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Dominique Jacobs

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THE TURNOVER OF SUCCINATED PROTEINS IN THE ADIPOCYTE FOR THE TREATMENT OF DIABETES

By

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Submitted in Partial Fulfillment of the Requirements for Graduation with Honors from the South Carolina Honors College

May, 2017

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Dedication

I would like to dedicate this thesis to my parents Lisa and Sam, as well as my sisters, Gia and Juliet for always believing in me. Your support is the reason why I push myself to work hard and be the best student/person I can be. Thank you for always counseling me and being the greatest role models throughout my entire journey.
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I would like to express my sincerest gratitude to the following people for welcoming me into the Frizzell lab as an undergraduate with little prior research or lab experience. I am thankful for their continued guidance and collective mentorship through every step of my project. With their help, I have learned an enormous amount of material and have grown as both a scholar and student researcher.

To my Thesis Director, Dr. Frizzell,

Thank you for giving me this opportunity and serving as an amazing role model and teacher throughout this process. Thank you for having patience with me and trusting me in your lab.

To my Second Reader, Dr. Piroli and soon-to-be PhD graduate, Allison Manuel,

Thank you for always answering many questions and concerns in the lab. Thank you for treating me like I was part of the team from the beginning. Your assistance and guidance is sincerely appreciated.
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List of Abbreviations

2SC................................................................................................................2-(S-Succino)cysteine

Atg....................................................................................................................Autophagy-Related Proteins

CHOP............................................. CCAAT/Enhancer Binding Protein (C/EBP) Homologous Protein

ER......................................................................................................................Endoplasmic Reticulum

RIPA....................................................................................................................Radio Immunoprecipitation Assay Buffer

ROS....................................................................................................................Reactive Oxygen Species

T2DM..................................................................................................................Type 2 Diabetes Mellitus

UPR....................................................................................................................Unfolded Protein Response

UPS.....................................................................................................................Ubiquitin Proteasome System
Chapter I

General Introduction

1.1 Epidemiology of Type 2 Diabetes Mellitus

In the United States 29.1 million people have diabetes, or 1 out of every 11 people. The rate of new cases of diagnosed diabetes has begun to fall, but the numbers are still very high. 86 million people are living with prediabetes, a serious health condition that increases a person’s risk of developing type 2 diabetes mellitus (T2DM) and other chronic diseases (1). Unfortunately, 90% of those 86 million people are unaware that they are at risk. The health and economic costs for both types of diabetes are enormous: more than 20% of health care spending is committed to people with diagnosed diabetes. Type 2 diabetes is characterized by an inability to use insulin, a hormone released by the pancreas in response to feeding, properly. Under normal circumstances insulin allows glucose to enter cells, where it can be used for energy production. When the body cannot use insulin effectively, glucose accumulates in the blood and pervades tissues that do not require insulin for glucose uptake, including the retina, kidneys and nerves. High blood sugar levels can lead to heart disease, stroke, blindness, kidney failure and the amputation of toes, feet, or legs as a result of cumulative damage over time. Diabetes was the seventh leading cause of death in the United States in 2013, and is the leading cause of kidney failure, lower-limb amputations and adult-onset blindness (1).

Both obesity and being overweight are important risk factors for T2DM. Almost 1 in 5 (12 million) children and 1 in 3 (78 million) adults in the U.S. struggle with obesity, leading to $147 billion spent on obesity-related health care costs each year. The median percentage of overweight or obese adults with diabetes was 85.6% in 2014. With millions of people being at
risk, it is paramount to address the economic, environmental and biological factors that contribute to T2DM (1).

1.2 Adipose Tissue and Type 2 Diabetes Mellitus

While T2DM is known to affect many organs and tissues, the liver is an important contributor to the chronic blood glucose levels observed in the absence of insulin action. However, the involvement of adipose tissue (fat), another insulin sensitive tissue, has been much less studied. Until recently, the role of fat in the development of obesity and its consequences was considered to be passive; adipocytes were considered to be little more than storage cells for triglycerides. However, adipocytes are now accepted as critical components of metabolic control with distinct endocrine functions. Adipocytes are present in a network in adipose tissue that also includes the stromal vascular fraction; comprised of macrophages, neutrophils, eosinophils, and endothelial cells (2).

The absence of adipocytes, as occurs in lipodystrophies, is both physiologically and metabolically detrimental (3). For example, the absence of functional adipose tissue in patients with Berardinelli-Seip Syndrome, results in hyperlipidemia and extra-adipose lipid accumulation. Notably these patients lack the satiety regulating hormone leptin (which is produced exclusively by adipocytes). Consequently, individuals consume excessive calories in the absence of adipose tissue storage, resulting in hepatic steatosis. Mental retardation and psychiatric disturbances are present in a variable proportion of affected patients (4). Therefore, the maintenance of healthy amounts of adipose tissue is essential for normal physiology.

Adipocyte hormones play a role in the regulation of metabolism, energy intake, and fuel utilization. Adipose tissue secretes a large number of proteins, termed adipokines, which act in
an endocrine manner to control metabolic functions (3). As an example, the hormone adiponectin is abundantly expressed in adipose tissue. This hormone enhances insulin sensitivity in the muscle and liver and increases free fatty acid (FFA) oxidation in muscle (3). In humans, reduced plasma adiponectin concentrations correlate with hyperinsulinemia and insulin resistance (3). Considering the necessity of adiponectin and leptin for extra-adipose tissue function, it is important that the adipocytes have a functional capacity for adipokine secretion.

In healthy individuals, the body responds to the ingestion of a meal by secreting insulin to facilitate glucose uptake into insulin sensitive tissues, such as the adipose tissue. If there is an excess of nutrients, the adipose tissue can expand and store the surplus calories in the form of triglycerides (TGs). In the obese/diabetic individual, the hypertrophied adipocytes appear to have maximized their capacity to store excess fuel, resulting in the storage of fat in peripheral tissues such as the liver and the muscle (2). In addition to the challenge of storing fuel excess, the early challenge of hyperinsulinemia may antagonize adipocyte function due to hyper activation of insulin-sensitive pathways, such as protein synthesis, culminating in defective protein homeostasis and intracellular stress (5). Recent evidence suggests that this and other factors may result in adipocyte dysfunction early in the cascade of events that contribute to the development of T2DM (5).

1.3 Adipocyte Metabolic Stress

It is critical that we understand the mechanism of adipocyte dysfunction during the progression of diabetes due to its major role in energy metabolism. At a biochemical level there are several proposed mechanisms underlying adipocyte metabolic stress. Increased fuel flux in
the adipocyte increases cellular oxygen consumption leading to increased production of reactive oxygen species (ROS) in T2DM (6). This production of ROS can lead to peroxidation of lipids forming the α, β-unsaturated aldehydes 4-hydroxy and 4-oxotrans-2,3 nonenal. These react with cysteine, histidine and lysine residues to irreversibly modify proteins by carbonylation (7). In addition to this mitochondrial derived oxidative stress, there is also evidence of endoplasmic reticulum (ER) stress in the adipocyte in diabetes. Chronic stimulation of cells with high glucose and insulin results in a continued demand on the cellular proteostatic machinery. When adipocyte proteostasis is overwhelmed, either due to demand or the accumulation of misfolded or oxidized proteins this results in the induction of the unfolded protein respond (UPR) and ER stress (8, 9, 10). The UPR is a normal homeostatic response to the accumulation of misfolded proteins in the ER. Upon initiation of the UPR, a glucose-regulated protein dissociates from the ER membrane to help chaperone misfolded proteins. Oxidoreductase proteins can then interact with the misfolded proteins in order to catalyze disulfide bond formation and correct folding, thereby relieving the pressure on the ER. The presence of unresolved ER stress, notably the accumulation of the pro-apoptotic protein CHOP, has been documented both in vitro and in human adipose tissue in diabetic conditions (11, 12).

1.4 Adipocyte Mitochondrial Stress

Alongside oxidative and ER stress, mitochondrial stress has been documented in adipocytes matured in high glucose and in the adipose tissue of diabetic mice (13). In particular, the chemical modification of proteins by fumarate as a result of mitochondrial stress has been uniquely described in the adipocyte in diabetes (14). The Krebs cycle intermediate fumarate reacts irreversibly with cysteine residues in protein, in a process termed protein succination, to
form 2-(S-Succino)cysteine (2SC) (Figure 1.1) (15). The levels of succinated proteins are increased in adipocytes matured in 30 mM glucose vs. 5 mM glucose, and in the adipose tissue of diabetic mice. The increase in 2SC in diabetes is a direct result of tissue accumulation of fumarate in response to nutrient excess and the resultant mitochondrial stress (16,17).

Mechanistically, this increase in stress is a product of the increased ATP/ADP ratio in glucotoxic conditions. As a consequence of respiratory control, the electron transport chain is inhibited, simultaneously increasing the mitochondrial membrane potential and the NADH/NAD⁺ ratio. This cascade of events increases fumarate and protein succination, due to the NAD⁺ dependent enzymes of the Krebs cycle being inhibited (14) (Figure 1.2). The analysis of several succinated proteins in adipocytes has demonstrated that succination is associated with impaired protein structure or function (13). For example, succination inhibits secretion of adiponectin oligomers, which correlates with reduced serum adiponectin in type 2 diabetes (13). While the effect of succination on several adipocyte proteins has been studied in detail, we are only beginning to understand how succinated proteins are regulated or turned over within the cell.

In this thesis, I will investigate how succinated proteins are turned over within the cell by studying two major cellular degrading pathways: the ubiquitin proteasome system (UPS) and autophagy (18). I expect that these studies will reveal more information on the factors that regulate succinated protein turnover. In particular, I expect to confirm that while ubiquitinated proteins may accumulate when the proteasome is inhibited, succinated proteins will be turned over by a different mechanism. In addition, I hypothesize that Cathepsin B inactivity in diabetic cells will further prevent the turnover of succinated proteins through the autophagic pathway.
Figure 1.1 Formation of 2-((S-Succino)cysteine (2SC). The reaction of fumarate with cysteine-residues on proteins to form 2-((S-Succino)cysteine (2SC).
Figure 1.2 Mitochondrial Stress Increases Protein Succination. A proposed sequence of events resulting from high glucose concentration (glucotoxicity) in type 2 diabetes including the glucose-induced increase in ATP/ADP, mitochondrial membrane potential, NADH/NAD⁺. Inhibition of the Krebs cycle increases fumarate concentrations, allowing free thiol groups on cysteine residues to react with fumarate forming succinated proteins. Accumulation of succinated proteins in the ER inhibits protein folding, assembly, post-translational modification, transport and secretion, leading to ER stress.
Chapter 2
The Turnover of Succinated Proteins

2.1 Introduction

In the cell there are two protein degradation pathways: the ubiquitin proteasome system (UPS) and the lysosomal degradation system (also referred to as autophagy) (Figures 2.1 & 2.2) (19, 20). These pathways are important for the removal of damaged or unwanted proteins into small polypeptides, and also help maintain amino acid pools and energy balance, either during acute starvation for the UPS, or in the course of chronic starvation for autophagy. Additionally, these pathways constitute essential components of the cellular control of protein quality that sense misfolded or damaged proteins, tagging them for degradation (18). Failure of the proteolytic systems to maintain basal cellular turnover or to accommodate to the degradative requirements of cells under stress conditions leads to altered cellular homeostasis, compromising energetic balance and promoting intracellular accumulation of damaged components (21). Studying these systems is essential to develop a better understanding of protein homeostasis, particularly in the context of chemically modified proteins.

Cells exert tight control over the synthesis and elimination of important proteins. The UPS is primarily responsible for the degradation of cytosolic, ER, nuclear, and short-lived proteins. Proteins to be eliminated are marked by the covalent attachment of multiple copies of an 8.5 kDa protein ubiquitin to be degraded on a lysine residue (19, 21). The ubiquitin-protein complex is transported to the proteasome, where the ubiquitin chain is removed, allowing the target protein to be unfolded by an ATP-dependent process. It is then sent to the interior of the proteasome, where it is degraded by three threonine proteases (Figure 2.1) (19). There are
many different topologies of the ubiquitin chain and each serves as distinct binding surfaces for
different classes of ubiquitin-binding proteins. The first ubiquitin is attached to a lysine residue
in the substrate to generate an isopeptide bond; however, cells can generate heterogeneous
chains whereby ubiquitin molecules are linked to different internal lysine residues within a
single chain or in which more than one ubiquitin molecule is attached to a single ubiquitin
forming branched chain (21). The complexity of these chains can be of varying lengths with
varying attachment points. Different modes of ubiquitination determine different cellular fates
of a protein. For example, ubiquitin, a tag once thought to be exclusive to proteasome
degradation, could also be used for cargo selection by some forms of autophagy (21).

Autophagy is primarily responsible for the degradation of most long-lived or aggregated
proteins (18). Compared to the proteasome, it has a much broader proteolytic role because it
can degrade whole organelles such as mitochondria (mitophagy) or peroxisomes (pexophagy)
(21). Autophagy is characterized by the formation of double-membrane vesicles known as
autophagosomes, which sequester cytoplasm. It is involved in cell growth, survival,
development, and death, and has been implicated in human physiopathologies such as cancer,
neurodegenerative disorders, myopathies, heart and liver diseases, and gastrointestinal
disorders (18). In the first step of autophagy a phagophore surrounds items to be degraded. As
the phagophore is forming, more ‘cargo’ is added and the membrane continues to grow until it
becomes an autophagosome. The genes encoding the basic components of the machinery are
autophagy-related genes (Atgs) (Figure 2.2) (22). Fusion of the autophagosome with the
lysosome involves microtubules and proteins that contribute to modulate the fusion process
(21). For instance, microtubule-associated protein light chain 3 (LC3-I) is converted to LC3-II via
lipidation with phosphatidylethanolamine and is attached to the autophagosome. The vesicle
can then fuse with the lysosome of adipocytes, which contains hydrolases capable of protein
degradation: cathepsin B (cysteine protease) and cathepsin D (aspartic protease) (21,23).
Lysosomal destabilization associated with activation of cathepsin B occurs in both cultured
adipocytes exposed to saturated fatty acids in vitro, as well as in vivo in adipocytes from obese
mice fed a high fat diet (23).

We have demonstrated that 2SC is produced endogenously in rats and is excreted in the
urine (16), suggesting that there is an intracellular mechanism that can facilitate the removal of
succinated proteins. We investigated the turnover of succinated proteins in the 3T3-L1
adipocyte cultured in high glucose conditions versus normal glucose conditions and found that
when adipocytes are matured in 30 mM glucose for 4 days and then switched to 5 mM glucose
for the remaining 4 days of maturation, 2SC levels no longer accumulate and are similar to the
levels observed in adipocytes that matured in 5 mM glucose (Figure 2.3A&B) (11). This indicates
that unhealthy cells (30 mM glucose) can become normal cells (5 mM) with a healthy diet. To
determine the intracellular mechanism by which 2SC is being degraded, we inhibited either the
proteasome or the lysosome. To inhibit protein degradation, MG132 (a proteasome inhibitor)
or chloroquine (a lysosomal inhibitor) was used (Figure 2.3A&B). Figure 2.3A&B suggests that
with chloroquine present, when the adipocytes were switched to normal glucose, turnover of
succinated proteins was prevented (Figure 2.3B), indicating that the lysosome was responsible
for the degradation of succinated proteins. In contrast, succinated proteins did not accumulate
in the presence of MG132, suggesting the proteasome was not involved.
Although previous studies found no increase in 2SC levels with MG132 treatment, we never detected ubiquitination to determine if the proteasome was completely inhibited. In this thesis I will investigate if the proteasome was inhibited in these studies. I will also explore potential lysosomal inhibitors as strategies to confirm that lysosomal inactivity leads to increase succination.
Figure 2.1 Schematic protein degradation by the ubiquitin proteasome system. Misfolded or aggregated proteins are ubiquitinated in an ATP-dependent manner. The ubiquitinated protein is recognized by the proteasome, which then degrades that protein into its constituent parts and recycles the ubiquitin molecules for marking other proteins for degradation (11,19).
Figure 2.2 Induction of autophagy and lysosomal degradation. Schematic of autophagic degradation from phagophore formation to autophagolysosome formation. Atgs assist in the formation of the autophagic vesicle and Atg3 and Atg7 mediate lipidation of LC3-I, forming LC3-II, which is incorporated in the autophagosomal membrane (11,20).
Figure 2.3 Turnover of succinated proteins. Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. A subset of adipocytes were matured in 30 mM glucose for 4 days and then switched to 5 mM glucose for the remaining 4 days of maturation (30→5). They were then treated with or without (A) 17.5 µM MG132 or (B) 25 µM chloroquine. Protein (A) 30 µg and (B) 40 µg was separated by 1-D PAGE and detection of 2SC was performed using a polyclonal anti-2SC antibody. Western blotting for LC3 was assessed to confirm inhibition of lysosomal degradation. MW markers are shown in kDa and β-Tubulin is shown to demonstrate equal protein loading (11).
2.2 Results

Considering the preliminary data that proteasomal inhibition does not lead to increased succination, the confirmation of MG132 action on inhibiting the turnover of ubiquitinated proteins was next investigated. Adipocytes were matured for 4 days in 30 mM glucose and then switched to 5 mM glucose for the remaining 4 days of maturation. On the 6th day, they were treated with +/− 50 µM MG132 for the remaining 2 days (lanes 7-9). Figure 2.4 confirms a pronounced increase in ubiquitination in cells treated with MG132. After confirmation of proteasomal inhibition, we repeated the experiment with the above adipocytes, next probing for changes in protein succination with the anti-2SC antibody. The results showed no significant increase in succination with MG132 treatment, despite the fact that ubiquitin accumulation demonstrated that the proteasome was inhibited. This data suggested that proteasomal inhibition does not prevent the degradation of succinated proteins (Figure 2.5), solidifying earlier observations in our laboratory (Figure 2.3A (lanes 10-12)).

Because the above experiments use variable concentrations of MG132 (50 µM, figure 2.4 and 17.5 µM, figure 2.3A), we next tried two intermediate concentrations, with lower toxicity than 50 µM, to confirm that the proteasome is not involved in succinated protein turnover. We also added the MG132 after 4 days of maturation, instead of day 6 of maturation, to be more similar to the preliminary results where the proteasome was inhibited for a longer time period (Figure 2.3A). Adipocytes were matured for 4 days in 30 mM glucose and then switched to 5 mM glucose +/- 25 µM (lanes 10-12) or 35 µM (lanes 13-15) MG132 for the remaining 4 days of maturation. 30 mM glucose adipocyte and 50 µM MG132 matured on day 6 (same samples from figure 2.4 (lanes 16-17)) were used as negative and positive controls (for
increased ubiquitination). Unlike figure 2.4 and the positive control included (lane 17), we did not detect increased ubiquitination in the cells treated with MG132 compared to the 5 mM and 30 mM glucose lanes (Figure 2.6). This suggests that the proteasome is not inhibited at these concentrations after 4 days of treatment. In addition, there was limited evidence of increased succination in the presence of MG132 after 4 days treatment, further suggesting that any interference with the proteasome did not impact protein succination (Figure 2.7). Fumarase knock down adipocytes (in which succination is significantly increased) were used as positive controls for increased succination.

Considering our preliminary data and the increase in succination following lysosomal inhibition (with chloroquine), we next wanted to examine the total protein levels of Cathepsin B. The adipocytes were matured in 5 mM or 30 mM glucose for 8 days, and a subset of adipocytes were matured in 5 mM glucose and transduced with the scrambled control or the fumarase knockdown lentivirus for 8 days. We had predicted that the large increase in succination in fumarase knockdown cells and high glucose cells compared to scrambled controls and 5mM glucose cells might lead to increased cathepsin B levels. This prediction is based on a previous observation that Cathepsin B activity levels are decreased in high glucose conditions, therefore protein levels may be increased in an attempt to compensate for a loss in protein activity. Figure 2.8 shows no change in Cathepsin B protein levels when probing with Cathepsin B antibody, however, the antibody detected non-specific bands in addition to the predicted molecular weight of 44, 37, 27, or 24 kDa.

Since earlier work in our laboratory had demonstrated that Cathepsin B activity is decreased in high glucose conditions, and this may contribute to accumulation of succinated
proteins in high glucose, we next sought to use a Cathepsin B inhibitor (ca-074 Me) to
determine if it would increase protein succination. The adipocytes were matured in 5 mM or 30
mM glucose for 8 days. A subset of these were treated with 25 µM Cathepsin B inhibitor on day
4 of maturation (for 4 days). The data showed no increase in 2SC accumulation in the
adipocytes with the added inhibitor versus the 30 mM glucose adipocytes (Figure 2.9). We then
tried an alternative experiment where the cells were allowed to begin to turn over the
succinated proteins in 30→5 mM conditions for one day before adding the inhibitor at day 5
(Figure 2.10). This was done so that the recovering cells might begin to increase Cathepsin B
activity and protein turnover, before being inhibited chemically again and demonstrating the
importance of this enzyme. Adipocytes were matured in 5mM or 30mM glucose for 8 days. A
subset of adipocytes were matured in 30 mM glucose for 4 days and then switched to 5 mM
glucose for the remaining 4 days of maturation (30→5). They were then treated with or
without 25µM Cathepsin B inhibitor on day 5 of maturation for a total of 72 hours. Figure 2.10
demonstrates that this still did not impact total 2SC levels versus the high glucose control. In
order to determine if cathepsin B was inhibited we next investigated the levels of LC3 as an
indicator of reduced autophagic flux (Figure 2.11). LC3 levels did not increase in the presence of
the Cathepsin B inhibitor, suggesting that the compound may not have fully inhibited lysosomal
activity.

We next investigated the effect of a compound known as Emodin on total levels of 2SC
in adipocytes (Figure 2.12). Emodin has previously been shown to promote autophagic flux, and
therefore it might also be expected to decrease protein succination. We hypothesized that this
might be a therapeutic option to assist cellular turnover of succinated proteins. The adipocytes
were matured in either 5mM glucose or 30mM glucose for a total of 8 days. A subset of these were treated with either 25μM Emodin or 50μM Emodin on day 2 of maturation for a total of 6 days. Following protein collection and quantification, an immunoblot for total 2SC levels (Figure 2.12) suggests that the higher 50 μM Emodin treatment slightly reduced succination levels in the cells. This suggests that Emodin may have therapeutic potential for diabetic adipocytes. We next investigated the levels of LC3 to ensure this decrease in succination is due to lysosomal activity. Adipocytes were matured in 5 mM glucose for 8 days. They were then treated with or without 25 μM chloroquine on day 7 of maturation. A subset of adipocytes were matured in 30 mM for 8 days. They were then treated with 50 μM Emodin on day 2, 4 and 6 of maturation and +/- 25 μM chloroquine on day 7 of maturation. Figure 2.13 shows no increase in LC3 levels with chloroquine added to the Emodin adipocytes, suggesting that Emodin does not promote autophagic flux.
Discussion

The formation of S-(2-Succino)cysteine (2SC) is an irreversible chemical modification as the reaction of fumarate with cysteine forms a thioether bond, and there are no known enzymes that can cleave this bond. Protein succination is increased in 3T3-L1 adipocytes that have been matured 30 mM glucose vs. 5 mM glucose (Figure 2.3, 2.5, 2.7, 2.9, 2.10, 2.12). We were interested in determining how succinated proteins were being degraded in the adipocyte as we previously had shown that turnover does occur, at least when the lysosome is inhibited by chloroquine (Figure 2.3A (lanes 7-9)). At the same time we also noted that succination did not accumulate with the addition of a proteasomal inhibitor (MG132); however, inhibition of the proteasome was not confirmed by increased ubiquitin levels, similar to the chloroquine induced increase in LC3-I and LC3-II.

We suspected that the proteasome might be capable of degrading at least some succinated proteins as we had primarily identified succinated cytosolic and ER proteins (15). Initially we wanted to detect if the earlier data (in figure 2.3A) was significant by confirming that the proteasome was being inhibited, and we were able to demonstrate significant increases in ubiquitination in cells where 50 µM MG132 was added for 2 days, confirming proteasomal inhibition. Importantly, there was no increase in succination concomitant with the increased ubiquitin levels, suggesting that succinated proteins are not tagged by ubiquitin for degradation in the proteasome. However, since our data with chloroquine suggested that the turnover of succinated proteins occurred slowly (~4 days to lower succination levels) and 50 µM is toxic for >2 days, we matured adipocytes in 25 and 35 µM for 4 days. Unfortunately, we did not detect efficient inhibition of the proteasome in these cells, based on the ubiquitin
immunoblotting (Figure 2.6). The 4 day incubation at these concentrations did not reduce cell viability or protein levels, but the proteasome was not inhibited therefore we hypothesize that prolonged exposure to MG132 may result in the diversion of ubiquitinated proteins to the lysosome for compensatory degradation, preventing any detectable changes in ubiquitination or succination. Overall, the data suggests that the slow turnover of succinated proteins combined with a limited time frame for testing proteasomal inhibition prevents the accurate assessment of the proteasomal role in succinated protein turnover.

Since our observations suggested that the proteasome does not play a detectable role in succinated protein turnover, we further examined the regulation of autophagy in the adipocytes and the mechanism related to the lysosomal degradation of succinated proteins. Since the primary hydrolase present in the lysosome is Cathepsin B, and we had previously published data that a cysteine residue is succinated in high glucose (15), we determined that Cathepsin B should be a relevant target for further study. In unpublished studies, we have also observed that Cathepsin B activity is reduced in cells matured in high glucose versus low glucose. Therefore, we wanted to investigate if the chemical inhibition of Cathepsin B would augment the increase in succinated proteins in high glucose.

We first investigated total levels of Cathepsin B protein by using a Cathepsin B antibody to see what would happen in cells known to have increased succination (Figure 2.8 (lanes 4-6 & 10-12)). We hypothesized that Cathepsin B protein levels might be increased to compensate for a dysfunctional succinated protein. Fumarase knock down cells (lanes 10-12) have increased succination because the shRNA interferes with mRNA processing, leading to fumarate
accumulation. Despite several attempts, the antibody did not reliably detect active cathepsin B, and no clear differences were observed across the cell lysates tested (Figure 2.8).

We next investigated if direct inhibition of Cathepsin B would impact the accumulation of succinated proteins in high glucose treated adipocytes. The Cathepsin B inhibitor CA-074 Me is a potent and irreversible cell permeable inhibitor. We expected to see an increase in 2SC levels upon treatment with the Cathepsin B inhibitor versus the 30 mM glucose adipocytes if Cathepsin B is significant for succinated protein degradation directed to the lysosome (Figure 2.9). Adding a Cathepsin B inhibitor to cells with increased 2SC protein levels (30 mM glucose adipocytes) should inhibit the lysosome further to have maximal succination. However, Figure 2.9 shows no increase in 2SC protein levels with Cathepsin B inhibitor compared to the 30 mM glucose adipocytes. It may be probable that cells in high glucose are already undergoing full inhibition; therefore they cannot be inhibited any further.

In an alternative approach we next matured cells in high glucose for 4 days, then switched to normal glucose to promote succination turnover and functional recovery of Cathepsin B activity. We anticipated that the addition of the inhibitor on day 5 might suspend cathepsin B activity and demonstrate the significance of this specific enzyme in succinated protein turnover. Unfortunately there was no difference with the 30→5 alone versus 30→5 Cathepsin B inhibitor (Figure 2.10), nor did LC3 levels suggest that the lysosome was inhibited (Figure 2.11). It is possible that the concentration of Cathepsin B inhibitor used was not sufficient to inhibit the lysosome completely and in future studies, this experiment will be conducted in association with the measurement of cathepsin B activity. It may also be possible that the inhibitor (CA-074 Me) never bound successfully to Cathepsin B; therefore, Cathepsin B
was never properly inhibited. This may be due to the methyl ester of the E-64 derivative CA-074. CA-074 alone is the first highly selective inhibitor of cathepsin B and exploits the exopeptidase activity of cathepsin B, unique among the other members of the cathepsins. CA-074 and its analogs bind to the occluding loop at the P’ site (24). Its inactive methyl ester CA-074Me, the compound we used, was designed to overcome poor cell permeability of the parent compound (24). However, this methyl ester may have prevented the conversion to the reactive compound.

While Cathepsin B chemical inhibition did not seem to inhibit autophagy, we wanted to try a different compound that might promote autophagy and succinated protein turnover. Emodin (1,3,8-trihydroxy-6-methylantraquinone) is a natural anthraquinone derivative found in various Chinese medicinal herbs (25). Traditionally, it has been used as an active constituent of many herbal laxatives. However, in the last few years, significant progress has been made in studying the biological effects of Emodin at cellular and molecular levels and it is emerging as an important therapeutic agent (25). Studies show that Emodin ameliorates cisplatin-induced apoptosis of rat and renal tubular cells in vitro through modulating AMPK/mTOR signaling pathways and activating autophagy (26). Cisplatin (cis-diamminedichloroplatinum II) is a chemotherapeutic reagent that is widely used for the treatment of malignant cancers. Also, aloe-emodin-mediated photodynamic therapy induces autophagy and apoptosis in human osteosarcoma cell line Mg-63 through the other significant signaling pathways (27). Due these findings, we wanted to detect if Emodin might also promote succination turnover in our 3T3-I adipocytes. If Emodin can promote autophagy, 2SC levels should be decreased. Figure 2.12 shows that the cells treated with the higher 50 µM concentration of Emodin have slightly
decreased levels of succination on multiple proteins versus cells treated with 30mM and no
drug. Therefore, Emodin appears to have utility as an agent that may reduce protein
succination. However, Figure 2.13 displays that 50 µM Emodin did not promote autophagic flux
as LC3 did not increase with inhibition of the lysosome (by chloroquine). This suggests that
although Emodin reduced 2SC levels at 50 µM, it is not because of its autophagic capabilities. It
is possible that Emodin may lower fumarate levels and measuring these levels may propose a
possible role in regulating metabolism.

In conclusion, we have shown that short-term inhibition of the proteasome sufficient to
increase ubiquitinated proteins does not increase succinated protein accumulation. This further
confirms our observations that succinated proteins may not be ubiquitinated and degraded
through the proteasome. While we were unable to further confirm that Cathepsin B has a
prominent role in the lysosomal mediated degradation of succinated proteins, our results still
confirm that succinated proteins can be turned over when cells are returned to normal glucose
conditions. In addition, further studies on the use of emodin as a therapeutic agent are
warranted, particularly in animal models of diabetes. Because it reduces succination, (not
through autophagy) it is possible that emodin acts in other manners to modulate adipocyte
metabolism. Further studies will be required to investigate this.
**Figure 2.4 Confirmation of proteasomal inhibition.** Adipocytes were matured in 5mM or 30mM glucose for 8 days. A subset of adipocytes were matured in 30 mM glucose for 4 days and then switched to 5 mM glucose for the remaining 4 days of maturation (30→5). They were treated with 50 µM MG132 on day 6 of maturation for a total of 48 hours. 30 µg of protein was separated by 1-D PAGE and detection of ubiquitin was performed using a ubiquitin antibody P4D1. MW markers are shown in kDa and coomassie blue stain is shown below to demonstrate equal protein loading.
Figure 2.5 Turnover of succinated proteins by the proteasome. Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. A subset of adipocytes were matured in 30 mM glucose for 4 days and then switched to 5 mM glucose for the remaining 4 days of maturation (30→5). They were then treated with 50 µM MG132 on day 6 of maturation for a total of 48 hours. 30 µg of protein was separated by 1-D PAGE and detection of 2SC was performed using a polyclonal anti-2SC antibody. MW markers are shown in kDa and coomassie blue stain is shown below to demonstrate equal protein loading.
Figure 2.6 Confirmation of proteasomal inhibition. Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. A subset of adipocytes were matured in 30 mM glucose for 4 days and then switched to 5 mM glucose for the remaining 4 days of maturation (30→5). They were then treated with or without 25 µM or 35 µM MG132 matured on day 4 of maturation for a total of 94 hours. 30 mM glucose adipocyte and 50 µM MG132 matured on day 6 (samples from figure 2.4) were used as positive controls. 5 µg of protein was separated by 1-D PAGE and detection of ubiquitin was performed using a ubiquitin antibody P4D1. MW markers are shown in kDa and coomassie blue stain is shown below to demonstrate equal protein loading.
Figure 2.7 Turnover of succinated proteins by the proteasome. Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. A subset of adipocytes were matured in 30 mM glucose for 4 days and then switched to 5 mM glucose for the remaining 4 days of maturation (30→5). They were then treated with or without 35 µM MG132 on day 4 of maturation for a total of 94 hours. 5µg of fumarase knock down adipocytes serve as a positive control for increased protein succination. 30 µg protein was separated by 1-D PAGE and detection of 2SC was performed using a polyclonal anti-2SC antibody. MW markers are shown in kDa and coomassie blue stain is shown below to demonstrate equal protein loading.
Figure 2.8 Investigating Cathepsin B total protein levels. Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. A subset of adipocytes were matured in 5 mM glucose and transduced with the scrambled controlled or transduced with the fumarase knockdown lentivirus for 8 days. 20 µg of protein was separated by 1-D PAGE and detection of Cathepsin B was performed using a Cathepsin B antibody. MW markers are shown in kDa and coomassie blue stain is shown to demonstrate equal protein loading.
Figure 2.9 Investigating Cathepsin B inhibition. Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. They were then treated with 25 μM Cathepsin B inhibitor on day 4 of maturation. 30 μg protein was separated by 1-D PAGE and detection of 2SC was performed using a polyclonal anti-2SC antibody. MW markers are shown in kDa and coomassie blue stain is shown below to demonstrate equal protein loading.
Figure 2.10 Turnover of succinated proteins by the lysosome via Cathepsin B inhibitor.

Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. A subset of adipocytes were matured in 30 mM glucose for 4 days and then switched to 5 mM glucose for the remaining 4 days of maturation (30→5). They were then treated with or without 25µM Cathepsin B inhibitor matured on day 5 of maturation for a total of 72 hours. 5µg of fumarase knock down adipocytes serve as a positive control for increase protein succination. 30 µg protein was separated by 1-D PAGE and detection of 2SC was performed using a polyclonal anti-2SC antibody. MW markers are shown in kDa and coomassie blue stain is shown below to demonstrate equal protein loading.
Figure 2.11 Confirmation of lysosomal inhibition via LC3. Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. A subset of adipocytes were matured in 30 mM glucose for 4 days and then switched to 5 mM glucose for the remaining 4 days of maturation (30→5). They were then treated with or without 25µM concentration of Cathepsin B inhibitor matured on day 5 of maturation for a total of 72 hours. 5µg of fumarase knock down adipocytes serve as a positive control for increase protein succination. 30 µg protein was separated by 1-D PAGE and detection of LC3 was performed using a LC3 antibody. MW markers are shown in kDa and coomassie blue stain is shown below to demonstrate equal protein loading.
Table

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>5</th>
<th>30</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor</td>
<td>-</td>
<td>25μM Emodin</td>
<td>50μM Emodin</td>
</tr>
</tbody>
</table>

**Figure 2.12 Emodin reduces protein succination.** Adipocytes were matured in 5 mM or 30 mM glucose for 8 days. They were then treated with 25 μM Emodin or 50 μM on day 2 of maturation. 30 μg protein was separated by 1-D PAGE and detection of 2SC was performed using a polyclonal anti-2SC antibody. MW markers are shown in kDa and coomassie blue stain is shown below to demonstrate equal protein loading.
Figure 2.13 Emodin does not promote autophagic flux. Adipocytes were matured in 5 mM glucose for 8 days. They were then treated with or without 25 µM chloroquine on day 7 of maturation. A subset of adipocytes was matured in 30 mM glucose for 8 days. They were then treated with 50 µM Emodin on day 2, 4 and 6 of maturation. One of those subsets was also treated with 25 µM Chloroquine on day 7 of maturation. 30 µg protein was separated by 1-D PAGE and detection of LC3 was performed using a LC3 antibody. MW markers are shown in kDa.
Chapter 3

Methods

Materials. Unless otherwise noted, all chemicals were purchased from Sigma Aldrich.

Preparation of 2SC antibody was prepared by Eurogentec. 3T3-L1 murine fibroblasts were purchased from American Type Culture Collection and used for cell culture. Polyvinylidene fluoride (PVDF) was purchased from GE Healthcare. Sodium dodecyl sulfate (SDS) and Tween-20 were purchased from Fisher Scientific. Criterion TGX Precast Gels were purchased from Bio-Rad.

Protein Extraction from Adipocytes. All cellular protein was collected at day 8 of maturation by lysing the cells in radioimmunoprecipitation assay buffer (RIPA) and sonicated 3 times for 12-15 seconds each. Ice cold acetone, 9x the Volume, was then added to the samples, vortexed, and then sat on ice for 10 minutes. The samples were then vortexed again and centrifuged at 2000 rpm for 10 minutes at 4° C. The acetone was decanted and the samples were re-suspended in 0.5-1 mL of RIPA buffer and sonicated 3 times each for 10 seconds each. The samples were stored at -70° C until further analysis. The protein concentration was determined using the Lowry assay.

Western Immunoblotting. Samples were prepared using 25-40µg for cell lysates with the addition of 5-7µL 4X Laemmli loading buffer. The samples were then boiled at 95° C, flash centrifuged then loaded on 12% or 18% gels and electrophoresed at 200 V for 60 minutes. The protein was transferred to a PVDF membrane in transfer buffer at 250 mA for 100 min or 40 mA at 4° C. The membrane was ponceau stained then blocked in 5% non-fat milk or 5% bovine serum albumin (BSA) according to the manufacturer’s instructions. Membranes were probed
using primary polyclonal anti-2SC. Antibodies for Ubiquitin and LC3 were from Cell Signaling.

Cathepsin B and β-tubulin were from Santa Cruz. Pierce™ ECL 2 Western Blotting Substrate was used and followed by detection of chemiluminescence using photographic film.
References


2 Rosen, D., Spiegelman, B. What we talk about when we talk about fat. *Cell* 2014; 256: 20-44


Appendix A

Buffer Preparations

**RIPA Buffer**

The buffer was prepared in 200 mL stocks containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA. On day of use, 2 mM sodium orthovanadate, 2 mM sodium fluoride and protease inhibitor (1:1000) was added to the buffer. The buffer was stored at 4°C.

**Running Buffer**

One liter of 10X stock was prepared containing 250 mM Tris-HCl, 1920 mM glycine and 10% SDS.

**Transfer Buffer**

One liter of 10X stock was prepared containing 250 mM Tris-HCl, 1920 mM glycine. Methanol was added at 20% for transfer process.

**Wash Buffer**

One liter of 10X stock was prepared containing 200 mM TrisOHCl, pH 7.4. Tween-20 was added at 0.05% to 1X wash buffer.
Appendix B:

Lowry Assay

Pipette the specific amounts of reagents into the microplate in the order listed. All samples are prepared in duplicates.

Table B.1: Preparation of BSA standard curve for the Lowry assay.

<table>
<thead>
<tr>
<th>Probe</th>
<th>BA (µL)</th>
<th>H2O (µL)</th>
<th>Copper Reagent (µL)</th>
<th>Incubation</th>
<th>Folin-Ciocolteu (µL)</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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<td>30 minutes at 37°C</td>
</tr>
<tr>
<td>Sample</td>
<td>5</td>
<td>15</td>
<td>20</td>
<td>30 minutes at 37°C</td>
<td></td>
<td>30 minutes at 37°C</td>
</tr>
</tbody>
</table>

**Stock BSA**

Dissolve 50 mg BSA (Bovine Serum Albumin) in 10 mL deionized water =5 mg/mL stock/working solution.

Working Solutions: Dilute 400 µL of 5mg/mL stock in 600 µL water =2mg/mL solution

Table B.2: Preparation of copper reagent for Lowry assay.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Working Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper Sulfate 1% (w:v)</td>
<td>100 µL</td>
</tr>
<tr>
<td>Sodium Tartrate 2% (w:v)</td>
<td>100 µL</td>
</tr>
<tr>
<td>Sodium Carbonate 10% (w:v) in 0.5 M NaOH</td>
<td>2 mL</td>
</tr>
</tbody>
</table>

**Folin-Ciocolteu Phenol Reagent:** Purchased as a 2 N stock solution. For working solution at 500 µL of stock solution to 5.5 mL of water. Read absorbance at 660nm.
Appendix C

Western Blotting

*Gel Electrophoresis*

1. After determining the protein content from the Lowry assay, 20-30 µg of protein was dissolved in water and 5 µL of Laemmli loading buffer was added.

2. Boil the samples for 15 min at 95˚ C then flash centrifuge.

3. Remove tape and comb from Bio-Rad pre cast Criterion gel and place in cassette.

4. Fill the cassette tank and gel with Tris/Glycine/SDS running buffer.

5. Load the samples into their individual lanes and 8 µL of marker into your lane of choice.

6. Run the gel at 200 V for 60 min.

*Wet Transfer*

1. Remove the gel from the precast and cut to size.

2. Soak the gel in Tris/Glycine/Methanol transfer buffer for 15 minutes.

3. Charge the PVDF membrane for ~30 sec in methanol. Soak the 2 pieces of blotting paper, 2 sponges, and the membrane in Tris/Glycine/Methanol for 15 min.

4. Assemble the transfer apparatus, starting with the black side first. Keep all materials soaking in transfer buffer during the assembly.

5. Place the sponge flat on the black side, followed by a piece of blotting paper. Next place the gel on top followed by the PVDF membrane. Roll out any air bubbles between the gel and membrane using a roller. Finally, put the remaining piece of blotting paper on top of the membrane and the sponges on top.

6. Assemble the apparatus and transfer at 250 mA for 100 min or 40 mA for at least 12 hrs.

7. Remove the membrane from the apparatus and wash 3 times with nanopure water.

8. Place the membrane in ponceau stain for 1 min then wash with nanopure water to visualize the bands. Inspect the membrane for equal loading and where bubbles formed during the transfer process. Was the ponceau stain off the membrane with Tris-HCl was buffer.
9. Block the membrane 5% non-fat dry milk of 5% BSA for at least 1 hr.

**Immunostaining for 2SC**

1. Prepare 1% milk by diluting the 5% milk 1:4 in Tris-HCl wash buffer.
2. Add 2SC antibody to the milk in 1:8000 dilution. Incubate for at least 1 hr on rocker.
3. Pour off the antibody and wash the membrane 3 times in wash buffer for 5 min each.
4. Add secondary antibody anti-rabbit to 1% milk, 1:6000 dilution and incubate for 1 hr at room temperature.
5. Pour off the milk and wash the membrane 3 times for 5 min each in wash buffer.

**Developing**

1. Prepare ECL solution by adding solution B to solution A in a 1:40 dilution.
2. Add the ECL to the membrane and incubate for 5 min. Place the membrane in the cassette and cover with plastic wrap.
3. In the dark room, place a piece of X-ray film over the membrane then develop. Inspect the film after it has been developed and adjust the exposure times accordingly.

**Buffers**

1. Stock Running Buffer: 10X SDS – 30.3 g Tris base, 144 g glycine and 10 g SDS dissolved to 1 L water.
   Working solution = 100 mL of 10X stock diluted to 1L water

2. Stock Transfer Buffer: 10X transfer – 30.3 Tris base and 144 g glycine dissolved to 1 L water
   Working solution = 150 mL of 10 X stock and 300 mL of methanol diluted to 1 L water

3. Stock Wash Buffer: 10X Wash- 24.4 g Tris base dissolved to 1 L water, pH 7.4
   Working solution = 100 mL of 10X stock and 500 µL Tween-20 diluted to 1 L water