) and no detectable phosphorylation of p38 (Figure 14). Whereas in subcutaneous adipose tissue, p-38 activation was greater in all the high fat groups, but only the SARA group reached statistical significance (5.8 fold increase relative to LFD) (Figure 15). In skeletal muscle, there was a 2-fold increase in p38 activation in the tricep muscles of the LARD group, and 3.1 and 3.3-fold increase in COM and SARA groups, respectively (Figure 16 & 17). There was no detectible activation of JNK in either skeletal muscle or liver (Figures 16-18).
Figure 14: Markers of inflammation in gonadal adipose tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a ‘*’. 
Figure 15: Markers of inflammation in the subcutaneous adipose tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a ‘**’. 
Figure 16: Markers of inflammation in the tricep muscle tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a ‘**’.
Figure 17: Markers of inflammation in the soleus muscle tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a ‘*’.
Figure 18: Markers of inflammation in the liver tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a **.
Chapter Five: Discussion

This study was conducted to evaluate potential negative health effects relating to insulin sensitivity and inflammation of naturally occurring dietary CLA\textsubscript{t10,c12}, given the studies showing that supplemental doses of this isomer has a negative impact on insulin resistance\textsuperscript{90-94}. To that end, we produced a 60\% high fat diet with the fat component composed entirely of butter produced from milk collected from dairy cows suffering from SARA – a condition that can sometimes result from the dairy industry production practice of feeding a high grain, low forage diet ration to milk-producing cows\textsuperscript{101-103}. This butter represents the most CLA\textsubscript{t10,c12}-enriched source of naturally occurring dietary fat for humans. We compared the effects of the high CLA\textsubscript{t10,c12} butter against a commercially available butter, and included a custom non-SARA butter as control against differences that may arise from custom versus commercially produced butter products.

This study did not produce statistically significant differences in the AUC between any treatment groups in the evaluation of whole body glucose homeostasis using glucose, insulin and pyruvate tolerance tests. The single exception was a statistically significant \((p < 0.05)\) effect of the LARD diet in the pyruvate tolerance test due to an exceptionally strong response by two of the five subjects in that group (AUC for the lard group were 54.8, 69.8, 136.1, 514.5 and 515.0). However, this experiment had a small sample size (\(n=5\) per group) and 5 treatment groups, which reduced the power of the statistics produced by this experiment\textsuperscript{106}. Despite the low power of the statistics and their inconclusive nature, there were some consistent trends across the tolerance tests.
At the end of 8 weeks, all rats were euglycemic. After 8 weeks of high-fat feeding, there was a consistent trend across all the tolerance tests. The lard-based diet resulted in the greatest deviation of whole body insulin sensitivity relative to the low fat diet in each of the glucose (Figure 8), insulin (Figure 10) and pyruvate tolerance tests (Figure 12). That the lard-based diet had the greatest impact on whole body glucose homeostasis was expected, and served as the positive control in this experiment. The small sample size (n=5 per group) and large number of treatment groups rendered any comparison to the other treatment groups inconclusive. Despite this fact, there was another trend among the butter-based diets that was consistent across all three tolerance tests. The SARA group demonstrated the most glucose intolerance in the GTT (Figure 8), the least insulin sensitivity in the ITT (Figure 9), and had the greatest hepatic glucose production in the PTT (Figure 10) of the butter-fed groups; however, these results only reached statistical significance at the single time point of the ITT where there was the greatest drop in blood glucose (Table 4). No statistical differences were demonstrated in whole body glucose homeostasis between commercial and non-SARA butter in any of the tolerance tests (Table 6). Due to the low power of the statistics resulting from group mean analysis, a Student’s t-test was performed between the commercial and NonSARA butters to more clearly analyze the difference between these groups, since the NonSARA group was included as a control against potential variability due to custom, small-batch butters (Table 6). There were no significant differences between the blood glucose values of the COM and NonSARA groups in any tolerance test ($p=0.6610$ for the GTT, $p=0.2881$ for the ITT, $p=0.830$ for the PTT). In future studies, it may be possible to eliminate the non-
SARA group. All groups had nearly identical food intake (Figure 4) and there was no significant difference in the final mean body weight of the groups, though the LFD group had the lowest final body weight (Figure 2). Caloric intake and fat mass are unlikely to be responsible for the effects on whole body glucose homeostasis seen in high fat-fed rodents. Though the statistics in this experiment were inconclusive, the consistent trends across all three tolerance tests alludes to the possible existence of a treatment effect that may have the potential to be more robust with a larger sample size.

Generally, a 60% high fat diet composed mainly of lard is a commonly used model, and will typically produce systemic and tissue-specific insulin resistance after 8 weeks. In the current study, not even the positive control (LARD) produced the expected degree of insulin resistance. This is likely due to the small sample size (n=5) used in this experiment, gender may have a confounding effect as well. Most studies examining high fat diet-induced insulin resistance use male rats, although the Sprague-Dawley strain is commonly used in metabolic studies. In fact, a replicate of this study failed to produce any difference in body weight or whole body glucose homeostasis between any group (including LFD), even after 22 weeks of high fat feeding (See Appendix A).

MAPKs are thought to be centrally involved in the signaling pathways that mediate interference with insulin signaling. MAPKs - and JNK in particular - can directly interfere with insulin signaling by reducing the activity of IRS-1 via serine phosphorylation, and can be involved in upregulating pro-inflammatory genes that can negatively impact insulin sensitivity. MAPK activation may therefore indicate
cell intrinsic stress involved in early insulin resistance development (ER stress), 2) the potential inflammatory nature of the fatty acid profile of the diet by mediating signaling through TLR4, and 3) the intensity of tissue inflammation by transducing pro-inflammatory cytokines commonly involved in insulin resistance such as TNFα. We examined MAPK quantity and activation in five metabolic tissues: gonadal and subcutaneous adipose tissue, tricep and soleus muscle, and in the liver. We expected to see the greatest activation of MAPKs in adipose tissue, anticipating that both cell intrinsic metabolic stress and inflammation would originate there. However, we saw limited evidence for the involvement of MAPKs in any of the five tissues we examined. The mechanism underlying the modest insulin resistance induced by high fat feeding in this study is unclear, based on the measurements that were taken.

High dairy intake has been associated with a reduced risk for T2D\textsuperscript{108,109} without increasing the risk of cardiovascular disease\textsuperscript{110}; however, a meta-analysis concluded that low fat dairy was more effective than high fat dairy consumption\textsuperscript{111}. A recent study demonstrated that high consumption of low fat dairy improved fasting insulin by 9% and insulin resistance by 11% in healthy obese individuals\textsuperscript{112}. Few studies focus on butter as the sole source of dietary fat. One interesting study demonstrated that healthy men consuming a high fat, butter-based diet supplemented with 5.5 g/day \text{CLA}_\text{mix} showed elevated markers of lipid peroxidation compared to butter alone, but no significant differences in fasting insulin, glucose or insulin resistance; however, there was no non-butter control used in this study\textsuperscript{113}. 

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Dairy and butter fat are complex mixtures, and which components impact insulin sensitivity and metabolism remains unknown. The simple addition of milk to the diet in a high sucrose-induced model of insulin resistance was sufficient to improve the insulin sensitivity of rats\textsuperscript{114}. There are other components of butter fat that have a marked effect on insulin resistance and inflammation. The supplementation of butyric acid (a short-chain fatty acid found in milk fat) in the diet of obese, high fat-fed mice improved insulin sensitivity (reduced fasting glucose, insulin and improved insulin tolerance) as well as increased thermogenesis, fatty acid oxidation, mitochondrial function and biogenesis in skeletal muscle\textsuperscript{115}. Butyrate attenuated proinflammatory signaling (reduced activation of MAPKs and activity of NF\textsuperscript{k}B) and the production of proinflammatory cytokines (TNF\textalpha, MCP-1, IL-6) in adipocytes co-cultured with macrophages\textsuperscript{116}. These mechanisms were mediated by the butyrate-induced suppression of lipolysis by down-regulating triglyceride lipase, hormone-sensitive lipase and fatty acid binding protein-4 in adipocytes\textsuperscript{116}. Additionally, butyrate enhanced the production of anti-inflammatory cytokines (IL-10 and IL-4) and reduced proinflammatory IL-2 and IFN-\gamma in stimulated human peripheral blood monocytes\textsuperscript{117}.

In conclusion, the outcome of this study does not suggest an increased risk of insulin resistance in healthy rodents from the higher than normal CLA\textsubscript{t10,c12} levels that may result from dairy industry production practices. The decreasing impact on insulin resistance of CLA\textsubscript{t10,c12} as the dose used makes up a smaller proportion of total dietary fat\textsuperscript{79,80}, and the modest effects on insulin resistance of pharmaceutical doses in human clinical trials\textsuperscript{90-94}, indicate that slightly elevated CLA\textsubscript{t10,c12} content of dairy products are unlikely to pose a
health risk to healthy individuals. However, clinical trials show that the effects of CLA_{10,12} are more potent in those already overweight or obese than in lean individuals\textsuperscript{93,94,97,98}. Therefore it may be prudent to evaluate the effects of SARA butter in a model of established obesity, or for high risk groups such as those with existing insulin resistance or type 2 diabetes.
Chapter Six: Integrative Discussion

The purpose of the study was to evaluate potential negative health effects relating to insulin sensitivity and inflammation of consuming dairy products with increased CLA\textsubscript{t10,c12} content by evaluating whole body glucose homeostasis and MAPK activation. Elevated CLA\textsubscript{t10,c12} content of dairy products can result from production practices that increase the incidence of SARA in milk-producing dairy cows. This study was able to evaluate the general question of the hypothesis, but also had some significant shortcomings that should be accounted for in future studies of this type.

Aside from the small sample size (n=5), the main shortcoming in this study was that the positive control group did not produce the anticipated results, which complicates the interpretation of the lack of MAPK activation. In theory, MAPK activation should be able to capture changes in cell metabolism occurring at various points from earlier to later stages of high fat diet-induced insulin resistance: ER stress, FFA signaling through TLR4, pro-inflammatory cytokine signaling and inflammation. Further analysis would be required to determine which factors were responsible for MAPK activation, however due to the lack of MAPK activation detected in the current study, no further analysis was carried out.

High fat feeding is a widely used model to induce insulin resistance in metabolism studies; the effects of a high fat diet develop rapidly\textsuperscript{118,119}. Deterioration of glucose
tolerance in mice is measurable by the third day of a high fat diet\textsuperscript{27,26,118,119}; hepatic insulin resistance appears as early as one week and skeletal muscle insulin resistance as early as three weeks after the initiation of a high fat diet varying from 45% to 60% kcal from fat\textsuperscript{26,27,119,120-122}. In humans, a high fat diet composed of 45% of kcal from fat reduced insulin sensitivity by 11% in less than one month\textsuperscript{123}; a 60% fat diet resulted in elevated fasting glucose and reduced suppression of hepatic gluconeogenesis after only 5 days\textsuperscript{124}. In the current study, the positive control (LARD) diet did not produce the degree of whole body insulin resistance that was expected after eight weeks of high fat feeding.

Males are represented more frequently than females as the animal and human subjects in metabolism studies, although the Sprague-Dawley strain of rat is commonly used\textsuperscript{125,107}. The blunted effects of the LARD diet on whole body glucose homeostasis in the current study may be due to gender differences in fat storage and adipose tissue metabolism. In humans, as in rats, females generally have a greater fat mass as a percent of total body weight than males\textsuperscript{126,127}. Females may have metabolic adaptations to manage higher adiposity that makes them less susceptible to small perturbations of adipose tissue dysfunction\textsuperscript{128-130}. As a result, females may have a higher threshold of tolerance to high fat intake and/or increasing adiposity before the negative impacts become apparent\textsuperscript{128-130}. Since the current study was looking at relatively short-term effects, there may not have been sufficient time to allow significant metabolic dysfunction to develop. Additionally, the pattern of fat storage is different between genders. Females tend to store excess fat in subcutaneous adipose tissue (SAT) depots, whereas males tend to store excess fat in visceral adipose tissue (VAT)\textsuperscript{127,131}. Abdominal adiposity, and VAT in particular, is
more highly correlated with T2D risk and the development of insulin resistance than any other adipose tissue depot\textsuperscript{3,127}. These results highlight the importance of acknowledging the sex differences in health studies. Applying knowledge gained from health studies focused on a single gender may not serve the entire population. In order to obtain results more comparable to the body of scientific literature, it may be helpful to repeat this study using male rats – if indeed gender is the variable that accounts for the discrepancy in the current study.

Two alternate modifications of this study could produce clearer results in the future. Firstly, a larger sample size would be useful in order to evaluate molecular mechanisms of involved in the very early stages of insulin resistance. For instance, tissue harvesting could be divided into 2 groups: one control group sacrificed in a fasted state and a treatment group that received an insulin injection prior to sacrifice. This design would allow for comparisons in endogenous insulin signaling by examining targets in the phosphorylation cascade that transduces the effects of extracellular insulin, such as the activation of IRS-1 or downstream targets including PKB, AS160 or GLUT4 translocation. Such a design would allow for the evaluation of any tissue type chosen (adipose depots, different skeletal muscles, liver, cardiac muscle). Alternatively, muscle strip explants or cultured adipose tissue explants from each treatment group could be employed for similar purposes.

Additional measurements need to be taken in future studies to identify the cause of MAPK activation once the model has been adjusted to produce insulin resistance and
increasing adiposity. For instance, IRE-1 activation or intracellular calcium levels could indicate ER stress. A measure of plasma cytokines, adipokines, and free fatty acids would also be useful in interpreting potential causes of MAPK activation, as would TLR and TNFR activation.

Although the results of this study do not suggest that high dietary intake of CLA_{10,12} pose a health risk to healthy individuals, further study may be warranted. Clinical trials show that the effects of CLA_{10,12} are more potent in those already overweight or obese than in lean individuals. Therefore it may be prudent to evaluate the effects of SARA butter in a model of established obesity, or for high-risk groups such as those with existing insulin resistance or type 2 diabetes.
References


6. Tuomilehto J: Type 2 diabetes is a preventable disease: Lifestyle is the key. Journal of Medical Sciences 2010, 3:82–86


Febbraio MA, Bruce CR: Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding. Diabetologia 2013, 56:1638–1648


44. Davis JE: Development of obesity-induced inflammation and insulin resistance: The role of adipose tissue, fatty acids, and toll-like receptors. 2008, 1–171


61. Stephens JM, Lee J, Pilch PF: Tumor Necrosis Factor-alpha -induced Insulin Resistance in 3T3-L1 Adipocytes Is Accompanied by a Loss of Insulin Receptor Substrate-1 and GLUT4 Expression without a Loss of Insulin Receptor-mediated Signal


76. de Carvalho EBT, de Melo ILP, Mancini-Filho J: Chemical and physiological aspects of isomers of conjugated fatty acids. Ciência e Tecnologia de Alimentos 2010, 30:295–307


85. Chung S, Brown JM, Provo JN, Hopkins R, McIntosh M: Conjugated Linoleic Acid Promotes Human Adipocyte Insulin Resistance through NFkB-dependent Cytokine


Appendix A

An identical replicate of the feeding trial in this study was performed but the time was extended. Twenty two weeks consuming a high fat diet did not produce any significant difference in the final body weight or growth curves between any of the 5 treatment groups (Figure xix). In fact, the group consuming the low-fat diet (10% kcal from fat) was the second largest by body weight after 22 weeks. Additionally, there was no difference in whole body glucose homeostasis between groups (data not shown).

However, there is precedent for these results in the literature evaluating the sex differences in fat metabolism in rodents. A recent study showed in a direct comparison of male and female Sprague-Dawley rats consuming a high fat diet that female rats are resistant to high fat diet-induced obesity as compared to males\textsuperscript{129}. Additionally, female rats did not respond to the anti-obesity treatment in the study that reduced adiposity in male rats\textsuperscript{129}. This suggests differential fat metabolism between genders in their response to high fat diets.

There may be other genetic factors that may be important to consider if a study endeavors to focus on the metabolic mechanisms involved in mediating the effects of a high fat diet rather than evaluate the effects of a high fat diet on a population level. For instance, it has been observed previously that up to 50% of Sprague-Dawley rats are resistant to diet-induced obesity regardless of gender\textsuperscript{132}, and small genetic differences within the same inbred rodent strains can have significant impacts on metabolism\textsuperscript{133,134}. Sprague-Dawley
rats respond differently to exercise-induced weight loss depending on whether they were resistant to the effects of high fat feeding\textsuperscript{135}. Dourmashkin et al. (2006) have developed a model for the prediction of which Sprague-Dawley rats are likely to develop obesity to account for this issue; early weight gain on a high fat diet is correlated with an obesity prone phenotype, including increased lipoprotein lipase activity in adipose tissue, but reduced activity in skeletal muscle, promoting fat storage over oxidation\textsuperscript{136}. In the current study, such considerations may not have been relevant, since the goal was to evaluate the effect of SARA butter on a population, rather than the specific metabolic actions of CLA\textsubscript{10,c12}, however it would be important to sort out responders and non-responders to a treatment in order to isolate the mechanisms at play. Indeed a comparison of the different effects of a treatment between responders and non-responders would likely offer valuable insight into the relevant mechanisms for effective treatment.
Figure xix: Growth curve of female Sprague-Dawley rats fed either a low or high fat diet for 22 weeks.
Appendix B

Figure xx: Individual AUC for the pyruvate tolerance test of twenty five female Sprague-Dawley rats