THE EFFECT OF THE ANTI-INFLAMMATORY DRUG SODIUM SALICYLATE IN MATURE PERIPARTURIENT DAIRY CATTLE AND IMMORTALIZED BOVINE MAMMARY EPITHEILAL (MAC-T) CELLS

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Abstract

During the transition period, 3 wk before to 3 wk after calving, dairy cows experience a variety of sudden hormonal and metabolic shifts that could result in metabolic disorders or diseases, which can be detrimental to the productive life and longevity of the cow. Cows undergo a negative energy balance, where they cannot consume enough feed to meet their energy requirements. To make up this deficit, cows mobilize adipose tissue in the form of non-esterified fatty acids (NEFA) which are transported to the liver and are either used for fuel or stored as triglycerides. High levels of circulating NEFA can lead to endoplasmic reticulum (ER) stress, which is linked to inflammation. This low-grade inflammation can compromise cell function. To mitigate this inflammation, sodium salicylate, a non-steroidal anti-inflammatory drug (NSAID), was given to mature (3+ parity) cows for 7 d after parturition via their drinking water. Blood was collected daily and a glucose turnover assay was performed. Liver, muscle, and adipose tissue was collected on d 7. Overall, it appeared that SS increased insulin sensitivity and depressed gluconeogenesis post-transcriptionally. Multiple *in vitro* studies were performed on immortalized bovine mammary epithelium (MAC-T) cells to determine the action of SS when ER stress was induced with palmitate (PALM). Treatment with SS did not mitigate, and in some cases exacerbated, the ER stress response. The addition of bovine serum albumin (BSA), a common component of cell culture media, may alter reactive oxygen species (ROS) measurements due to its antioxidant property. Overall, SS seems to alter metabolic processes and the cellular response to stress.

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Chapter 1 - Literature Review

The transition period is one of the most problematic periods that a dairy cow will ever undergo. During this period, defined as 3 wk before to 3 wk after calving (Gummer 1995), the cow experiences endocrine changes due to calving and metabolic changes due to a sudden increase in energy demand to synthesize milk, as well as being switched to a high-energy lactating cow diet (Drackley, 1999; Ingvartsen, 2006; Mulligan and Doherty, 2008). These sudden and drastic changes can result in disorders or diseases that can be detrimental to the productive life and longevity of the cow. Goff (2008) stated that cows need a strong immune system, normal calcium levels, and must suffer no significant decreases in feed intake to have a successful transition period. If these three physiological statuses can be maintained, a cow can have a relatively smooth transition period. Likewise, a failure of any one of these 3 aspects can put a cow at risk for succumbing to 1 or many disorders.

The profitability of a cow during her lactation is determined by how successful her transition period is (Drackley, 1999). According to Leblanc (2010), it is estimated that 30-50% of dairy cows will experience a metabolic disorder or infection during the transition period. Transitional disorders can cost between $224 and $494 per cow per case due to milk loss, veterinary fees, cost of treatment, extra labor to tend to the sick cow, and delays in rebreeding (Guard, 2008). The high prevalence coupled with the high cost of treatment for transition disorders has made it imperative to identify methods to mitigate these disorders. This review will discuss metabolic challenges that cows undergo and their link to systemic inflammation, and the use of sodium salicylate, a non-steroidal anti-inflammatory drug, as a possible method to moderate this chronic inflammation as a method for increasing transition period success.

Metabolic Changes and Transition Disorders

During the 3 weeks postpartum, cows are at a high risk of succumbing to 1 or more disorders related to the metabolic shifts that cow is experiencing. The most common disorders include ketosis, milk fever, fatty liver, rumen acidosis, displaced abomasum, metritis, and mastitis (Ingvartsen, 2006; Mulligan and Doherty, 2008). These disorders are all interconnected, so that a cow suffering from one disorder has a higher chance of succumbing to another disorder.

Milk fever is known as a 'gateway disease' due to its high correlation to other metabolic disorders (Mulligan and Doherty, 2008), while fatty liver is considered a secondary disease of other disorders that inhibit feed intake (Ingvartsen, 2006).

Cows experience a large increase in energy demand after parturition due to the onset of lactation. Often, the cow is unable to consume enough energy to meet these demands and enters a state known as negative energy balance (NEB). This NEB can be exacerbated since cows endure an almost 20% decrease in voluntary DMI around parturition as well as undergoing a decrease in plasma glucose concentrations (Grummer, 1995). To compensate for the energy shortage, the cow will mobilize fatty acids from adipose tissue. These fatty acids are released into plasma as non-esterified fatty acids (NEFA). In the liver, NEFA can be taken up and completely or partially oxidized to provide energy to the animal or be converted to triglycerides (TG). Since the majority of the circulating glucose is being diverted to the mammary gland, the energy that these fat-derived fuels supply is crucial for meeting the energy demand of the rest of the body.

Partial oxidation of NEFA results in the creation of ketone bodies, which are released from the liver and used by peripheral tissues as a source of energy. Ketosis occurs when there are relatively high levels of ketones in the blood coupled with low circulating glucose (Ingvartsen, 2006). In the United States, 2-20% of cows experience clinical ketosis during the first month of lactation, while the incidence of those undergoing subclinical ketosis is as high as 34% (Ingvartsen, 2006). Over-conditioned cows that have large adipose tissue deposits are at a much higher risk of ketosis, as are cows that suffered ketosis in a previous lactation (Bobe et al., 2004; Ingvartsen, 2006).

When the uptake of NEFA into the liver exceeds the amount of NEFA that the liver can completely or partially oxidize, NEFA will be converted to triglycerides. These triglycerides will accumulate in the liver and, in large quantities, can impair hepatic function and may hinder glucose synthesis (Bobe et al., 2004). This accumulation of TG in the liver results in what is known as fatty liver syndrome and nearly 50% of cows experience some degree of this during the transition period. Obese cows, which have large stores of adipose tissue, are at a much higher risk for developing fatty liver because they have an increased mobilization of NEFA from adipose tissue and greater depression of feed intake at calving. This can lead to a more severe negative energy balance and demand even more NEFA to be mobilized (Bobe et al., 2004).

Fatty liver and ketosis are highly correlated, meaning that a cow with fatty liver is at a much higher risk of ketosis and cows suffering from ketosis are at a higher risk of having a fatty liver (Bobe et al., 2004). Fatty liver can compromise immune function by hindering the release of inflammatory mediators such as leukocytes, lymphocytes, and neutrophils and increases circulating proinflammatory cytokines (Bobe et al., 2004). This can render a cow unable to fight off infection such as mastitis.

There are a plethora of other physiological shifts that occur at parturition that have the potential to induce a transition disorder. Insulin resistance occurs when cells are unable to respond to the normal actions of insulin. High levels of circulating NEFA have been linked to insulin resistance, indicating that transition period cows may be undergoing a state of insulin resistance (Hayirli, 2006). Reducing the effectiveness of insulin seems to facilitate the funneling of available glucose to the mammary gland to be used as energy for milk synthesis (Giesy et al., 2012). Approximately 85% of the total glucose supply is diverted to the mammary gland (Sordillo et al., 2009).

Cows may also experience hypocalcemia and, in severe cases, milk fever due to the mobilization of calcium to meet the demands for milk production (Leblanc, 2010). On top of coping with these drastic metabolic changes, cows experience immunosuppression (Goff, 2008; Leblanc, 2010). There can be a 25-40% decline in neutrophil and lymphocyte function and if a cow suffers from a metabolic disease, this can increase to as high as 60-80% loss of immune function (Goff, 2008).

At the onset of lactation, there is a struggle to meet the energy demand of lactation during a period of immunosuppression and a failure to meet this demand can result in metabolic disorders that will exacerbate the cow's already weakened immune system. These challenges predispose her to other disorders that can have large negative economic impacts, hinder whole-lactation productivity, and reduce cow longevity in the herd.

Systemic Inflammation and its Relationship to the Transition Cow

The dairy cow possesses a robust immune system that helps restore order after being invaded by pathogens or experiencing harmful stimuli. While the immune system fights whatever stimuli activated a response, it also has to control the extent of the response so as to not damage surrounding tissue and cells. The immune system is comprised of innate and acquired

immunity. Innate immunity provides the initial, non-specific response to a pathogen. Components that are involved in the innate response include leukocytes, cytokines, eicosanoids, and non-immune cells such as epithelial and endothelial cells (Sordillo et al., 2009). Acquired immunity is a more specific response that is elicited when an infectious agent successfully evades the innate immune response. If the animal encounters that pathogen again, immunological memory will render a heightened state of immune activity that is faster and stronger than what occurred during the first encounter (Janeway et al., 2005).

Inflammation can be classified as either acute or chronic. Acute inflammation is characterized by vasodilation, release of eicosanoids and cytokines, and clinical symptoms that include redness, swelling, heat, and pain at the site of infection. This acute inflammation is a positive response since it quickly eliminates the offending pathogen and does not usually result in the damage of nearby tissues and cells. Conversely, chronic or low-grade inflammation can be detrimental to the normal function of the animal and damage tissues and cells. This inflammation is initiated by excess or severe shortage of nutrients and has been dubbed by some as meta-inflammation (Hotamisligil, 2006).

Meta-inflammation in the Transition Dairy Cow

The regulatory pathways of immune and metabolic functions of an organism are closely related. This could be because the functional units that control the immune and metabolic functions came from common ancestral structures (Hotamisligil, 2006). In fact, the metabolic and immune cells are close to each other in the liver and in adipose depots (Hotamisligil, 2006). Chronic inflammation was first linked with obesity and type 2 diabetes with the discovery that tumor necrosis factor-α (TNFα) was overexpressed in obese mice (Hotamisligil et al., 1993). TNFα is a proinflammatory cytokine that also promotes insulin resistance. This illustrated that an inflammatory response can alter metabolic function, in this case insulin action, during obesity.

Transition cows possess characteristics similar to type-2 diabetes, including a state of low-grade inflammation right after parturition (Humblet et al., 2006), mobilization of fat stores, and elevated NEFA levels (Contreras and Sordillo, 2011). As previously alluded to, obese cows are at a higher risk of contracting a metabolic disorder because of the elevated NEFA levels. This is similar in humans, where obese people have a much higher chance of succumbing to type 2 diabetes (Hotamisligil, 2010).

Lipids and the Inflammatory Response

Though the true mechanism for how lipids cause an inflammatory response is not known, there are several possible pathways by which lipids could initiate a response. When NEFA enter the cell and are not used for energy, they can be transported to the ER or the Golgi apparatus where they can attach to proteins and be used in the cell membrane (Contreras and Sordillo, 2011). Once in the cell, fatty acids can bind to receptors and regulate gene expression. One example of this is the ability of lipids to regulate peroxisome proliferator-activated receptors (PPAR). PPAR modulate the inflammatory response in many cells such as adipocytes (Contreras and Sordillo, 2011). Another example of lipids influencing receptor binding is the activation of Toll-like receptors (TLR), especially TLR4. TLR4 activation can lead to an inflammatory response as well as an upregulation of proinflammatory cytokines (Sordillo et al., 2009; Contreras and Sordillo, 2011; Baker et al., 2011).

Another way lipids can orchestrate an inflammatory response is through the biosynthesis of lipid mediators such as spingolipids, ceramides, and eicosanoids (Hannun and Obeid, 2008; Sordillo et al., 2009). Eicosanoids are regulators of inflammatory response whose precursors include thromboxanes, prostacyclins and prostaglandins. These precursors come from the polyunsaturated fatty acids (PUFA) that are metabolized either through the cyclooxygenase (COX) or lipooxygenase (LOX) pathways (Sordillo et al., 2009). There are two isoforms of COX; COX1 is expressed in most tissues and synthesize low levels of prostaglandins and COX2 is associated with the biosynthesis of proinflammatory mediators (Amann and Peskar, 2002; Sordillo et al., 2009; Contreras and Sordillo, 2011).

A third way lipids can initiate an inflammatory response is by inducing endoplasmic reticulum (ER) stress. The ER is responsible for many activities in the cell including protein folding and storage of calcium. ER stress can be caused by myriad physiological actions including high levels of fatty acids, much like what occurs in a transition cow. ER stress leads to a protein folding deficit, where the protein folding capacity of the ER is at maximum capacity or the folding process is interrupted and cannot meet the demands of the cell. This can cause an accumulation of unfolded or misfolded proteins to accumulate in the cell (Zhang and Kaufman, 2008; Hotamisligil 2010). In order to cope with this stress, the accumulation of these misfolded proteins initiates the unfolded protein response (UPR). The main objective of the UPR is cell survival; the UPR works to restore the ER to homeostasis. In the event that the UPR fails to

return the ER to homeostasis, the UPR will initiate apoptosis to protect the host from the large amount of misfolded proteins (Zhang and Kaufman, 2008; Hotamisligil, 2010; Tabas and Ron, 2011).

The UPR cascade has three starting points, which include inositol-requiring 1α (IRE1), double-stranded RNA-dependent protein kinase (PKR)- like ER kinase (PERK), and activating transcription factor 6 (ATF6; Zhang and Kaufman, 2008). During normal ER function, these sensors are inactive due to the attachment of immunoglobulin-heavy-chain-binding protein (BiP or GRP78). BiP has a high affinity for misfolded and unfolded proteins, so when there is a large build-up of these, BiP will detach from the three arms and attach to the misfolded proteins, thus activating the UPR (Hotamisligil, 2010).

Once BiP is detached, PERK is phosphorylated, allowing it to phosphorylate eukaryotic initiation factor 2α (eIF2α). This phosphorylation inhibits the synthesis of new proteins in an attempt to slow down the buildup of the misfolded and unfolded proteins (Zhang and Kaufman, 2008). The phosphorylation of eIF2α also allows the translation of activating transcription factor 4 (ATF4), which then enters the nucleus of the cell and upregluates target genes involved with amino acid biosynthesis, oxidative stress response and apoptosis (Hotamisligil, 2010).

When the IRE1 branch is activated, IRE1 auto-phosphorylates. IRE1 then splices X-box-binding protein-1 (XBP-1) into its active form, referred to here as XBP-1s. XBP-1s is a key factor for transcription during ER stress. XBP-1s travels to the nucleus where it induces transcription of genes that code for ER chaperones and enzymes involved with protein folding, maturation, and export (Zhang and Kaufman, 2008). ATF6 follows a similar pathway to IRE1 when activated. ATF6 migrates to the Golgi apparatus and is cleaved by site-1 protease (S1P) and S2P. The remaining fragment of ATF6 then goes to the nucleus and follows the same pathway as XBP-1s (Hotamisligil, 2010).

If these three branches of the UPR are unable to revert the ER back to homeostasis through the alteration of cell transcription, the UPR will then initiate apoptosis, which is mainly mediated by CCAAT/enhancer-binding protein homologous protein (CHOP). Initiation of apoptosis is a defense mechanism to protect the host from cells that are functionally compromised. Cells that are exposed to severe and chronic stress signals, such as inflammatory cytokines and high fatty acid or glucose levels, are often subjected to apoptosis.

The UPR is linked with the nuclear factor-κB (NF-κB) and JUN N-terminal kinase (JNK) inflammatory pathways. The branches of UPR can directly initiate an inflammatory response or act indirectly through accumulation of reactive oxygen species (ROS). As previously mentioned, the ER stores a large amount of calcium. When there is an accumulation of misfolded and unfolded proteins, calcium begins to leak out of the ER and into the mitochondria. Calcium disrupts normal functions by depolarizing the mitochondrial membrane and disrupts the electron transport chain (Zhang and Kaufman, 2008). This leads to an increase of ROS production, and a subsequent onset of oxidative stress. High levels of ROS will initiate the NF-κB and JNK pathways since ROS are important mediators in these pathways.

When at rest, NF-κB is bound by inhibitor of κB (IκB), which does not allow NF-κB to leave the cytosol (Baker et al., 2011). The NF-κB pathway can be initiated either by the UPR response, high levels of inflammatory cytokines, or TLR activation. Upon initiation, the inhibitor of κB kinase (IKK) complex is activated and will phosphorylate IκB molecules, thereby releasing NF-κB (Zhang and Kaufman, 2008). Once freed, NF-κB will upregulate specific genes that are involved with cell proliferation and release cytokines that will activate an immune response (Baker et al., 2011).

The JNK pathway is largely initiated by the IRE1 branch of the UPR response. Once IRE1 is activated, it recruits TNF-α-receptor-associated factor 2 (TRAF2), which then recruits and activates JNK (Zhang and Kaufman, 2008). Once activated, JNK phosphorylates serine residues in insulin-receptor substrate 1 (IRS1). This inhibits the phosphorylation of IRS-1 tyrosine residues, which limit IRS-1 function and can lead to insulin resistance (Zhang and Kaufman, 2008). Ozcan and colleagues (2004) demonstrated this when they induced ER stress in mouse hepatocytes. The liver cells that were stressed had increased levels of serine-phosphorylated IRS-1. When cells were treated with a synthetic inhibitor of JNK, serine-phosphorylation of IRS-1 was reversed.

Systemic Inflammation Involvement in Transition Disorders

There are many ways in which different transition cow disorders can initiate an inflammatory response. The infectious diseases, such as metritis and mastitis, directly initiate an acute response to combat the invading pathogens. Elevated levels of lipolysis in the adipose tissue will increase the circulating NEFA concentration. High levels of fatty acids can induce ER stress, which in turn can initiate the NF-κB and JNK inflammatory pathways and lead to

systemic inflammation and insulin resistance. High amounts of fatty acids can also activate TLR receptors and COX pathways, leading to a release of proinflammatory cytokines, which can also initiate ER stress and exacerbate inflammation.

During the transition period, cows can be experiencing symptoms similar to type-2 diabetes due to the hallmark insulin resistance, high concentrations of circulating NEFA, and resulting chronic inflammation. Unlike type 2 diabetes however, cows also experience low blood glucose (Grummer, 1995). This inflammation will produce inflammatory cytokines, such as TNFα, which can then in turn cause ER stress, creating a vicious cycle that can easily spin out of control and damage the host cells through excessive production of ROS, as well as the cytokines themselves doing damage when they are circulating in large quantities.

Use of Non-Steroidal Anti-Inflammatory Drugs to Mitigate Systemic Inflammation

There are many management schemes to mitigate adipose mobilization to limit NEFA release that can be employed on dairy farms. These range from using weight management of dry cows, to a gradual step up from a low energy to a high energy diet to a blanket treatment of all fresh cows with glucose precursors for a few days after calving. All of these have varying degrees of success depending of the farm and implementation of the strategy.

Given that researchers have discovered chronic inflammation plays a role in metabolic disturbances, a different approach to achieving a smooth transition period could be mitigating the inflammation itself. If proinflammatory cytokine production could be limited, there would be less stimulation of ER stress and, in turn, less ROS production and less cell damage regardless of the amount of circulating NEFA. This may also alleviate metabolic dysfunction since inflammation has been linked to insulin resistance and to compromised cell function.

Non-steroidal anti-inflammatory drugs (NSAIDs) can be used to block endogenous inflammation. NSAIDs are classified into groups based on their chemical structure or mode of action. The drugs discussed in this review are aspirin and sodium salicylate (SS), which are found in the salicylate group of NSAIDs.

Actions of Sodium Salicylate

Aspirin and SS, though in the same class, vary slightly in structure and function. Aspirin contains an acetyl group, and when consumed, 50% of aspirin is almost immediately de-

acetylated to salicylic acid (Amann and Peskar, 2002). Aspirin is sometimes termed as a 'bifunctional drug' because the acetyl group and the salicylic acid impact different pathways, essentially acting like two drugs in one (Rainsford, 2004). In contrast, SS is a salt form of salicylic acid and does not contain an acetyl group.

Sodium salicylate has a myriad of effects including being anti-inflammatory, antipyretic, and an analgesic. There has been a wide variety of research done with SS to determine its mechanistic actions; however, there are many conflicting reports (Amann and Peskar, 2002).

COX-1 and COX-2 Inhibition

Arachidonic acid is converted to prostaglandin by COX enzymes (Amann and Peskar, 2002). Arachidonic acid is derived from dietary sources of linoleic acid (C18:2; Rainsford, 2004). Prostaglandins are precursors to eicosanoids, which are involved in the inflammatory response. Aspirin is an effective COX inhibitor because it carries an acetyl group that is released after ingestion and binds to a serine at the active site of the COX enzyme (Roth et al., 1975; Vane, 1994). This blocks the active site, impeding the binding of arachidonic acid, and therefore irreversibly inhibiting prostaglandin synthesis and the subsequent inflammatory response (Amann and Peskar, 2002). Sodium salicylate does not possess the acetyl group required to block the active site on the COX enzyme, making SS a weak COX inhibitor (Kopp and Ghosh, 1994). This was confirmed by Smith and others (1975), who found that aspirin, but not SS, reduced the rat foot swelling induced by carrageenan when arachidonic acid (an inflammatory precursor via the COX pathway) was concurrently administered. The anti-inflammatory effects of SS must therefore be due to SS altering a different inflammatory pathway.

Aspirin and SS, after being consumed, are converted to salicylic acid after the loss of either the acetyl or sodium group. It is believed that salicylic acid is responsible for the majority of the anti-inflammatory effects exhibited by both drugs. Whittle and colleagues (1980) demonstrated that aspirin and SS have similar anti-inflammatory potencies by indicating that the dose to reduce carrageenan-induced rat paw edema by 50% (ED_{50}) for aspirin was 130 mg/kg and 140 mg/kg for SS. Since the only thing aspirin and SS have in common structurally is the salicylic acid, this finding supports the belief that salicylic acid is responsible for the anti-inflammatory effects demonstrated by these drugs.

Uncoupling of Oxidative Phosphorylation

Salicylate, but not aspirin, has been shown to activate adenosine monophosphate-activated protein kinase (AMPK) in human embryonic kidney cells (Hawley et al., 2012). When activated, AMPK switches off adenosine triphosphate (ATP) consuming processes and turns on ATP-generating pathways (catabolism). Activation of AMPK usually occurs when ATP synthesis in the mitochondria is inhibited, which leads to increased levels of AMP and ADP (Smith and Smith, 1966; Hawley et al., 2012). There is evidence that salicylate impairs the integrity and respiration function of mitochondria; therefore, it is possible that SS may also alter mitochondrial ATP synthesis by altering oxidative phosphorylation (Chung et al, 2003; Battaglia et al., 2005). Cronstein and colleagues (1994) demonstrated that SS diminishes intracellular ATP in human umbilical vein endothelial cells (HUVEC). This decrease in ATP formation then induced the release of adenosine into the extracellular fluid. Cronstein and others (1999) speculated that uncoupling of oxidative phosphorylation could enhance ATP catabolism, thus releasing adenine nucleotides which are converted to adenosine extracellularly. Adenosine is an anti-inflammatory agent, so it is thought that this adenosine pathway could be responsible, at least in part, for the anti-inflammatory effects exhibited by SS (Amann and Peskar, 2002).

Inhibition of the NF-κB Pathway

As reported previously, NF-κB is an important transcription factor involved with the inflammatory response. In its inactive state, NF-κB resides in the cytoplasm bound to IκB. Activation of NF-κB occurs when IKK, made up of IKK-α and IKK-β, phosphorylate IκB, which then releases NF-κB.

Kopp and Ghosh (1994) found that SS inhibited NF-κB activation. Further investigation revealed that SS prevented the release of NF-κB from IκB by interfering with the pathway involved with the phosphorylation of IκB. Yin and colleagues (1998) further defined the inhibition mechanism by finding that SS specifically targets and irreversibly binds to IKK-β, but not IKK-α. Therefore, SS inhibits the NF- κB pathway by blocking the ability of IKK to phosphorylate IκB, thereby inhibiting the release of NF-κB.

Link Between Inflammation and Insulin Resistance

Insulin resistance plays a major role in the development of many metabolic health disorders including obesity, type 2 diabetes, hypertension, and cardiovascular disease (Kim et al., 2001). Insulin resistance is when tissue becomes less sensitive to insulin and fails to respond as

it normally would in the presence of insulin. Though the mechanisms that lead to insulin resistance are unknown, a link has been established between high levels of circulating free fatty acids and insulin resistance (Boden, 1997). It is believed that elevated levels of plasma fatty acids interfere with the insulin signaling in muscle by inhibiting phosphorylation of insulin receptor substrate (IRS) proteins, more specifically IRS-1 (Morino et al., 2006; Park et al., 2007). Hepatic insulin resistance is believed to be linked to fatty acids reducing the insulin-stimulated tyrosine phosphorylation of IRS-2 (Morino et al., 2006).

Low-grade, or meta-inflammation, is a hallmark of type 2 diabetes and other metabolic disorders (Hotasmisligil, 2006). High levels of circulating fatty acids can initiate the UPR, which leads to the activation of inflammatory pathways including the NF- κB pathway. It thus stands to reason that inflammation may have a part in insulin resistance. This link between low-grade inflammation and insulin resistance has been confirmed (Xu et al., 2003). Arkan and others (2005) performed a study where they fed a high fat/diabetes-inducing diet to a set of mice that lacked IKK-β enzymes in the hepatocytes and a set of mice lacking the enzymes in myeloid cells. Mice that lacked IKK-β enzymes in the hepatocytes were protected from hepatic insulin resistance but still suffered from muscle and adipose insulin resistance. Mice that lacked IKK- β in the myeloid cells were systemically protected from insulin resistance. These results suggest that the NF-κB pathway is directly involved with fat-induced insulin resistance.

Since salicylate inhibits inflammation through blocking IKK-β, salicylates have been evaluated as a potential therapeutic agent for type 2 diabetes. There have been many reports that indicated that aspirin and SS prevent insulin resistance in obese or diabetic rats, mice, and humans (Kim et al., 2001; Yuan et al., 2001; Hundal et al., 2002; Park et al., 2007). In addition to increased insulin sensitivity, Hundal and colleagues (2002) observed a 22% decrease in hepatic glucose production and a 24% decrease in fasting plasma glucose in human type 2 diabetic patients after being treated for 2 weeks with 6.2 g/d of aspirin. This could partially be caused by an increased uptake of glucose by peripheral tissues due to the increase of insulin sensitivity.

Use of Sodium Salicylate in Transition Dairy Cattle

As stated previously, dairy cows undergo myriad metabolic changes around the time of parturition including an increase in circulating free fatty acids, low-grade inflammation, and

insulin resistance. Some transition disorders and decreased milk production have been linked to these alterations, making the transition period an important economic period for the dairy farmer. By limiting the extent of inflammation and insulin resistance, there may be the potential to stop a metabolic transition disorder.

Some research has been done in regard to using SS for its analgesic properties in calves during dehorning and castration, but little has been done to investigate the use of SS in the transition period. Some work with other NSAIDS, such as ketoprofen and meloxicam (neither of which is in the salicylate family) have been done in transition dairy cows but the focus of these studies was on alleviation of pain associated with dystocia and the subsequent impact on milk production and reproduction (Richards et al., 2009; Newby et al., 2013).

Flunixin meglumine, a member of the fenamate NSAID family, has been tested in transition cows. Shwartz and others (2009) found that treating transition cows with flunixin for the first 3 d after calving had no effect on milk yield or plasma glucose levels, but did depress feed intake.

In a previous study, SS was administered via water (1.95 g/L) to cows for 7 days after calving (Farney et al., 2013a, 2013b). Treatment with SS had no effect on feed or water intake but decreased the pro-inflammatory cytokine TNF-α in plasma, indicating that SS did suppress inflammation. Insulin sensitivity was increased in SS treated cows (measured via the RQUICKI equation) and mature (lactation 3+) cows experienced hypoglycemia on d 7. This hypoglycemia was not accompanied by a decrease in mRNA expression of hepatic gluconeogenic genes, indicating that the increased insulin sensitivity could potentially be increasing the uptake of glucose by peripheral tissues. Treated cows also experienced a 'rebound' increase in plasma NEFA and BHBA after they were removed from treatment. Cows on treatment also experienced a greater degree of negative energy balance than the control cows. Even though the SS cows seemed to be more metabolically distressed, they had greater milk production than the control cows. In fact, 3+ lactation SS cows had a 21% higher 305-d milk yield than the control cows; however, the mechanism underlying this is unknown. It is possible that SS may alter mammary gland function that in turn increased whole-lactation milk production. This study has left many questions as to the exact mode of action of SS on glucose metabolism in ruminants as well as its impact on mammary gland function.

Conclusions

Transition dairy cows undergo many metabolic changes similar to that of type 2 diabetes including increased plasma fatty acids, meta-inflammation, and insulin resistance. Fatty acids can cause an inflammatory response, which has been linked to fat-induced insulin resistance. Many approaches have been taken in transition dairy cattle to reduce the level of circulating NEFA but another potential approach to decrease the amount of metabolic disorders is to mitigate inflammation. One potential way to limit inflammation is to use NSAIDs, such as sodium salicylate. There are still many unanswered questions as to the mode of action of SS on glucose metabolism and its ability to increase whole-lactation milk production in mature cows.

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Chapter 2 - Sodium Salicylate Decreases Glucose Turnover Rate in Periparturient Dairy Cows, Likely Through Enhanced Liver Insulin Sensitivity

Abstract

Low-grade inflammation has been implicated as a contributor to metabolic disease during the transition to lactation. In previous work, administration of sodium salicylate (SS) for 7 d led to hypoglycemia in mature dairy cows in early lactation. The purpose of this study was to identify the mode of action underlying this response to SS. Twenty mature (3+ parity) cows were assigned alternately at time of calving to either control (CON) or SS treatments. CON treatment received a molasses carrier in drinking water while the SS received 2.5 g/L SS with the molasses carrier in drinking water for 7 d after parturition. Blood samples were collected daily. A glucose turnover assay was performed on d 7, followed by liver, muscle, and adipose tissue biopsies. Results were analyzed in the MIXED procedure of SAS with significance declared at $P < 0.05$. There were no treatment effects on DMI ($P = 0.98$) or water intake ($P = 0.61$). Tumor necrosis factor alpha (TNFa) mRNA expression was decreased by SS in adipose tissue ($P = 0.09$) but not in muscle ($P = 0.97$) or liver ($P = 0.52$). Plasma haptoglobin was not altered by treatment ($P = 0.34$). Though treatment did not alter plasma glucose or insulin concentrations, RQUICKI, a measure of insulin sensitivity, and plasma glucagon tended to be increased by SS ($P = 0.08$). Insulin:glucagon was increased by SS ($P = 0.01$). Cows on SS had a 25% decrease in glucose turnover rate ($P = 0.05$) compared to control cows. There were no differences in mRNA expression of gluconeogenic genes in liver or of GLUT4 transporters in any of the tissues. These results indicate that SS may increase insulin sensitivity in mature fresh cows. This increase in sensitivity of insulin could explain the lower glucose turnover rate because of increased post-transcriptional inhibition of gluconeogenesis by insulin during SS treatment.

Introduction

Dairy cows undergo a variety of physiological changes during the transition period, 3 wk before to 3 wk after calving (Gummer 1995). These changes include endocrine changes due to calving and metabolic changes due to a sudden increase in energy demand to synthesize milk, as well as being switched to a high-energy lactation diet (Drackley, 1999; Ingvartsen, 2006; Mulligan and Doherty, 2008). Transition cows also exhibit symptoms similar to type-2 diabetes including mobilization of fat stores, elevated plasma non-esterified fatty acid (NEFA) levels (Contreras and Sordillo, 2011), and low-grade inflammation (Humblet et al., 2006) following parturition.

Both high levels of circulating plasma NEFAs and chronic, low-grade inflammation have been linked to insulin resistance in humans and lab animals. Insulin resistance, the decreased sensitivity of tissue to the presence of insulin, plays a major role in the development of many metabolic health disorders including obesity, type 2 diabetes, hypertension, and cardiovascular disease (Kim et al., 2001). It is believed that elevated levels of plasma fatty acids interfere with the insulin signaling in muscle through inhibiting phosphorylation of insulin receptor substrate (IRS) proteins, particularly IRS-1 (Morino et al., 2006; Park et al., 2007). Hepatic insulin resistance is believed to be linked to fatty acids reducing the insulin-stimulated tyrosine phosphorylation of IRS-2 in humans (Morino et al., 2006).

Inflammatory pathways including the NF-κB pathway are also stimulated during the transition period and can impair many metabolic functions (Zhang and Kaufman, 2008). There are a number of stimuli for this activation including endoplasmic reticulum (ER) stress, high levels of circulating lipids, and infection (Hotamisligil, 2010). Arkan and others (2005) fed a high fat/diabetes-inducing diet to a set of mice that lacked IKK-β enzymes in the myeloid cells. The role of IKK-β is to activate NF-κB, which will then stimulate an inflammatory response (Baker et al., 2011). Mice that lacked IKK- β in the myeloid cells were systemically protected from insulin resistance. These results suggest that the NF-κB pathway is directly involved with fat-induced insulin resistance.

In dairy cows, reducing the effectiveness of insulin seems to facilitate the funneling of the available glucose to the mammary gland to be used as energy for milk synthesis (Giesy et al., 2012). This diversion of almost 85% of the circulating glucose to the mammary gland

contributes to a severe negative energy balance and the cow is forced to mobilize more adipose tissue to make up the difference, which can lead to a variety of metabolic disorders (Sordillo et al., 2009).

One potential method to increase systemic insulin sensitivity is to mitigate inflammation. The supplementation of sodium salicylate (SS), a non-steroidal anti-inflammatory drug (NSAID), may decrease insulin resistance and the disruptive effects that inflammation may have on other metabolic processes. Sodium salicylate, though a weak cyclooxygenase (COX) inhibitor, has been shown to inhibit the NF-κB pathway by impairing the ability of IKK- β to activate NF-κB (Yin et al., 1998). In a previous study (Farney et al., 2013a; Farney et al., 2013b), SS was given to cows for 7 d postpartum. Mature cows (3+ parity), but not younger cows, exhibited hypoglycemia on d 7 through an unknown mechanism. The purpose of this study was to investigate the mechanism behind this hypoglycemia in the mature cows and to determine the extent to which SS alters glucose kinetics and insulin sensitivity.

Materials and Methods

Animals, Treatments, and Care

All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee (protocol # 3182). Cows were housed in the Kansas State University Dairy Unit Tie-Stall Facility for the duration of the trial. Twenty mature cows (3rd parity) were enrolled 4 to 36 h after parturition and were alternately assigned to 1 of 2 treatments for 7 d. Cows that experienced dystocia (calving difficulty 3+) or had multiple births were not enrolled. The first treatment was 2.5 g/L sodium salicylate (SS) with a molasses carrier (0.15 g/L) that was delivered to the cow via the drinking water supply. The second treatment was a control (CON) treatment, which supplied the cow with 0.15 g/L of the carrier molasses only in drinking water. After the 7-d treatment, cows were switched to normal water and removed from the tie-stall barn to rejoin the herd within 72 h.

Upon calving, all cows were given 1 bottle of Milk Fever CP (Aspen Veterinary Resources Ltd., Liberty, MO) subcutaneously and 454 g of the electrolyte mixture Fresh Cow YMCP (TechMix Inc., Stewart, MN) orally. One tube of CMPK Gel Plus (Vet Plus Inc., Menomonie, WI) was given orally once daily for the first 3 d postpartum. Urine ketones (ReliOn Ketone Strips, Wal-Mart Inc., Bentonville, AZ) and temperatures were monitored daily. Cows

with high urinary ketone concentrations (≥80 mg/dL) for 1 d or moderate ketones (40-80 mg/dL) for 2 consecutive days were treated per farm protocol. Cows exhibiting a temperature above 103.0°F were treated with antibiotics. Cows diagnosed with a displaced abomasum before d 7 were removed from study, though all data collected before removal was used for analysis.

Cows were milked 3 times daily at 8-h intervals. Cows were fed *ad libitum* twice daily at 630 h and 1600 h. Feed and water intake were recorded daily. Diet DM was 50.1%; it contained 17.43% CP, 38% NDF, and 23.6% ADF on a DM basis. Body weight (BW) and body condition scores (BCS) were recorded on d 1 and d 9.

Procedures and Sample Collections

Blood was collected into EDTA (6 mL) and sodium fluoride (6 mL) tubes (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ) from coccygeal vessels on d 1-7. The samples were then immediately centrifuged at 2,000 x *g* for 15 min and plasma was stored at -20°C until analyzed. Milk samples were collected 3 times daily on d 4-7.

On d 5, cows were fitted with a guidewire-style jugular catheter (Mila International Inc., Erlanger, KY). Under local anesthesia, a 14-gauge needle was used to access the jugular vein. A 45-cm long guidewire was threaded through the needle. The catheter (20 cm) was then strung onto the wire and was guided into the jugular vein. The catheter was flushed twice daily with 5 mL of a sterile solution of 3.5% sodium citrate to prevent clotting.

On d 7, a glucose turnover assay was performed. Beginning with the morning feeding, cows were fed equal amounts of the diet every two hours to meet steady-state requirements. At 1230 h, 1 g of U-^{13}C-glucose (99 atom %, Sigma Chemical Co., St. Louis, MO) dissolved in 50 mL saline was infused into the jugular vein via catheter in a bolus dose: syringes were weighed immediately before and after infusion to determine the exact amount administered. Jugular blood samples were collected 10 min before infusion and at 10, 20, 30, 40, 50, 60, 90, and 120 min post-infusion.

Immediately after the completion of the glucose turnover assay, liver, muscle, and adipose tissue biopsies were performed. Liver samples were taken as described previously (Morey et al., 2011) Approximately 200 mg of liver tissue (10 biopsies) were collected, snap-frozen in liquid nitrogen, and stored at -80°C until analyzed.

For the adipose tissue biopsies, the area between the tailhead and pin bone was used. The area was clipped, scrubbed, and a local anesthesia was applied (lidocaine hydrochloride; Agri

Laboratories Ltd., St. Joseph, MO). A 3 cm-long incision was made with a #22 blade (Feather Sterile Surgical Blade; GF Health Products Inc., Atlanta, GA). Subcutaneous adipose tissue was collected from the incision site with sterile forceps and surgical scissors. Approximately 5 g of tissue was collected, snap-frozen in liquid nitrogen, and stored at -80°C until analyzed. After tissue collection, the incision site was stapled closed.

Muscle tissue biopsies were collected at the first lumbar vertebra. The area was aseptically prepared and a local anesthesia was given (lidocaine hydrochloride; Agri Laboratories Ltd.). A 12-gauge needle was used to initially breech the skin to allow the biopsy needle access to the muscle tissue. A 14-gauge, 9 cm-long biopsy needle (SABD-1409-15-T; US Biopsy, Franklin, IN) was inserted through the initial hole, perpendicular to the Longissimus dorsi lumborum muscle fibers. Approximately 300 mg of muscle tissue was extracted, snap-frozen in liquid nitrogen, and stored at -80°C until analyzed.

Plasma Analysis

Plasma samples were analyzed for glucose (kit #439-90901; Wako Chemicals USA Inc., Richmond, VA), insulin (#10-1201-01; Mercodia AB, Uppsala, Sweden), BHBA (#H7587-58; Pointe Scientific Inc., Canton, MI), non-esterified fatty acids (NEFA-HR; Wako Chemicals USA Inc.), glucagon (# GL-32K, Millpore, Billerica MA), TNFa (Farney et al, 2011), lactate (#L7596-50; Pointe Scientific Inc.), and haptoglobin (Cooke and Arthington, 2013).

Glucose turnover test samples were analyzed for total glucose concentration to ensure steady-state requirements were met and then were analyzed for U-^{13}C-glucose to determine the enrichment of plasma glucose (Metabolic Solutions, Inc., Nashua, NH). Glucose was extracted and converted to aldonitrile pentaacetate derivative (Tserng and Kalhan, 1983), and negative chemical ionization GC/MS (Hewlett-Packard 5890) was used to analyze derivatized samples. The isotopic composition of the glucose was determined by monitoring unlabeled (M+0: m/z = 328) versus U-13C-labeled (M+6: m/z = 334) glucose derivatives. Enrichment of plasma glucose for each animal was fitted to an exponential decay curve using the following equation: $Et = E0 \times e^{-kt}$, where t = time relative to infusion (min), Et = enrichment of plasma glucose (U-^{13}C-glucose: unlabeled glucose ratio) at time t, E0 = enrichment at time t = 0, and k = rate constant (min-1). After using the best-fit equations to determine k and E0, the total glucose pool was calculated by this equation: $G = M \div E0$, where G = total glucose pool (g) and M = mass of tracer infused (g). Plasma glucose turnover rate (GTR, g/min) was calculated by the equation

GTR = G × k. Samples collected 10 min prior to infusion of U-^{13}C-glucose were also analyzed to verify the lack of natural occurrence of the M+6 isotopomer.

The revised quantitative insulin sensitivity check index (RQUICKI) was used as an indirect measure of insulin sensitivity. The following equation was employed: RQUICKI = 1/[log (Gb) + log (Ib) + log (FFAb)] (Perseghin et al., 2001). This equation has been validated for the use in dairy cattle (Holtenius and Holtenius, 2007).

Tissue Sample Analysis

Total RNA was isolated from all tissue types (RNeasy Lipid Tissue Mini Kit; Qiagen Inc., Valencia, CA) and RNA was quantified via spectroscopy (NanoDrop Technologies Inc., Wilmington, DE). All adipose samples and 5 randomly selected muscle and liver samples were analyzed for RNA integrity (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA). Complementary DNA was synthesized from RNA (High Capacity cDNA Reverse Transcription kit; Applied Biosystems, Foster City, CA). The synthesized cDNA was then used in quantitative real-time PCR in duplicate using SYBR green fluorescent detection (7500 Fast Real-Time PCR system, Applied Biosystems). All genes and primes are listed in Table 2.1. All sample values were normalized against the geometric mean of RPS9, RPS15, and β-actin values and the results are expressed as fold changes of threshold cycle ($2^{-\Delta Ct}$) value relative to control.

Statistical Analysis

Measurements are expressed as means ± S. E. Statistical analysis was carried out in the Mixed Procedure of SAS (version 9.3 SAS institute Inc., Cary, NC). Results were modeled with the fixed effects of treatment, day, and treatment × day interaction. Repeated measures within cow were modeled with an autoregressive (AR[1]) covariance structure. Day 1 plasma samples were taken before cows were put on treatment, so d1 values were used as a covariate just for the analysis of plasma metabolites. Several variables (all mRNA results from tissue except adipose GLUT4 and HSD11B1 and muscle PGC1a) were natural log-transformed for statistical analysis to achieve normal residual distributions, and reported means were back-transformed. Tendencies were declared at $P < 0.1$ and significance declared at $P < 0.05$.

Results

Water and Dry Matter Intake

There was no difference between treatments in water ($P = 0.61$) intake, indicating that there was little to no palatability issues with the SS treatment (Fig. 2.1). There was also no difference in DMI ($P = 0.98$; Figure 2.1).

BW and BCS

Day 1 BCS for CON and SS cows were 3.6 ± 0.17 and 3.47 ± 0.16 respectively (trt x day $P = 0.58$). Day 9 BCS for CON cows was 3.29 ± 0.16 and 3.29 ± 0.17 for SS cows (trt $P = 0.76$). There was no difference in BW between CON and SS treatments on d 1 (776 ± 21 kg vs. 777 ± 22 kg; $P = 0.97$) or d 9 (739 ± 21 kg vs. 735 ± 22 kg; $P = 0.89$). On average across treatments, cows lost 0.25 ± 0.11 BCS units and 40 ± 15 kg of BW.

Blood Metabolites and Glucose Turnover Test

Plasma NEFA, BHBA, and lactate concentrations were not affected by treatment (all $P > 0.32$; Fig. 2.2). Neither haptoglobin, an acute phase protein, nor TNFa, a proinflammatory cytokine, were altered by treatment ($P > 0.34$; Fig. 2.2).

Glucose and insulin levels were similar across treatments ($P = 0.25$ and $P = 0.13$ respectively; Fig. 2.3). However, RQUICKI, a measure of insulin sensitivity which takes into account glucose, insulin, and NEFA concentrations, tended to be greater (more sensitive) in SS treated cows ($P = 0.09$; Fig. 2.3). Glucagon, a stimulator of gluconeogenesis, tended to be elevated by SS treatment ($P = 0.08$; Fig. 2.4). The glucagon:insulin ratio was greater in CON cows ($P = 0.01$; Fig 2.4). The glucose turnover rate, an indicator of gluconeogenesis, was 25% less in SS treated cows than CON cows ($P = 0.055$; Fig. 2.4).

mRNA Abundance

Inflammatory cytokine tumor necrosis factor alpha (TNFa) mRNA abundance was decreased by SS in adipose tissue ($P = 0.09$) but not in muscle ($P = 0.97$) or liver ($P = 0.52$; Table 2.2). Treatment had no effect on hydroxysteroid (11-beta) dehydrogenase 1 (HSD11B1), which converts cortisone to cortisol, in any of the 3 tissues ($P > 0.67$). Insulin-dependent glucose transporter GLUT4 mRNA expression was also unaltered by SS treatment in these tissues ($P > 0.63$). Liver gluconeogenic genes glucose-6-phosphatase (G6PC), pyruvate carboxylase (PC) and peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC1a) mRNA abundance were not affected by treatment ($P > 0.67$).

Discussion

Treatment with SS did not significantly alter the inflammatory status of the cows between treatments based on the lack of change in plasma haptoglobin and TNFa levels, as well as muscle and liver TNFa mRNA. Contrary to our previous study, there were only numeric differences in plasma glucose levels and no hypoglycemia (Farney et al., 2013a).

High levels of circulating fatty acids can induce insulin resistance (Boden, 1997). It is believed that high levels of fatty acids cause a build-up of intracellular fatty acid metabolites such as ceramides which activate the serine/threonine kinase cascade, leading to the serine phosphorylation, and subsequent inhibition, of insulin receptor substrates (IRS-1 and IRS-2; Shulman, 2000). The inhibition of IRS-1 and IRS-2 leads to a decrease in insulin dependent glucose transport, specifically GLUT4, and thus insulin resistance. Increasing insulin sensitivity would reduce the inhibition of IRS-1 and IRS-2 and subsequently increase insulin dependent glucose transporters. Though RQUICKI did identify that SS treated cows had increased insulin sensitivity, there was no increase in GLUT4 mRNA in SS treated cows, although there may be differences at the protein level between treatments. Farney and colleagues (2013a) also saw an increase in RQUICKI on d 7 in SS treated cows.

One mechanism of SS is to impair the ability of IKK- β to activate the NF-κB inflammatory pathway (Yin et al., 1998). Arkan and colleagues (2005) also linked IKK- β to insulin resistance. Therefore, SS, which hinders IKK- β activity, may also increase insulin sensitivity. This has been confirmed by a variety of reports in obese or diabetic rats, mice, and humans (Kim et al., 2001; Yuan et al., 2001; Hundal et al., 2002; Park et al., 2007). In contrast, Farney and colleagues (2013a) did not see a difference in liver nuclear NF-κB abundance following SS treatment in cows, suggesting that there was not extensive impairment of IKK- β function. While the impairment of IKK- β may have had some role in the increased insulin sensitivity, there may be other mechanistic actions of SS that could contribute to the increased sensitivity of insulin.

Insulin inhibits gluconeogenesis; an increased response to insulin could explain why the glucose turnover rate decreased 25%. Glucose turnover rate is a good measure of gluconeogenesis because net portal appearance of glucose is minimal (Reynolds et al., 1988). Insulin has been shown to directly inhibit the pro-gluconeogenic genes PGC1a and G6P (Barthel and Schmoll 2003; Pugiserver et al., 2003). The fact that there was no difference in liver

gluconeogenic mRNA abundance indicates that the alteration in gluconeogenesis may occur post-transcriptionally.

Glucagon, a stimulator of gluconeogenesis, was increased in SS treated cows. Glucagon and insulin act inversely in regulating glucose metabolism; insulin suppresses while glucagon stimulates gluconeogenesis (Pilkis et al., 1988; Pilkis and Granner, 1992). The insulin:glucagon ratio was lower in SS treated cows, which may reflect a scenario where more glucagon was provided to override the more sensitized response to insulin. Even with higher glucagon concentrations and increased insulin sensitivity, gluconeogenic mRNA expression was not changed. Plasma glucose and insulin levels were similar in both treatments indicating that SS treated cows needed more glucagon to achieve the same level of glucose as CON cows, probably due to the increased sensitivity of insulin to hinder gluconeogenesis. In fact, Mamedova and colleagues (2013) found evidence that inflammatory signaling through toll-like receptor 4 (TLR4) promoted gluconeogenic gene expression in human hepatocellular carcinoma cells. If inflammation increases gluconeogenesis, it is probable that treatment with an anti-inflammatory agent would suppress gluconeogenesis. Although glucose and insulin levels in the current study were numerically lower in SS treated cows, there was no evidence of hypoglycemia, unlike a similar study (Farney et al., 2013a). One possible explanation for this discrepancy is the lack of power due to low animal numbers in the current study; 20 cows were used whereas Farney and colleagues (2013a) had 78 cows.

One mechanism of action of SS that could account for the post-transcriptional alteration in glucose metabolism is the ability of SS to activate AMP-activated protein kinase (AMPK; Hawley et al., 2012). It should be noted that the SS dose used by Hawley and colleagues (2012) was much higher than normally used *in vitro* (3+ mM v. 50-200 μM) in order to see a large, significant change. When activated, AMPK turns on catabolic (ATP generating) processes, such as fatty acid oxidation and glucose uptake, and turns off anabolic (ATP consuming) processes such as gluconeogenesis (Hardie, 2008). In the short-term, AMPK alters protein function, such as activating already existing GLUT4 transporters and deactivating gluconeogenic enzymes, and only alters gene expression in the long term (Hardie, 2008). The activation of already existing GLUT4 transporters could account for the numerically lower glucose concentrations in SS treated cows with no difference in GLUT4 mRNA expression. Rainsford (2004) suggested that SS treatment increased the conversion of pyruvate to lactate since the creation of glucose from

pyruvate was inhibited due to AMPK activation; however the current study did not detect any treatment difference in lactate levels. Activation of AMPK has also been linked to a decrease in insulin resistance, although the exact mechanism for this is unknown (Ruderman et al., 2003).

Conclusion

Treatment with the NSAID sodium salicylate increased insulin sensitivity in mature transition cows. Although there were no differences in plasma glucose or insulin levels, glucose turnover rate was decreased in SS treated cows. There was no detectible difference between treatments in mRNA expression of gluconeogenic genes or the insulin dependent glucose transporter GLUT4, indicating that the alteration SS had on glucose metabolism likely occurred post-transcriptionally, possibly through activation of AMPK.

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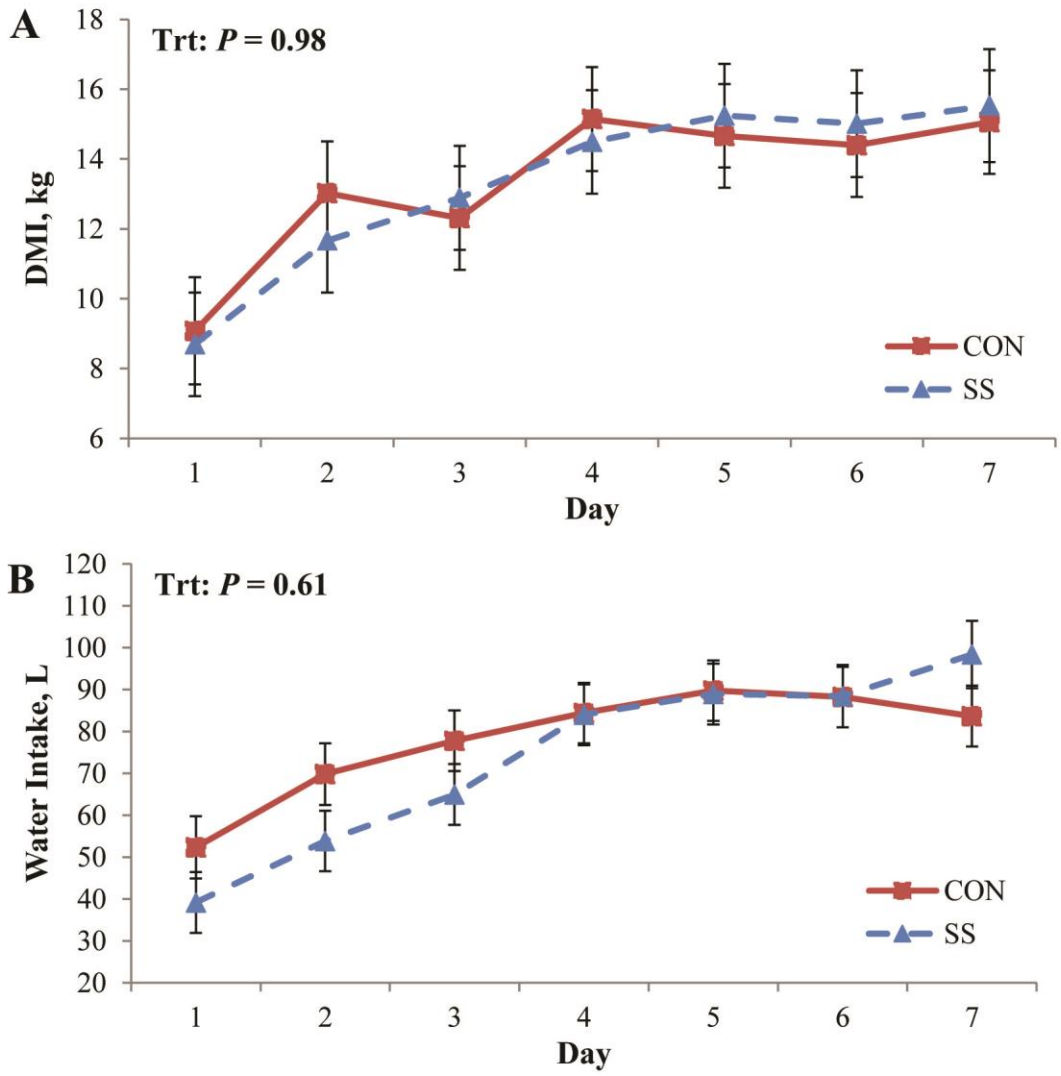
Table 2.1 Primers used for quantitative real-time PCR detection

Gene	Accession No.	Forward primer (5'-3') Reverse primer (5' -3')
RPS9	DT860044.1	GAACAAACGTGAGGTCTGGAGG ATTACCTTCGAACAGACGCCG
RPS15	NM_001024541.2	GGCGGAAGTGGAACAGAAGA GTAGCTGGTCGAGGTCTACG
β-actin	NM_173979.3	ACGACATGGAGAAGATCTGG ATCTGGGTCATCTTCTCACG
TNFα	NM_173966.1	AAGTAACAAGCCGGTAGCCCA CTTCCAGCTTCACACCGTTG
PGC1a	XM_005207759.1	TGCATGAGTGTGTGCTCTGT GCACACTCGATGTCACTCCA
PC	NM_177946.4	CTTCAAGGACTTCACTGCCACC GCCAAGGCTTTGATGTGCA
HSD11B1	NM_001123032.1	AGCATTGTGGTCGTCTCCTC TACGCAGCAACAAGTGGACA
GLUT4	NM_174604.1	TAGCCATCGTCACTGGCATC AGGAGGAGTGGCCATAAGGT
G6PC	NM_001076124.1	TGAGGATGGAGAAGGGAATG AACCAAATGGGGAAGAGGAC

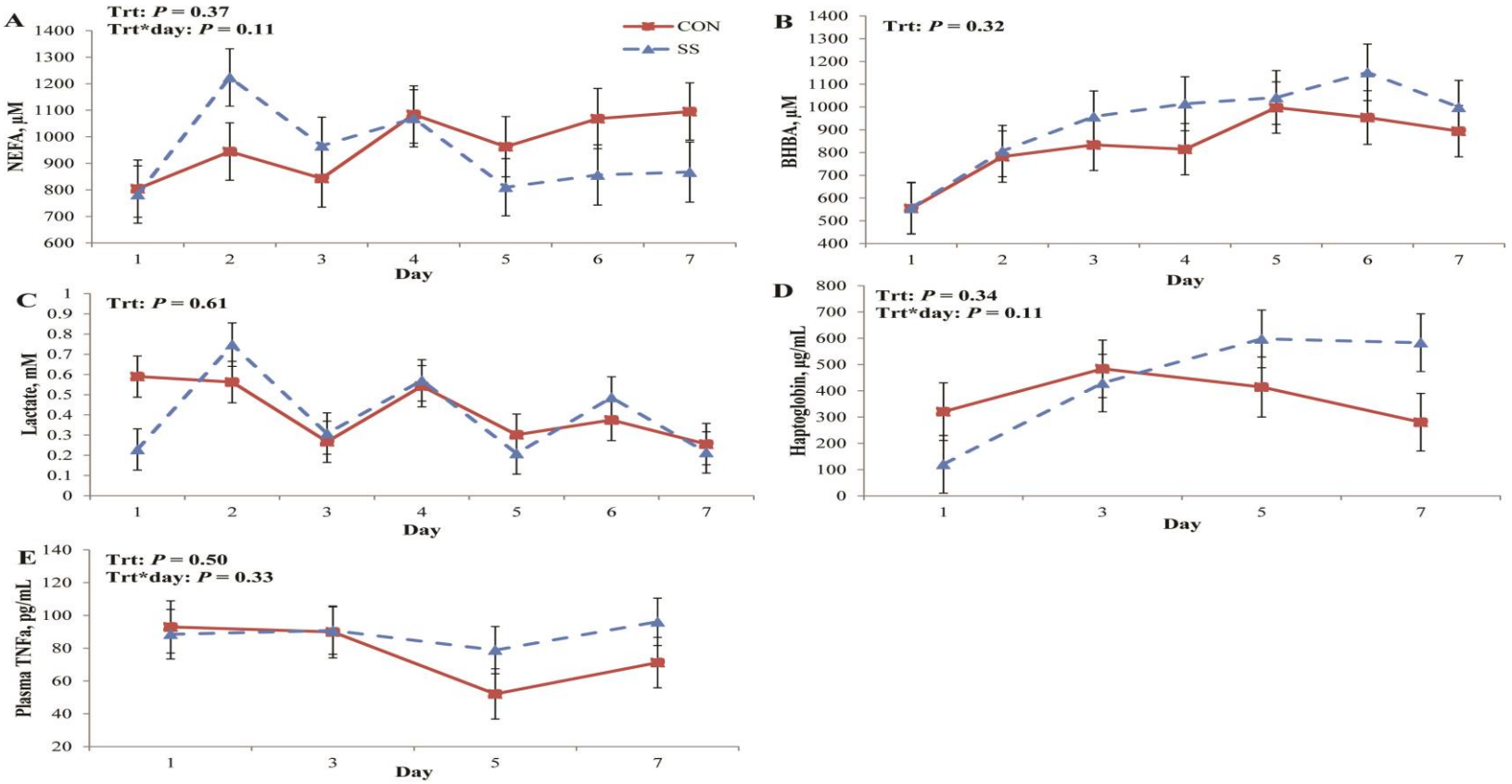
[1]RPS9, ribosomal protein subunit 9; RPS15, ribosomal protein subunit 15; TNFa, tumor necrosis factor alpha; PGC1a, peroxisome proliferator-activated receptor-gamma coactivator-1 alpha; PC, pyruvate carboxylase; HSD11B1, 11β-hydroxysteroid dehydrogenase type 1; GLUT4, glucose transporter type 4; G6PC, glucose-6-phosphatase.
[2]From NCBI Entrez Nucleotide Database (http://www.ncbi.nlm.gov/sites/entrez?db=nucleotide).

Figure 2.1 DM and Water Intake



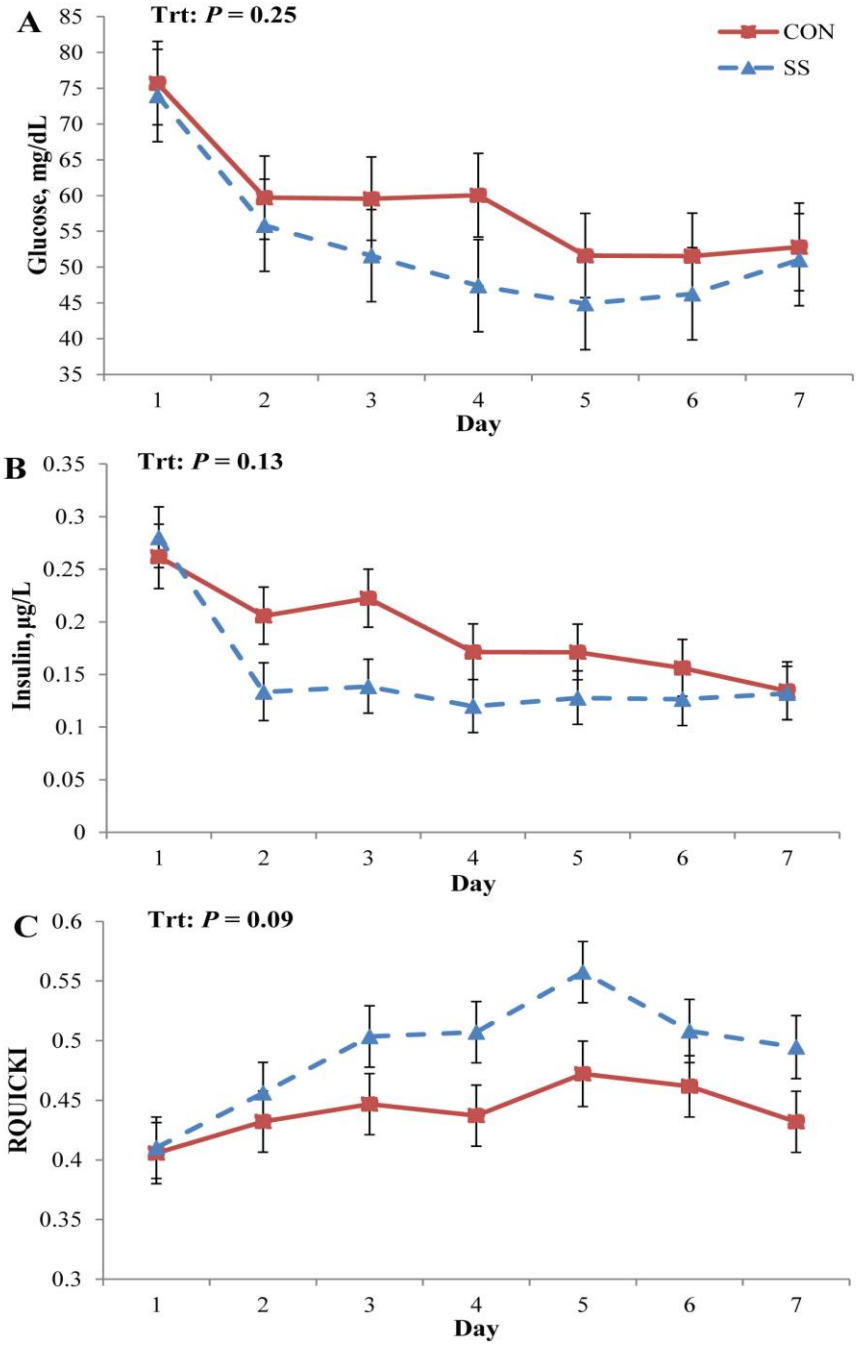
There were no differences in DM or water intake between treatments. It is interesting to note that cows on SS treatment had a numerically lower water intake (B) on days 1 through 3. This is probably due to palatability issues. Even though molasses was used to mask the bitter taste of SS, it is possible that it took the cows a few days to acclimate to the taste.

Figure 2.2 Plasma metabolites



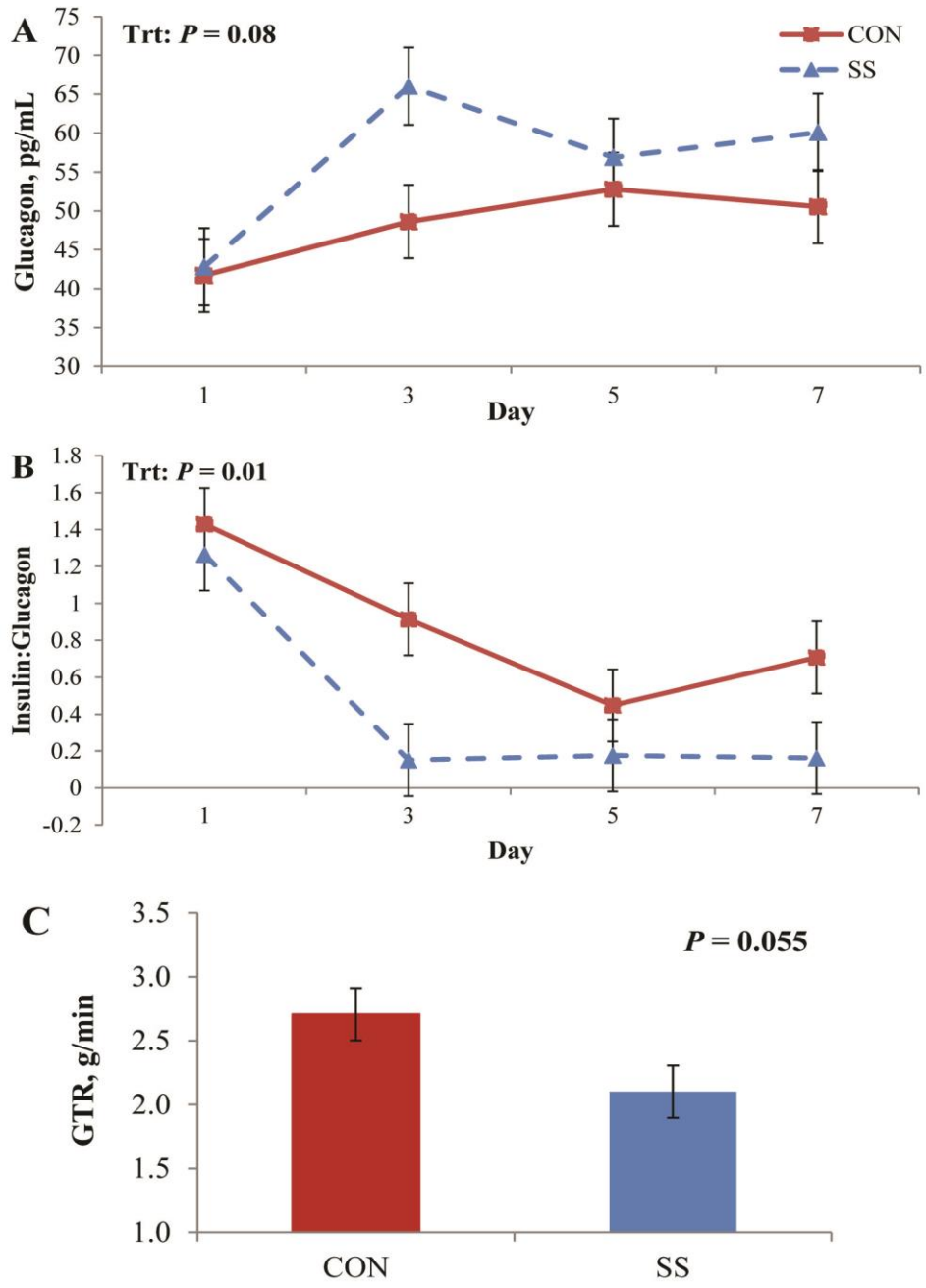
There were no differences in the plasma energy balance markers NEFA and BHBA between treatments (A and B). A suppression of hepatic gluconeogenesis could lead to an increase in lactate, however there were no differences in plasma lactate concentration between SS and CON treatments (C). Although SS is an anti-inflammatory agent, the plasma concentrations of the acute phase protein haptoglobin (D) and the inflammatory cytokine TNFa (E) were not altered by the treatment of SS.

Figure 2.3 Plasma glucose, insulin, and insulin sensitivity



There was no treatment effect on plasma glucose and insulin concentrations (A and B). Insulin sensitivity was measured using the RQUICKI equation, which takes into account plasma insulin, glucose, and NEFA levels. Cows treated with SS tended to have a higher RQUICKI (more insulin sensitive) than the CON cows (C).

Figure 2.4 Glucagon and glucose turnover rate



Glucagon, a hormone that stimulates gluconeogenesis, tended to be higher in SS cows (A). The insulin:glucagon ratio was lower in SS treated cows, indicating that SS cows had more glucagon per unit of insulin circulating in plasma (B). Glucose turnover rate (GTR), a measure of gluconeogenesis, was lower in SS cows, indicating that gluconeogenesis was being suppressed (C).

Table 2.2 mRNA fold differences in tissue

Tissue	Gene	mRNA Fold Difference[1]	*P*-Value
Muscle	TNFa	0.57 ± 0.38	0.41
	HSD11B1	0.28 ± 0.26	0.30
	GLUT4	0.82 ± 0.28	0.65
	PGC1a	0.28 ± 0.49	0.48
Adipose	TNFa	0.24 ± 0.35	0.09
	HSD11B1	1.20 ± 0.55	0.79
	GLUT4	1.52 ± 0.28	0.20
Liver	TNFa	0.42 ± 0.57	0.43
	HSD11B1	1.10 ± 1.92	0.96
	GLUT4	0.89 ± 0.91	0.93
	G6PC	1.42 ± 1.68	0.83
	PC	2.32 ± 2.50	0.62
	PGC1a	0.20 ± 0.47	0.14

[1]Fold differences of SS treatment when CON=1.

There was a tendency in adipose, but not liver or muscle tissue, for mRNA expression of the inflammatory cytokine TNFa to be lowered by SS treatment. Treatment with SS did not alter expression of gluconeogenic genes in the liver or glucose transporter GLUT4 in any tissue. There were not treatment differences in the expression of HSD11B1, which is involved in cortisol production and has been linked to insulin resistance in obesity.

Chapter 3 - Sodium Salicylate Does Not Alleviate Palmitate-Induced Endoplasmic Reticulum Stress in Immortalized Bovine Mammary Epithelial Cells

Abstract

Palmitate (PALM) has been shown to induce endoplasmic reticulum (ER) and oxidative stress in some cell types, whereas sodium salicylate (SS) and unsaturated fatty acids have been shown to mitigate these stresses. Therefore, we hypothesized that SS would counteract the effects of PALM in immortalized bovine mammary epithelial (MAC-T) cells. In the first experiment, MAC-T cells were treated with SS (50 μM), PALM (250 μM) or oleate (OLEIC; 500 μM), alone or in combination. To determine if PALM effects were due to ceramide (CER) synthesis, a second experiment was conducted where MAC-T cells were treated with PALM (250 μM), myriocin (MY; 1 μM; CER synthesis inhibitor), or both in the same basal media. In a third experiment, cells were treated with SS and PALM alone and in combination and were harvested at 3, 6, 12 and 24 h of incubation. An extracellular ROS assay was performed in experiment four where cells were treated as in experiment three but harvested at 180 min of incubation. The fourth experiment was replicated using human hepatocellular carcinoma (HepG2) cells. Results from replicate wells (6 to 12/treatment) were analyzed by ANOVA and treatment effects were declared significant at $P < 0.05$. PALM increased mRNA abundance of the ER stress response targets XBP-1 (4-fold, $P < 0.01$), ATF3 (46-fold, $P < 0.001$), and CHOP (34-fold, $P < 0.001$), but neither SS nor OLEIC affected these transcripts ($P > 0.10$). The XBP-1 transcript is also spliced during the ER stress-response. PALM increased the proportion of XBP-1 that was spliced (3.8 vs. 13.6 ± 2.2%, $P < 0.001$); this effect was not counteracted by SS or OLEIC. MY treatment ablated the XBP-1 mRNA and XBP-1 splicing responses to PALM ($P < 0.05$) but further increased CHOP and ATF3 mRNA abundance ($P < 0.001$). PALM had no effect on TBARS (a measure of oxidative products), but decreased extracellular ROS levels ($P < 0.001$). These results demonstrate that PALM induced ER stress in MAC-T cells, in part through CER effects. SS was not able to counteract the PALM effect.

Introduction

Chronic, low-grade inflammation is a hallmark of metabolic disorders, such as obesity and type 2 diabetes. This meta-inflammation has been linked to metabolic dysfunctions such as insulin resistance (Kim et al., 2001; Ozcan et al., 2004; Zhang and Kaufman, 2008; Hotamisligil, 2010; Back and Kaufman, 2012). Inflammation can be induced by a variety of pathways including the unfolded protein response (UPR), which occurs when the endoplasmic reticulum (ER) is stressed.

Endoplasmic reticulum stress occurs when the demand for protein folding cannot be met by the ER, causing an increase in the amount of misfolded and unfolded proteins that the ER releases into the host body. The purpose of the UPR is to protect the host by returning the stressed ER to homeostasis by altering cell function to make up for the protein folding deficit. In the event that this alteration does not resolve the protein shortage, the UPR initiates apoptosis to protect the host from the potential release of mass quantities of malfunctioning proteins, which can have toxic effects (Tabas and Ron, 2011; Moore and Hollien, 2012). A variety of stimuli can induce ER stress such as high levels of circulating free fatty acids (FFA), glucose, and proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α; Zhang and Kaufman, 2008).

The UPR cascade has three starting points, which consist of inositol-requiring 1α (IRE1), double-stranded RNA-dependent protein kinase (PKR)- like ER kinase (PERK), and activating transcription factor 6 (ATF6; Zhang and Kaufman, 2008). During normal ER function, these sensors are inactive due to the attachment of immunoglobulin-heavy-chain-binding protein (BiP or GRP78). BiP has a high affinity for misfolded and unfolded proteins, so when there is a large build-up of these, BiP will detach from the three arms and attach to the misfolded proteins, thus activating the UPR (Kaufman, 2002; Hotamisligil, 2010).

Once BiP is detached, PERK is phosphorylated, allowing it to phosphorylate eukaryotic initiation factor 2α (eIF2α). This phosphorylation inhibits the synthesis of new proteins in an attempt to slow down the buildup of misfolded and unfolded proteins (Zhang and Kaufman, 2008). The phosphorylation of eIF2α also allows for the translation of activating transcription factor 4 (ATF4), which then enters the nucleus of the cells and upregulates target genes involved with amino acid biosynthesis, oxidative stress response and apoptosis (Hotamisligil, 2010).

When the IRE1 branch is activated, IRE1 auto-phosphorylates. IRE1 then splices X-box-binding protein-1 (XBP-1) into its active form, referred to here as XBP-1s. XBP-1s is a key factor for transcription during ER stress. XBP-1s travels to the nucleus and induces transcription of genes that code for ER chaperones and enzymes involved with protein folding, maturation, and export (Zhang and Kaufman, 2008). ATF6 follows a similar pathway to IRE1 when activated. ATF6 migrates to the Golgi apparatus and is cleaved by site-1 protease (S1P) and S2P. The remaining fragment of ATF6 then goes to the nucleus and follows the same pathway as XBP-1s (Hotamisligil, 2010). In the event where the UPR is unable to revert the ER back to homeostasis through the alteration of cell transcription, the UPR will then initiate apoptosis, which is mainly mediated by CCAAT/enhancer-binding protein homologous protein (CHOP).

The UPR is linked with inflammatory pathways such as the nuclear factor-κB (NF-κB) and JUN N-terminal kinase (JNK). The branches of UPR can directly initiate an inflammatory response or act indirectly through accumulation of reactive oxygen species (ROS). The ER stores a large amount of calcium, but when there is an accumulation of misfolded and unfolded proteins, calcium begins to leak out of the ER and is taken up into the mitochondria. In the mitochondria, calcium disrupts normal functions by depolarizing the mitochondrial membrane and disrupting the electron transport chain (Zhang and Kaufman, 2008). This leads to an increase of ROS production, and a subsequent onset of oxidative stress. High levels of ROS initiate NF-κB and JNK pathways since ROS are important mediators in these pathways, which leads to an upregulation of specific genes involved with cell proliferation and the release of cytokines to activate an immune response (Baker et al., 2011).

Sodium salicylate (SS) is a member of the salicylate group of non-steroidal anti-inflammatory drugs (NSAIDs), of which aspirin is also a member. After consumption, SS is quickly converted to salicylic acid (Amann and Peskar, 2002). Though a weak cyclooxygenase (COX) inhibitor (Smith et al., 1975; Kopp and Ghosh, 1994), SS has been shown to inhibit NF-κB activation (Kopp and Ghosh, 1994, Yin et al., 1998) and uncouple oxidative phosphorylation, leading to an increase of extracellular adenosine release (Cronstein et al., 1999).

Evidence also suggests that NSAIDs may exhibit their anti-inflammatory effects by increasing the UPR. Tsutsumi and colleagues (2004) reported that CHOP, the apoptotic component of UPR, was involved in NSAID-induced apoptosis they observed in pig gastric

mucosal cells. They also noted that other UPR components such as XBP-1, ATF-6, and ATF-4 were upregulated in the presence of SS.

In this series of experiments, our aim was to assess the ability of SS to inhibit the UPR and inflammatory response, which was induced by the presence of the fatty acid palmitate (C16:0). We also assessed the ability of SS to decrease ROS production in ER stressed cells.

Materials and Methods

Immortalized bovine mammary epithelial (MAC-T) cells (Huynh et al., 1991) were graciously donated by Dr. Wendi Cohick from Rutgers University. Dulbecco's modified Eagle's medium (DMEM 1x; ref # 11965-092, Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS; cat #: S01520, Biowest, Kansas City, MO), 1% penicillin streptomycin (# 15070, Life Technologies), 1% insulin (# I9278; Sigma Aldrich, St. Louis, MO), and 2% bovine serum albumin (fatty acid free BSA; # A8806-5G, Sigma Aldrich) was used in all experiments. Cells were cultured at 37°C with 5% atmospheric CO_2. Before treatments, all cells were cultured for 24 h to reach at least 80% confluence. Cells were then washed with phosphate buffered saline (PBS) before fresh medium and treatment were added. After the designated incubation time, the medium was removed, and cells washed with cold PBS. Cells were frozen with Trizol, mixed with beta mercaptoethanol, and stored at -20°C until RNA was harvested unless otherwise stated.

Treatments

In the first experiment, cells were treated with sodium salicylate (SS; 50 μM), palmitate (PALM; 250 μM), or oleate (OLEIC; 500 μM), both alone and in combination and incubated for 24 h. For the second experiment, cells were treated with PALM (250 μM) or myriocin (MY; 1 μM) or both and incubated for 24 h. Myriocin is a known ceramide (CER) synthesis inhibitor (Chavez and Summers, 2012).

A third experiment was performed to monitor treatment effects over time. Cells were treated with SS (50 μM) or PALM (250 μM) or both. Cells were harvested after 3, 6, 12, and 24 hours of incubation. In a fourth study, cells were treated as in the third experiment with or without BSA, and medium was collected at 15, 30, 45, 60, 120, and 180 minutes of incubation.

Measurement of Oxidative Stress

Oxidative stress was quantified in two ways. The first method employed was measurement of thiobarbituric acid reactive substances (T-BARS Kit #10009055: Cayman Chemical Co., Ann Arbor, MI). Cells from experiment one were used for this analysis. Briefly, cells in Trizol were thawed and put into a 5 mL vial with 100 μL SDS solution and 4 mL of color reagent provided in the kit. The vials were then placed in boiling water for 1 h, incubated on ice for 10 min, centrifuged for 10 min at 1,600 x *g* at 4°C, then 150 μL from each vial was added to a 96-well plate and absorbance was read at 540 nm.

The medium from experiment four was used to measure extracellular oxidative stress with the acridan lumingen PS-3 assay (Uy et al., 2011). Briefly, the working reagent for this assay, ALPS-3 substrate, was created by mixing reagents A and B from western blotting kits in a 40:1 ratio (Amersham ECL Plus kit, GE Healthcare). Medium (50 μL) was pipetted in triplicate into a 96-well chemiluminescence plate. 50 μL of PBS and the ALPS-3 substrate were added to the medium and the plate was incubated in darkness for 5 min before analysis of chemiluminescence (Wallac Victor^2 1420 Multilabel Counter).

To validate results from the acridan lumigen PS-3 assay, we treated hepatocellular carcinoma (HepG2; HB-8065 purchased from ATCC, Manassas, VA) cells with SS (50 μM) or palmitate (250 μM) or both, either with or without BSA, and incubated the cells for 24 h. The medium was harvested and used in the acridan lumigen PS-3 assay.

Histochemical Assay

Hematoxylin and eosin (H&E) staining was performed at the 24 h time point of experiment three. Treated cells were washed with PBS, fixed with methanol for 10 min, then washed again with PBS. Fixed cells were incubated for 3 min in hematoxylin solution (35.2 g/L aluminum sulfate, 0.4 g/L sodium iodate). After 3 washes for 5 min each in PBS, the sections were allowed to air dry before glycerol was added. The image was visualized with a Nikon TMS microscope (Sheerin Scientific Co. Inc., Shawnee, KS) and images were taken using a digital camera (Nikon E995, Japan).

Transcript Abundance

For all experiments, RNA was extracted from cells in 1 mL ice-cold Trizol reagent (Qiagen RNeasy Lipid Tissue Mini Kit, Valencia, CA) according to manufacturer specifications and quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara,

CA). Total RNA amount was used to synthesize cDNA using a High-Capacity cDNA reverse transcription Kit (Applied Biosystems, CA).

Quantitative real-time PCR was performed in triplicate with 5% of the cDNA product in the presence of 200 nmol/L gene-specific forward and reverse primers using SYBR green fluorescent detection (ABI 7500 Fast, Applied Biosystems). Relative abundance of mRNA was quantified using the $2^{-\Delta Ct}$ method with an ABI Prism 7000 Sequence detector (Applied Biosystems, CA) instrument. All sample values were normalized against the geometric mean of RPS9 and RPS15 values. Results were expressed as fold changes relative to normalization transripts.

Statistical Analysis

Results from replicate wells (6 to 12/treatment) were analyzed by ANOVA (JMP 8.0, SAS Institute) to assess fixed effects of each treatment factor and interactions. Individual treatment contrasts were evaluated by Tukey's HSD. Several variables (XBP-1, XBP-1s, ATF3, CHOP, TBARS, TNFa, and IL-1B) were natural log-transformed for statistical analysis to achieve normal residual distributions, and reported means were back-transformed. Treatment effects were declared at $P < 0.05$ and tendencies at $P < 0.10$.

Results and Discussion

Experiment 1

In a 24-h period, PALM, a known ER stress inducer (Karaskov et al., 2006; Jeffrey et al., 2008), increased mRNA expression of total (active and inactive) XBP-1 ($P < 0.01$), while neither OLEIC, which has been shown to mitigate the ER stress effect of PALM in muscle cells (Peng et al., 2011), or SS were able to counteract this effect ($P > 0.46$; Fig. 3.1). Treatment with PALM also increased XBP-1s while SS and OLEIC again did not alter the effect of PALM (Fig. 3.1). The most prominent mediator of apoptosis, CHOP, also increased with PALM treatment ($P < 0.01$). Neither SS nor OLEIC were able to counteract the pro-apoptotic effect of PALM (Fig 3.1). These results give evidence that the MAC-T cells treated with PALM were in a state of ER stress. Oxidative stress, measured by TBARS, was not altered by PALM ($P = 0.32$). This was surprising, as it has been documented that PALM increases intercellular reactive oxygen species (ROS) production (Nakamura et al., 2009; Yuzefovych et al., 2010). There has been some work

indicating that SS may have antioxidant effects (Sagone and Husney, 1987; Haynes et al., 1993); however, TBARS was only decreased in the presence of SS and OLEIC in combination ($P < 0.01$; Fig 3.1).

Experiment 2

Palmitate is a common saturated fatty acid and plays a role in creating long chain fatty acids and sphingolipids. Palmitate can be taken up by the cell and converted to CER (Chavez and Summers, 2012). CER are a central part of sphingolipid metabolism and are generated in response to stress stimuli (Hannun and Obeid, 2008; Chavez and Summers, 2012). CER can induce and exacerbate ER and mitochondrial stress (Chavez and Summers, 2012). To determine if PALM-induced ER stress in MAC-T cells was due to CER synthesis or PALM itself, cells were treated with myriocin (MY), a compound that prevents the conversion of PALM to CER (Chavez and Summers, 2012). Treatment with MY mitigated the PALM effect by reducing mRNA abundance of total XBP-1, indicating that ER stress induced by PALM was due, at least in part, to CER synthesis (Fig. 3.2). MY treatment did not completely eliminate the PALM effect, so it is possible that PALM also induced ER stress through a CER-independent pathway. Conversely, MY in combination with PALM increased mRNA abundance of CHOP (Fig. 3.2). There has been some evidence that MY induced apoptosis in other cell types (Chen et al., 1999; Furuya et al., 2002). It is therefore possible that MY promoted apoptosis in MAC-T cells when the cells underwent CER-independent ER stress.

Experiment 3

The results from the first two experiments were only collected at one time point (24 h). The third experiment was designed to monitor changes in mRNA abundance that occurred prior to the 24 h time point. Interestingly, most of the large shifts in mRNA abundance of the target genes started to occur after 12 h of incubation (Fig. 3.3). PALM+SS had the highest mRNA abundance of total XBP-1 over the entire experiement ($P < 0.02$). The percent of XBP-1s exceeded 50% of total XBP-1 with treatment of PALM alone at 12h before falling to 12% at 24h. This confirms that PALM was again able to induce ER stress in the MAC-T cells. Treatment with SS increased the percent of XBP-1s by 7% over CON (22% v. 29%; $P = 0.02$). Activating transcription factor 3 (ATF3) was not affected by treatment, but was increased in all treatments over time (time: $P < 0.01$), and with PALM and PALM+SS treatments having a higher numeric

value at 24h. Activated by cell stress, ATF3 is also involved in the JNK pathway, which stimulates inflammatory signaling (Chen et al., 1996, Zhang et al., 2001).

The mRNA abundance of the inflammatory cytokines tumor necrosis factor alpha (TNFa) and interleukin-1 beta (IL-1B) was measured. Abundance of TNFa spiked at the 12h time point for PALM and PALM+SS treatments, with SS treatment causing a steady increase in mRNA abundance over time (all interactions $P < 0.01$; Fig. 3.3). PALM increased IL-1B over time ($P < 0.001$), with PALM+SS closely paralleling the trajectory of mRNA abundance over time, indicating that SS was unable to mitigate the effect of PALM on the cells. Since ER stress and inflammatory pathways are closely linked, cells with PALM-induced ER stress had higher abundance of inflammatory cytokines. It is interesting that SS, an anti-inflammatory agent, did not decrease the inflammatory cytokine abundance in stressed cells.

The apoptotic mediator CHOP mRNA abundance was increased with PALM ($P < 0.01$; Fig. 3.3). There have been some reports of SS inducing apoptosis in some cell types (Schwenger et al., 1997; Tsutsumi et al., 2004). Schwenger and colleagues (1997) identified that SS induces apoptosis through mitogen-activated protein kinase (MAPK) p38. ER stress activates many MAPK pathways including p38 and JNK. The activation of p38 MAPK phosphorylates (and activates) CHOP, leading to apoptosis (Cuadrado and Nebreda 2010; Darling and Cook, 2014). In this study, it is interesting to note that SS by itself did not have an effect on CHOP, but exhibited numerically higher mRNA abundance of CHOP when combined with PALM.

Overall, SS was unable to mitigate the ER stress induced by PALM, and in some cases, such as total XBP-1, it exacerbated the PALM effect (PALM x SS: $P < 0.001$). SS by itself had little effect on UPR transcripts; however in cells that are stressed, SS either failed to alleviate the stress or exacerbated it. An increase in XBP-1 and XBP-1s in PALM+SS cells indicates that the UPR was initiated to attempt to restore homeostasis. While SS may be assisting the cells to resolve the ER stress quicker, it is also possible that the effects of SS on this cell type may not be directly related to the UPR, but on a different component of the cell that is indirectly related to the UPR. For instance, SS may alter mitochondrial function, which does not have a direct role in ER stress; however the mitochondria may release reactive oxygen species (ROS), which can stimulate ER stress. There has been much speculation as to what mode(s) of action SS has on a cell, but results vary depending on the cell type, environment, and dose of SS (Amann and Peskar, 2002).

H&E staining of the cells from the 24 h time point is shown in Fig. 3.4. A visual assessment of the cells show that the SS-treated cells seem swollen compared to their CON counterparts. Both the PALM and PALM+SS treatments had fewer living cells, of which most are distended and misshapen.

Experiment 4

Endoplasmic reticulum stress can lead to increased production of reactive oxygen species (ROS). The ER stores large amounts of calcium. An accumulation of misfolded and unfolded proteins can cause this calcium to leak into the cytosol of the cell and concentrate in the matrix of the mitochondria (Zhang and Kaufman, 2008). This causes a depolarization of the mitochondrial membrane and leads to a disruption of electron transport and an increase of ROS production and subsequent oxidative stress, which can then go and further stimulate and exacerbate ER stress (Zhang and Kaufman, 2008). A TBARS assay for intracellular reactive oxygen metabolites was performed in experiment one, however this experiment was run to measure extracellular ROS levels by performing a newly developed assay that measures ROS levels in the medium (Uy et al., 2011).

This experiment was performed both with and without bovine serum albumin (BSA). Serum albumin has been shown to exhibit antioxidant properties and has a high binding affinity for long chain fatty acids (Roche et al., 2008), so the objective of this experiment was to determine if the common practice of including BSA in the cell medium had an effect on ROS assay results.

Surprisingly, PALM treated cells had lower extracellular ROS concentrations measured by the acridan lumingen PS-3 assay than CON and SS treated cells regardless of the presence of BSA ($P < 0.001$ for BSA and no BSA; Fig. 3.5). SS did not alter the PALM effect with or without BSA ($P = 0.57$). SS by itself lowered ROS concentrations only in the presence of BSA ($P < 0.001$). The presence of BSA only affected ROS levels in the SS treatment. There has been some debate as to whether SS has antioxidant properties (Amann and Peskar, 2002; Rainsford, 2004). These results indicate that SS had no extracellular antioxidant effect when BSA was not present. The creators of this assay stated that they have not identified which reactive oxygen species were measured by this assay (Uy et al., 2011), so it is difficult to determine the exact mechanisms that SS and BSA were influencing.

The low ROS concentrations in the PALM treated cells may be because at 180 minutes of incubation, PALM treatment may not have yet caused enough ER stress to elicit ROS release. In the time-dependent study (experiment 3), changes in ER stress markers were not seen until after 6-12 h of incubation. The results from the previous experiments already indicated that SS does not prevent or alter the responses to PALM, so it is not surprising that the SS+PALM treatment resulted in ROS concentrations similar to PALM.

To validate the unexpected ROS results obtained in the MAC-T cells, the experiement was repeated in HepG2 cells. In this case, extracellular ROS was measured after 24h of incubation. Without BSA, ROS concentrations in SS treated cells were about two-fold higher than CON ($P < 0.001$; Fig. 3.6). The ROS concentrations in the PALM and PALM+SS treated cells were similar with and without BSA (26.3 ± 2.1 vs. 25.5 ± 2.1 and 25.8 ± 2.1 vs. 24.5 ± 2.1 respectively). The presence of BSA reduced the concentration of ROS in CON media by half (14.2 ± 2.1 vs. 30.7 ± 2.1; $P < 0.001$). The apparent antioxidant properties of BSA could account for why SS ROS levels were much higher without BSA than with BSA.

There has been some work that has documented increased ROS production with SS treatment (Chung et al., 2003; Battaglia et al., 2005). It is believed that the reason for this increase in ROS production was due to SS compromising mitochondrial function by altering mitochondrial membrane potential (Chung et al., 2003). While this may be a factor contributing to higher ROS levels in SS-treated cells, it is interesting to note that the cells that were stressed with PALM, which presumably interrupted mitochondrial function by altering the electron transport system, had lower ROS, and that PALM seemed to protect the cells in the PALM+SS treatment from the SS compromising mitochondrial integrity.

Conclusion

Palmitate induces ER stress at least in part by driving ceramide synthesis. Sodium salicylate did not mitigate the PALM effects, and in some incidences, exacerbated the response. The concentration of extracellular ROS was altered with the inclusion of BSA, likely due to its antioxidant properties. It is important to note that ROS assay results can be altered by the inclusion of serum albumin, which is commonly used in cell culture media. Overall, SS seems to increase the UPR transcript genes when cells are undergoing ER stress, as well as increasing apoptosis in stressed cells to contain the amount of unfolded and misfolded proteins that could be

released and damage other cells and the host. SS treated cells had higher ROS levels (without BSA) than PALM in the HepG2 cells. The same response was not seen in the MAC-T cells, possibly due to the limited incubation period.

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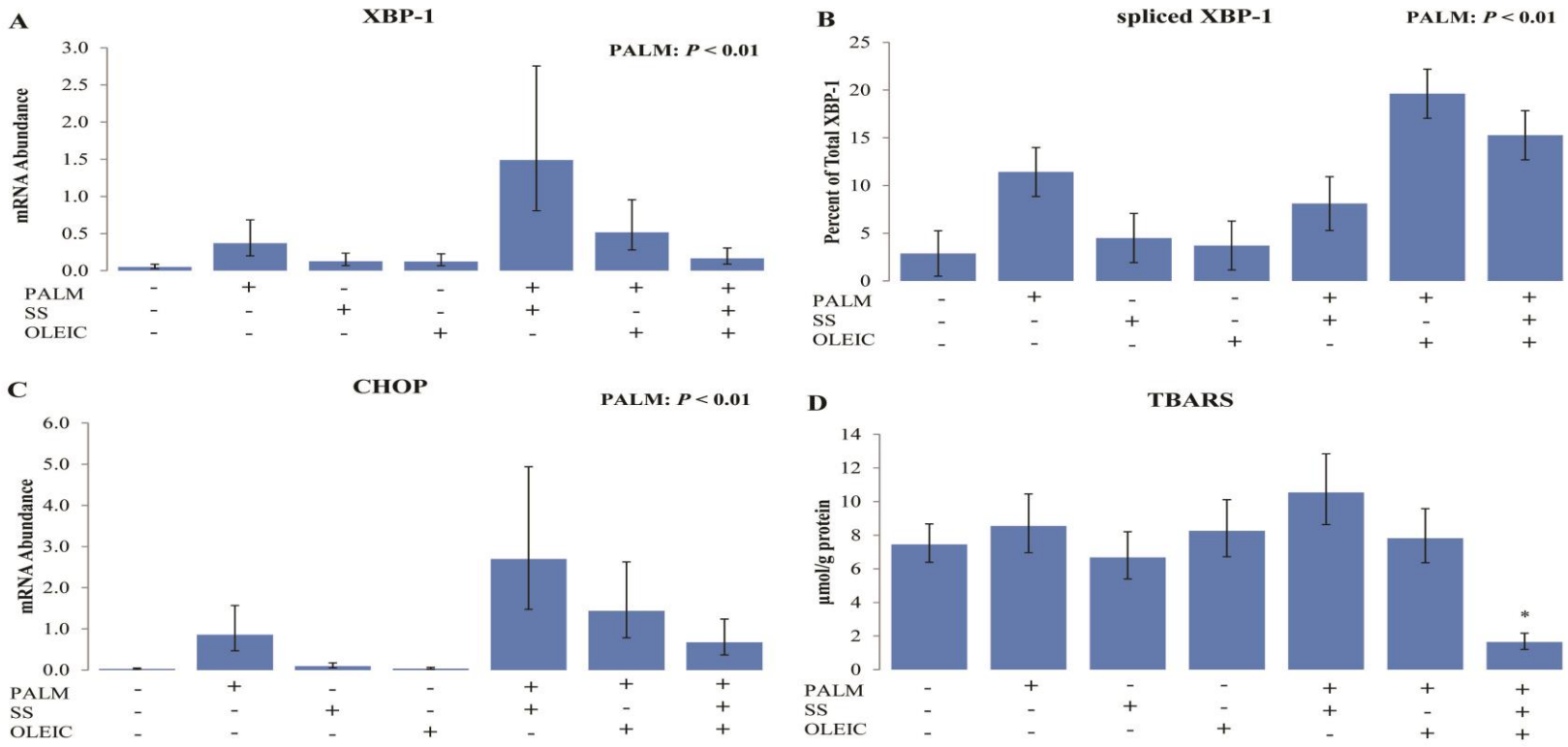
Table 3.1 Primers used for quantitative real-time PCR detection

Gene[1]	Accession No.[2]	Forward primer (5'-3') / Reverse primer (5' -3')
RPS9	DT860044.1	GAACAAACGTGAGGTCTGGAGG
		ATTACCTTCGAACAGACGCCG
RPS15	NM_001024541.2	GGCGGAAGTGGAACAGAAGA
		GTAGCTGGTCGAGGTCTACG
CHOP	NM_001078163.1	CAAACAGGAAATCGAGCGCC
		TGGCCTGAAATGGAAGTGCT
ATF3	NM_001046193.2	GAGTCCAGCTTCCAAAGGCT
		ATCACGTGGCTTCAAATGCG
TNFα	NM_173966.1	AAGTAACAAGCCGGTAGCCCA
		CTTCCAGCTTCACACCGTTG
IL-1B	NM_174093.1	GCCAACCTTCATTGCCCAGGTTT
		CAGGTACGGTTGCCATGGCTGT
XBP-1	NM_001271737	TTGTCACCCCTCCAGAACATC
		TCCAAGTTGAACAGAATGCCC
XBP-1s	NM_001271738	TGCTGAGTCCGCAGCAGG
		CATCAGAGTCCATGGGGAGA

[1]RPS9, ribosomal protein subunit 9; RPS15, ribosomal protein subunit 15; CHOP, CCAAT/enhancer-binding protein homologous protein; ATF3, activating transcript factor 3; TNFa, tumor necrosis factor alpha; IL-1B, interleukin-1 beta; XBP-1, xbox binding protein 1; XBP-1s, spliced XBP-1 (active form).
[2]From NCBI Entrez Nucleotide Database (http://www.ncbi.nlm.gov/sites/entrez?db=nucleotide).

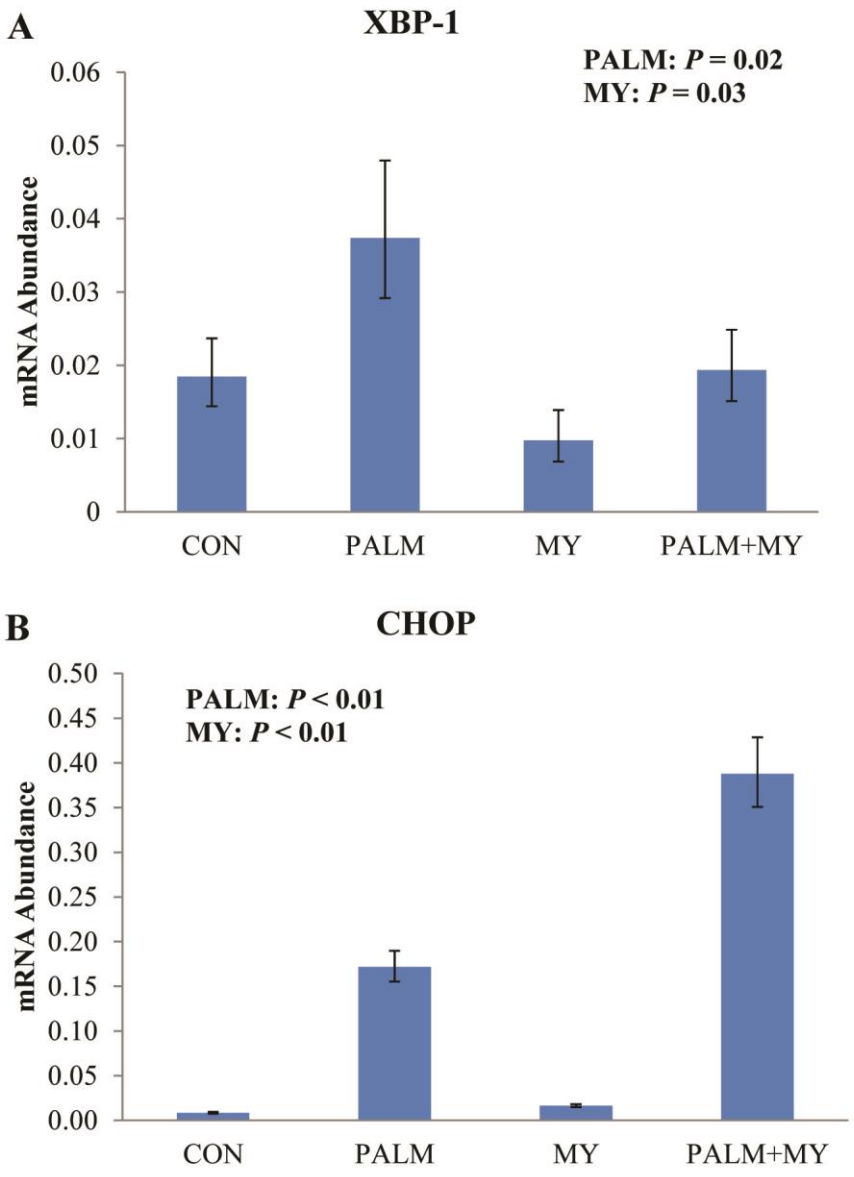
Figure 3.1 Effect of SS, PALM, and OLEIC



Panel A: PALM, but not SS or OLEIC, altered mRNA abundance of XBP-1 ($P < 0.001$ and $P > 0.82$ respectively). Neither SS or OLEIC mitigated the PALM effect ($P > 0.15$). Panel B: PALM treatment increased XBP-1s ($P < 0.001$), while SS and OLEIC did not ($P > 0.13$). Effect of PALM was not altered by SS or OLEIC ($P > 0.15$). Panel C: SS and OLEIC did not affect CHOP abundance ($P > 0.33$) or alter the PALM effect ($P > 0.12$). Panel D: PALM had no effect on TBARS ($P = 0.33$). The SS and OLEIC effect ($P < 0.001$) was probably driven by their synergistic effect when in combined with PALM (*).

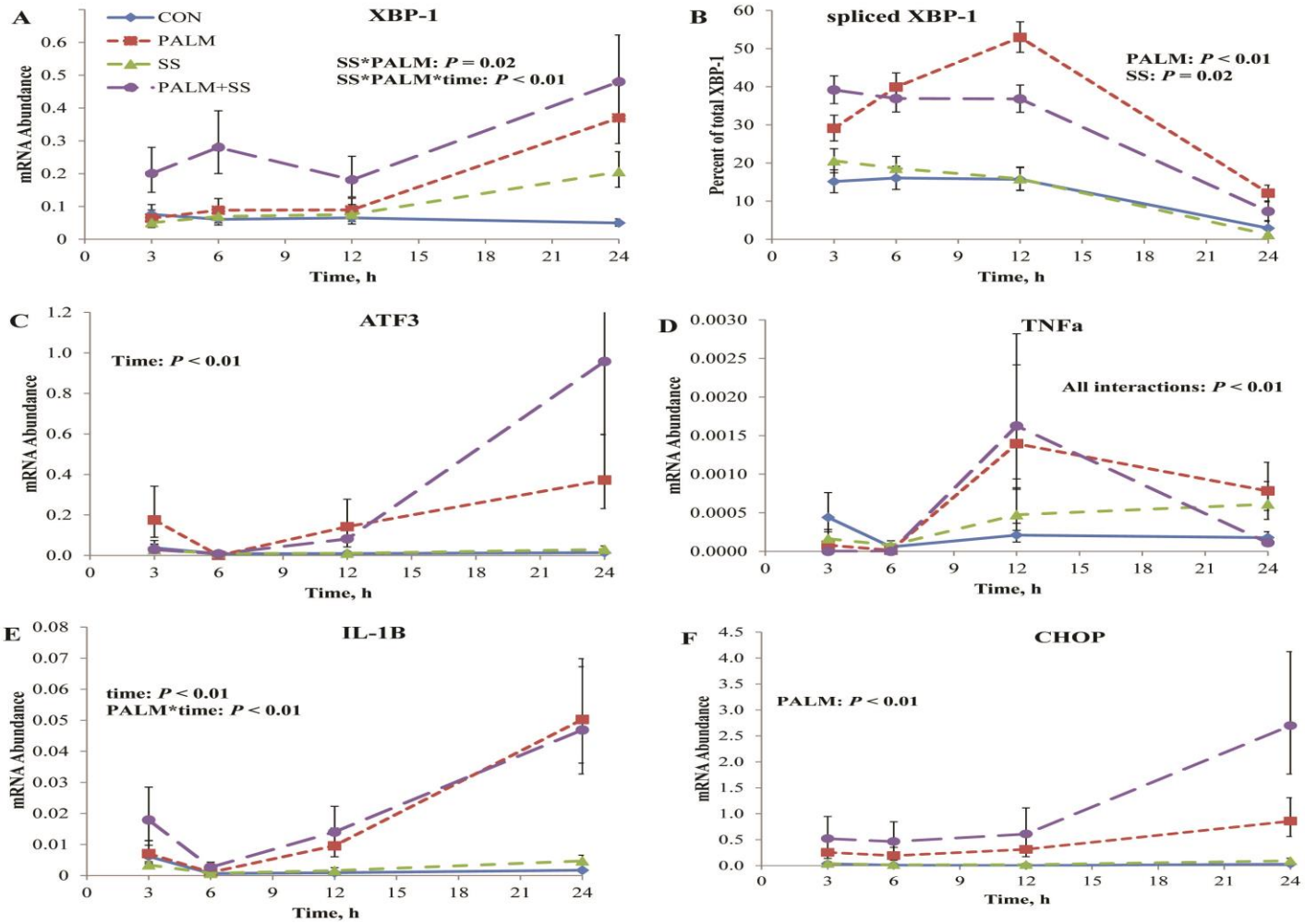
*$P < 0.001$

Figure 3.2 MY alters PALM effect



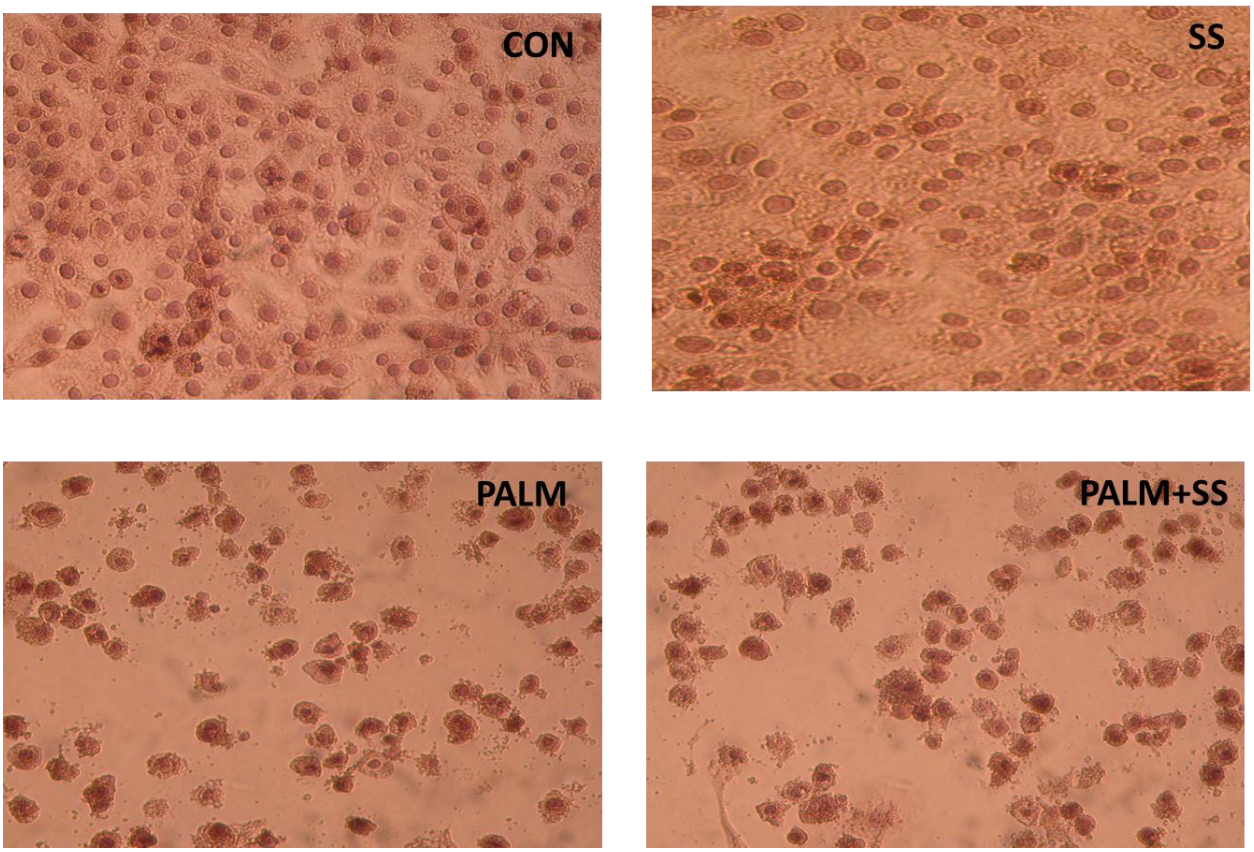
Panel A: MY, a CER synthesis inhibitor, decreases XBP-1 in the presence of PALM. Panel B: MY exacerbates the PALM effect on CHOP mRNA abundance.

Figure 3.3 MAC-T cells response to PALM and SS over time



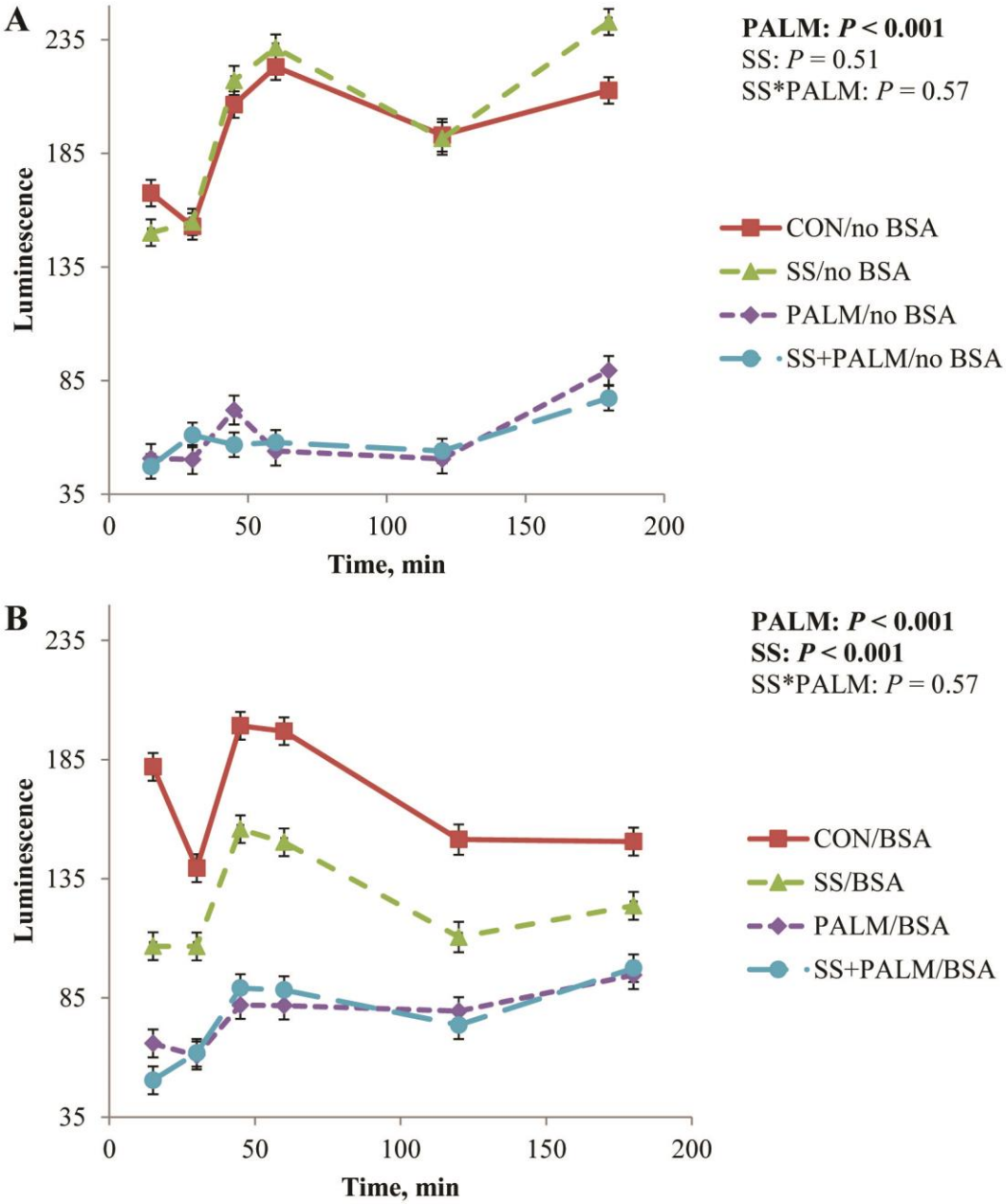
PALM increases UPR mediators and inflammatory cytokines (A-F). SS does not alter (C, D, E, F) and in some cases exacerbates the PALM effect (A). All interactions are not significant unless otherwise stated.

Figure 3.4 Histochemical Staining of MAC-T cells



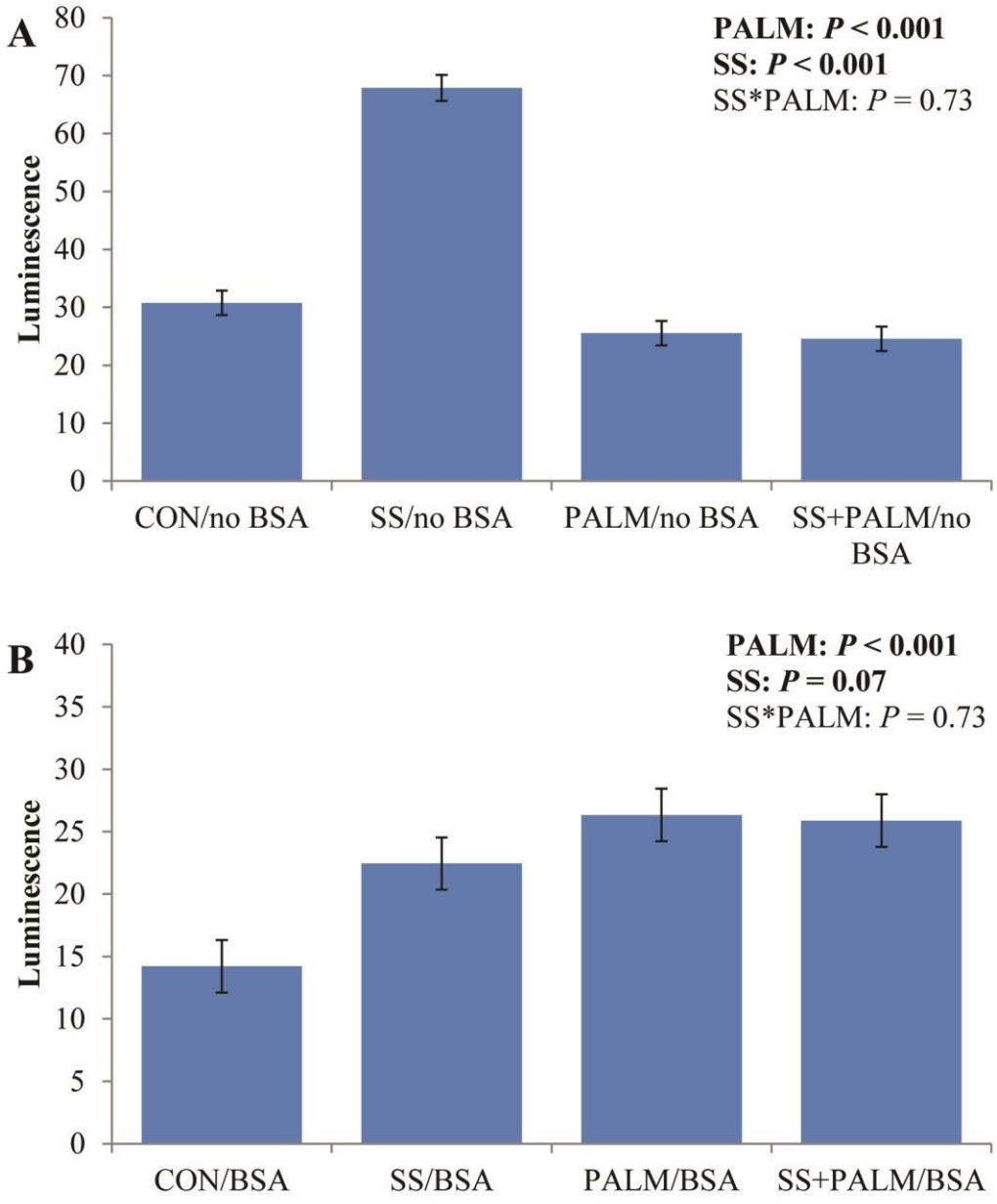
Hematoxylin and eosin (H&E) staining of MAC-T cells. Cells treated with SS seem to be swollen and distended. In the presence of PALM, there are few living cells, and those that are alive are misshapen. Cells in the presence of PALM+SS are also misshapen.

Figure 3.5 MAC-T cell extracellular ROS assay



PALM decreased extracellular ROS, and SS did not alter the PALM effect regardless of the presence of BSA. SS affected ROS levels only in the presence of BSA.

Figure 3.6 HepG2 cell extracellular ROS assay



PALM and SS effects on ROS were altered by BSA.