

HLAVINKA, KATHERINE, M.S. The Role of Reactive Oxygen Species in Insulin Resistance. (2013)
Directed by Dr. Yashomati M. Patel 42pp.

Insulin resistance and type II diabetes mellitus are major public health issues in the U.S.; more specifically insulin resistance is strongly correlated with obesity. Multiple factors influence insulin resistance such as hyperglycemic conditions and increased levels of reactive oxygen species (ROS). As oxygen radicals accumulate in adipocytes from increased glucose oxidation, they interfere with insulin signaling and affect glucose uptake. To determine if insulin sensitivity can be prolonged with the aid of antioxidants, adipocytes were maintained in a high glucose medium supplemented with antioxidants ascorbic acid or α -tocopherol and then tested for insulin sensitivity. Our studies reveal that ROS levels fluctuate in insulin resistant adipocytes (Day 11-21). We show for the first time that there is a decrease in ROS levels at Day 13, which then significantly increase at Day 15 and this trend continues every other day till Day 21. At Day 10 and 15 the ROS levels are high, but adipocytes have high GLUT4 expression at Day 10 and decreased expression on Day 15. By Day 15 there is increased phosphorylation of AKT but no change in IRS-1 phosphorylation. The levels of ROS were significantly decreased with the application of antioxidants. With decreased ROS levels there was overall increased phosphorylation of IRS-1 and AKT. The lipid content and distribution was not affected by ROS levels. In conclusion, the development of insulin resistance is effected by ROS, however the data indicates that there is a mechanism independent of AKT, or a target downstream of AKT that is affected and leading to the development of insulin resistance. ROS levels also regulate the expression of GLUT4 and thus GLUT4-mediated glucose uptake resulting in resistance to insulin signaling. Insulin resistance is affected by ROS levels differently if it is added exogenously, or if it is amassed

endogenously. Identification of factors that interfere with insulin signaling and thus type II diabetes, like ROS levels, can lead to the development of possible therapeutic treatments.

THE ROLE OF REACTIVE OXYGEN SPECIES
IN INSULIN RESISTANCE

by

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A Thesis Submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2013

Approved by

Committee Chair

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This thesis is dedicated to my loving and supportive family and my fiancé who have and continue to support me through my life's endeavors.

APPROVAL PAGE

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May 31, 2013 _____
Date of Acceptance by Committee

May 22, 2013 _____
Date of Final Oral Examination

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Yashomati M. Patel and committee members Dr. Zhenquan Jia, and Dr. Christopher Kepley for their guidance and support. I would also like to thank Dr. Jia's lab for the use of their EVOS imaging microscope and Dr. Steimle's lab for their help as well. I would like to thank Amit Patel, Joseph Ramos and Corrie Sapp for their assistance throughout this project. I would also like to thank the Biology Department at the University of North Carolina at Greensboro for the funding they have awarded to this project.

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CHAPTER I

INTRODUCTION

Background

Approximately one third of the adult population in the U.S. is clinically obese ¹. Even more alarming is that nearly 20% of the adolescent population is also obese ². Obesity-associated health problems such as type II diabetes mellitus, heart disease, high cholesterol, and various cancers have made obesity the seventh leading cause of death in the U.S. ¹. Obesity is not only a leading public health issue but also a major economic issue costing the U.S. \$147 billion in 2008 ³. One of the major obesity-associated diseases is type II diabetes; of patients with type II diabetes, 90% are clinically overweight ⁴. The hallmark of type II diabetes is that the body stops responding to insulin. As a key hormone regulator of energy homeostasis, insulin signals adipose and skeletal muscle tissue to uptake excess glucose from the blood. When adipocytes are constantly exposed to hyperglycemic conditions, excess energy is stored as triacylglycerol in increasing numbers and size of lipid droplets. As adipocytes continue to increase their triacylglycerol content, they increase in size, and can stop responding to insulin signaling thus stopping insulin stimulated glucose uptake. Without insulin stimulated glucose uptake into adipocyte and muscle tissues, plasma glucose levels remain chronically high, leading to the development of diabetes.

Glucose Uptake and Storage in Adipocytes

Numerous studies investigating insulin resistance have utilized the 3T3-L1 adipocyte cell culture model. 3T3-L1 preadipocytes are murine embryonic fibroblasts

that can be induced to differentiate into mature adipocytes. During differentiation the first lipid vacuoles form, once adipocytes are fully differentiated they contain many lipid vacuoles. After prolonged exposure to glucose the lipid droplets form and begin to coalesce into a unilocular fat droplet that pushes the nucleus towards the plasma membrane, giving it a “signet-ring” structure.

In the body, high glucose concentrations are sensed by pancreatic β -cells resulting in increased insulin secretion. Insulin stimulates adipose and muscle tissue to uptake glucose from the blood to lower the plasma glucose concentration back to normal levels. Insulin resistance develops when cells are constantly exposed to hyperglycemic conditions. Insulin resistance is a precursor to type II diabetes and occurs when the cells that respond to insulin require a higher concentration of insulin to stimulate glucose uptake.

Insulin stimulated glucose transport into adipose tissue is a crucial function of insulin signaling. Insulin binds to its tyrosine kinase receptor causing autophosphorylation of intrinsic tyrosine residues. The phosphorylated tyrosine residues recruit and phosphorylate insulin receptor substrate-1 (IRS-1) on tyrosine residues (Y608/632 in mouse, Y612/632 in humans), activating mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways^{5,6}. Specifically, PI3K catalyzes the formation of phosphatidylinositol-3, 4, 5-triphosphate (PIP₃), which is the allosteric activator of phosphoinositide-dependent kinase (PDK). PDK activates AKT and PKC stimulating translocation of the insulin responsive glucose transporter 4 (GLUT4) to the plasma membrane. The presence of GLUT4 in the plasma membrane facilitates glucose transport into the cell.

Activation of AKT is critical to glucose uptake and plasma glucose homeostasis. Adipocytes chronically exposed to high glucose levels stop responding to insulin and become insulin resistant. In high glucose concentrations adipocytes have lower AKT phosphorylation than those in lower glucose concentrations ⁷. With lower AKT phosphorylation, GLUT4 translocation and glucose uptake are down regulated in hyperglycemic conditions ⁷. Also, in high glucose or high insulin conditions it has been observed that there are decreased levels of GLUT4 either due to down regulation of gene transcription or post translational control of GLUT4 as insulin resistance occurs; decreased expression is coupled with desensitization of 3T3-L1 to insulin stimulated glucose uptake ⁸.

Oxidative Stress

Many factors influence the insulin signaling pathway, one of which is oxidative stress. Oxidative stress occurs when the rate at which reactive oxygen species (ROS) are generated exceeds the cell's capacity to scavenge them. Increased levels of ROS are associated with obesity and are produced during adipocyte differentiation ⁹. ROS are generated in response to growth factors and cytokines and act as natural messenger molecules, especially as a part of the proinflammatory responses; however this function is newly realized and not well understood ^{10,11}. ROS are predominantly produced by oxidative phosphorylation in the mitochondria and have been suggested as a stimulant in the progression of insulin resistance and diabetes. Most ROS are produced in the mitochondria during respiration in mitochondrial complexes I and III ¹². It has been shown that as adipocytes increase in size, the concentration of ROS increases in 3T3-L1 adipocytes ¹³. An increase in glucose, insulin, or free fatty acids can stimulate increased ROS production. Examples of ROS produced during glucose oxidation are the

superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the peroxy radical ($OH\bullet$) (figure 1) ¹⁴. Accumulation of ROS can be detrimental to the health of the cell, but can also serve as part of necessary functions in the physiology of the cell, such as the inflammatory response. ROS are radical forms of oxygen that readily react with, and cause damage to, cellular machinery, lipids, and nucleic acids ¹⁵.

Adaptive stress response pathways are naturally in place to combat the adverse effects of ROS. Typically, oxygen is used as the terminal electron acceptor in the electron transport chain in oxidative phosphorylation; on occasion it is not combined with hydrogen, but instead accepts an electron and becomes $O_2^{\bullet-}$. The enzyme superoxide dismutase (SOD) is produced in the cell to regulate the levels of $O_2^{\bullet-}$; it does this by catalyzing the conversion of $O_2^{\bullet-}$ into H_2O_2 (figure 1); H_2O_2 has the ability to diffuse through cellular membranes and is less reactive than $O_2^{\bullet-}$ ¹¹. Finally, catalase or glutathione peroxidase transforms H_2O_2 into water, rendering the radical $O_2^{\bullet-}$ inert (figure 1). Increased ROS production stimulates the activity of glutathione peroxidase, oxidizing H_2O_2 to water and thereby reducing glutathione from its oxidized form (GSH) to its reduced form (GSSG) (figure 1). Glutathione reductase then oxidizes NADPH to NADP+ and oxidizes glutathione back to GSH. NAD(P)H oxidase is another source of ROS production that attracts and stimulates macrophages in obese patients, causing injury to surrounding tissues ¹⁶. Other sources of ROS production include the p450 system, cytosolic enzymes and transition metals. Though present in bacteria and fungi, glucose oxidase is not produced in humans or murine adipocytes and does not contribute to the ROS in 3T3-L1 adipocytes ¹⁷.

In cases of hyperglycemia, oxidative phosphorylation occurs at higher rates, inadvertently producing higher amounts of $O_2^{\bullet-}$, and thus higher amounts of ROS. The

amplified concentration of ROS affects insulin stimulated glucose transport. In hyperglycemic conditions the production of ROS during differentiation increases both *in vivo* and *in vitro*^{7,18}. Studies have shown that when adipocytes become insulin resistant they produce and accumulate lower amounts of ROS, suggesting that with less glucose uptake, there is a lower production of ROS⁷.

Production and accumulation of ROS naturally occurs in adipocytes exposed to chronic high glucose conditions and occurs before the onset of insulin resistance¹³⁻¹⁵. Oxidative stress may play a causative role in insulin resistance because increased oxidative stress is a precursor to insulin resistance; yet, the direct role of oxidative stress is unknown¹⁵. Likewise, chronic insulin treatment, mimicking the necessity of higher insulin concentrations due to increased plasma glucose levels for insulin stimulated glucose uptake, has been found to increase intracellular $O_2^{\bullet-}$, H_2O_2 , and OH^{\bullet} in 3T3-L1 cells¹⁹. Once 3T3-L1 preadipocytes differentiate into mature adipocytes, there is insufficient data on the effects of ROS on the development of insulin resistance and how adipocytes deal with this stress. Insulin stimulates increased glucose uptake and thus increased cellular respiration and $O_2^{\bullet-}$ levels. If ROS play a major role in insulin resistance it may be possible to maintain insulin sensitivity if ROS are scavenged.

Antioxidant Function

Intrinsically, adipocytes counteract oxidative stress by inducing the activity of antioxidant enzymes like glutathione peroxidase, glutathione reductase, SOD, and catalase (figure 1). Adipocytes are also able to utilize antioxidant dietary compounds and vitamins to aid in the control of oxidative stress. Both ascorbic acid and alpha-tocopherol are dietary compounds known to function as antioxidants intrinsically through

the same endogenous pathways the cell uses to scavenge $O_2^{\bullet-}$, H_2O_2 , and $OH^{\bullet-}$ (figure 1).

L-ascorbic acid (vitamin C) is a water soluble vitamin that has antioxidant properties. In liver cells exhibiting ischemia, the addition of ascorbic acid causes an increase in SOD and glutathione activity²⁰. Vitamin C is especially useful in antioxidant functions because of its ability to regenerate antioxidants like catalase, glutathione peroxidase, and α -tocopherol (vitamin E) by accepting the free electron and converting from ascorbic acid into dehydroascorbate, which then breaks down^{21,22}. L-ascorbic acid and its reduced counterpart, dehydroascorbate, can be transported into a cell via simple diffusion, facilitated diffusion, and active transport mechanisms²³. There is little research on the known natural antioxidant vitamin C and its effects on insulin sensitivity in 3T3-L1 adipocytes.

Alpha-tocopherol, or vitamin E, is a fat soluble vitamin that also is a phenolic antioxidant. Vitamin E is stored in the lipid droplets of adipocytes. Alpha-tocopherol scavenges unpaired electrons from H_2O_2 or lipid peroxy radicals and mimics the action of glutathione peroxidase to diffuse the reactivity of these molecules. Though vitamin E and C have different solubility's, vitamin C has the ability to recycle vitamin E²². Vitamin E typically enters the cell via the LDL receptor pathway²⁴.

Effects of ROS on Insulin Stimulated Glucose Uptake

Scavengers of ROS could potentially prevent insulin resistance and type II diabetes⁹. Many studies have examined the effects of ROS on insulin stimulated glucose uptake in adipocytes using exogenously applied ROS e.g. H_2O_2 (figure 2a). What has not been examined is the role of endogenously produced ROS on the development of insulin resistance (figure 2b). Studies of exogenously added or applied

ROS have identified mechanistic effects of ROS on insulin stimulated glucose uptake. For instance, micro molar amounts of H_2O_2 applied to 3T3-L1 adipocytes were found to decrease insulin signaling and insulin mediated glucose metabolism^{18,25}. Increased oxidative stress was also found to down regulate GLUT4 expression in 3T3-L1 adipocytes^{25,26}. High levels of ROS inhibited insulin signaling by causing phosphorylation of IRS-1 serine residues^{27,28}. IRS-1 serine (S636) phosphorylation causes allosteric inhibition of the tyrosine residue phosphorylation and thus inhibition of IRS-1 activation by the insulin receptor²⁷⁻³⁰.

While previous studies have examined the role of exogenously added ROS, they have not investigated the impact of endogenously produced ROS. ROS overproduction is stimulated by high plasma glucose concentrations before insulin resistance occurs. As cellular respiration rates increase, there are higher levels of $O_2^{\bullet-}$ produced. The $O_2^{\bullet-}$ produced are scavenged by the adaptive stress response pathway. $O_2^{\bullet-}$ radicals have a very short half-life of 0.05 seconds and are very reactive; measuring levels of $O_2^{\bullet-}$ will give the best measure of ROS levels in differentiated adipocytes³¹. Examining the relationship between the production of ROS by glucose oxidation and how it affects glucose uptake and insulin signaling will provide insight to better understand how ROS induces insulin resistance.

Effects of Antioxidants on Insulin Stimulated Glucose Uptake

The use of an antioxidant to alleviate ROS accumulation in 3T3-L1 adipocytes may help regain insulin sensitivity in insulin-resistant adipocytes. One study found that applied antioxidants counteract the effects of exogenous H_2O_2 but do not affect the endogenous oxidative stress levels³². Another study found that by applying antioxidants that mimic the activity of glutathione and SOD prolonged insulin sensitivity and GLUT4

activity by 65%¹⁵. GLUT4 translocation and insulin sensitivity were also sustained in hyperglycemic conditions with the addition of antioxidants³³. In these and other studies antioxidants have been found to block the effects of exogenous H₂O₂, however little work has been done to alleviate endogenous H₂O₂ production. Antioxidants have only been shown to recover a portion of insulin sensitivity, but not full sensitivity³⁴. The addition of antioxidants to insulin resistant 3T3-L1 adipocytes could also alleviate insulin resistance, increase insulin signaling, and increase glucose uptake. With the antioxidants ascorbic acid and α-tocopherol, insulin-resistant 3T3-L1 adipocytes may maintain insulin sensitivity with added antioxidants in the presence of high glucose levels. Antioxidant treatment of ascorbic acid or α-tocopherol have been used in clinical studies to examine their ability to recover insulin sensitivity in insulin resistant or type II diabetic individuals³⁵⁻³⁸. However, these studies are not comparable to our study as the antioxidants are being given to individuals that are already insulin resistant; we are interested in examining the maintenance of insulin sensitivity with decreased ROS levels in high glucose conditions and examining insulin signaling and sensitivity in this environment before the onset of insulin resistance; we are not treating insulin resistant adipocytes but insulin sensitive adipocytes to determine this effect.

Lowering ROS levels, by adding antioxidants, may have a profound effect on insulin sensitivity in adipocytes. Decreasing ROS accumulation, with antioxidants, may allow insulin-resistant 3T3-L1 adipocytes to regain insulin sensitivity.

CHAPTER II

EXPERIMENTAL DESIGN (MATERIALS & METHODS)

Materials

Dulbecco's Modified Eagle's Medium (DMEM) tissue culture media was from Gibco (#11995-073). Serum supplements Bovine Calf Serum (#SH30072.03) and Fetal Bovine Serum (#SH30071.03) were from Hyclone. Penicillin Streptomycin was purchased from Gibco (#15140122). 3-isobutyl-1-methylxanthine (MIX) (#101024374), dexamethasone (DEX) (#D4902), and insulin (#15500) were obtained from Sigma-Aldrich. Trypsin-EDTA (1X) was from Gibco (#25300-054). Protein Extraction Buffer was made with PICII (#P5726) and PICIII (#P0044) from Sigma-Aldrich. Bovine Albumin (BSA) was obtained from MP Biomedical LLC (#810033). Ascorbic acid was derived from FisherScientific (#BP351-500) and α -tocopherol from Sigma-Aldrich (#258024). Primary antibodies pAKT(S473) (#9271S), AKT (#9272S), and Pan-Actin (#4968) were acquired from Cell Signaling, pIRS-1 Tyr612 (#OPA1-03183) from ThermoScientific, anti-rat carboxy-term IRS-1 (#06-248) from Upstate Biotechnology Incorporated, pan-actin from Celand Glut4 goat polyclonal (N-20) (#sc-1606) from Santa Cruz Biotechnology. Secondary anti-rabbit IgG antibodies were from SIGMA (#A6154-1ML) and donkey anti-goat IgG-HRP from Sanya Cruz Biotechnology (#sc-2020). The SuperSignal West Pico Stable Peroxide solution and SuperSignal West Pico Luminal/enhancer solutions were attained from ThermoScientific (#34077). Nitortetraxolium Blue chloride (NBT) was obtained from Sigma-Aldrich (#N6876). Phosphate Buffered Saline was obtained from FisherScientific (#BP399.500). Guava

ViaCount Reagent for FLOW Cytometer was from EMD Millipore Corporation (#4000-0040).

Cell Culture

Murine 3T3-L1 preadipocytes were grown to confluency in high glucose DMEM with 10% Bovine Calf Serum (BCS). Forty-eight hrs post confluency (Day 0) the 3T3-L1 preadipocytes were induced to differentiate in high glucose DMEM containing PenStrep and 10% Fetal Bovine Serum (FBS) with 0.52 mM 3-isobutyl-1-methylxanthine, 1 μ M dexamethasone, and 1.7 μ M insulin. On Day 4, the medium was changed to a high glucose DMEM, 10% FBS and 1 μ M insulin. The media was replaced every 2 days with DMEM-10% FBS. Cells were fed the day before being assayed.

Antioxidant Treatment Groups

Adipocytes were kept at a high glucose concentration and with added antioxidants α -tocopherol (40 μ M) and ascorbic acid (60 μ M) to the treatment groups from Day 7 through Day 15.

NBT Assay

The nitroblue tetrazolium (NBT) assay is used to quantify ROS produced in cells, specifically to measure the amount of superoxide anions produced in cells. NBT, a yellow solution, is membrane permeable and is reduced by the superoxide anion to formazan, a blue insoluble form of NBT. 3T3-L1 preadipocytes were differentiated. At the time of NBT testing, cells were washed 3 times at room temperature with 1X PBS. NBT (0.2%) in 1X PBS or 1X PBS was applied to the experimental and control samples respectively. The treated cells were incubated at 37°C and 5% CO₂ for 90 min. Following incubation, the treatments were aspirated and trypsin was added to cells for 60 sec. The cell suspension was centrifuged at 1400 rpm for 10 min. The supernatant

was discarded and 50% acetic acid in 1X PBS was added. Samples were sonicated. Samples were transferred to a 96 well plate and the absorbance was measured at 560 nm³⁹⁻⁴¹.

Total Protein Extraction

3T3-L1 preadipocytes were differentiated, on Day 7 cells were treated with or without antioxidants. For each treatment group at Days 10 and 15, cells were washed twice with 1X PBS. Following the washes, Pessin protein lysis buffer (616 μ L dH₂O, 70 μ L protease inhibitor (10X), 7 μ L PICII and 7 μ L PICIII) was prepared and added to cells.

The cell extract was rocked for 20 min at 4°C. Following rocking, the extract was centrifuged for 20 min at 6000 rpm at 4°C. After centrifuging, the protein extract was removed and stored at -20°C.

Immunoblotting

Approximately 20-30 μ g protein extract was subjected to SDS-PAGE. After electrophoresis, the proteins were transferred to Immobilon filters. The Immobilon filter was incubated in 4% BSA in 1X Tris-Buffered Saline – Tween 20 (1X TTBS) (0.05 M tris, 0.15 M NaCl, 1% Tween-20 at pH 7.5) for an h. Primary antibodies were incubated with the Immobilon filter at a dilution of 1:1000 overnight at 4°C. The primary antibody was then removed and the Immobilon filter was washed 3 times at increments of 7 min each, using 1X TTBS. A secondary antibody was then used at a 1:2000 dilution in 1X TTBS for 45 min. After 45 min, the Immobilon filter was washed 3 times at 7 min increments in 1X TTBS.

Enzymatic Chemiluminescence (ECL)

The SuperSignal West Pico kit was used to detect specific proteins on the Immobilon filter and imaged on the Bio-Rad ChemiDoc XRS imaging station.

Cell Viability Assay on FLOW Cytometer

3T3-L1 adipocytes were grown to confluency and treated as described above. At Days 10 and 15 cells were harvested using trypsin. Equal volumes of media was added to stop trypsinization of cells and the suspension was centrifuged at 5 for 5 minutes. The supernatant was removed and the cells were resuspended in 500 μ L of 1X PBS. A 1:20 dilution was made with the Guava ViaCount Reagent. Samples were vortexed and placed in the dark for 5 minutes and then assayed by flow cytometry.

Quantification of Lipid Droplets

Day 10 and 15 unstained adipocytes were imaged on an AMG EVOSxl imaging station using phase contrast microscopy at 40X and 100X. Lipid droplet diameters from 20 adipocytes per day, per treatment were counted and categorized by size on both Day 10 and 15. The diameter size categories measured were <6.25 μ m, 6.25-12.5 μ m, 12.5-25 μ m, and >25 μ m. The percent distribution of lipid droplets by size was calculated.

Statistical Analysis

Data are represented as \pm standard deviation of at least three independent experiments. Student's t-test was used for statistical analysis. Differences were considered statistically significant at $p < 0.05$.

CHAPTER III

RESULTS

ROS Levels Fluctuate After the Onset of Insulin Resistance

Previous studies have shown that initially there is an increase in ROS from Day 0 to 7 while differentiation occurs; however, little is known about the ROS levels in differentiated adipocytes as they become insulin resistant. The NBT assay was utilized to determine the levels of ROS produced in mature adipocytes. NBT is able to diffuse through the cell membrane and react with the superoxide anion to form formazan (a blue precipitate). In high glucose medium, increase cellular respiration occurs resulting in increased $O_2^{\bullet-}$ production; thus the NBT assay was optimal to measure $O_2^{\bullet-}$ levels as a representative sample of ROS levels in mature adipocytes. ROS levels were measured using the NBT assay every other day, from Day 7 through Day 21. As seen in figure 3, after differentiation, adipocytes produce relatively high levels of ROS (Day 7-11). While ROS levels remain fairly constant when adipocytes are insulin sensitive (up to Day 10), after Day 11 ROS levels fluctuate from low to high every other day (figure 3). At Day 13 ROS levels decrease to about half the levels experienced after differentiation on Day 10. The ROS then increase back to levels present in insulin sensitive adipocytes within two days. This significant rise in ROS indicates that adipocytes are experiencing oxidative stress and may be a major factor promoting insulin resistance. This experiment was repeated three times and a consistent flux of ROS levels was observed each time. These findings suggest that after Day 11 (at a time point when adipocytes are sensitive to insulin) ROS levels are not stable and may indicate the onset of insulin resistance.

Determination of Antioxidant Treatment Concentrations

Previous studies have shown that antioxidant treatment can decrease high levels of ROS. Thus we wanted to determine if antioxidants could reduce the high levels of endogenous ROS produced in insulin sensitive and resistant cells. To determine the effects of antioxidants on insulin resistant adipocytes we used two well characterized antioxidants, ascorbic acid and α -tocopherol. To determine the optimal concentration of ascorbic acid and α -tocopherol required to reduce ROS levels in differentiated adipocytes, we tested concentrations used previously for differentiating adipocytes^{42,43}. Thus, we assayed ROS levels at various antioxidant concentrations in insulin sensitive (Day 10) and insulin resistant (Day 15) adipocytes. Starting on Day 7 adipocytes were treated with varying ascorbic acid or α -tocopherol concentrations in dH₂O and DMSO respectively until Day 10 or 15. A viability assay was performed to verify that the vehicle or antioxidant concentrations were not detrimental to cell viability. We found that increasing concentrations of ascorbic acid or α -tocopherol had no effect on cell viability (figure 4a, b). As seen in figure 4c the ascorbic acid concentration (60 μ M) used previously in differentiating adipocytes was sufficient to significantly lower ROS levels in mature adipocytes compared to both untreated and vehicle (dH₂O) treated adipocytes. ROS levels were significantly decreased at 60 μ M ascorbic acid to approximately the same levels as high concentrations. Concentrations higher than 60 μ M elicited no further reduction of ROS, and at Day 15 actually enhanced the ROS production at the high concentration of 500 μ M (figure 4c). The optimal α -tocopherol concentration utilized on differentiating adipocytes (40 μ M) was shown to be ample enough to significantly reduce ROS levels in mature adipocytes compared to both untreated and vehicle (DMSO) treated adipocytes (figure 4d). Increasing concentrations of α -

tocopherol concentrations did not further enhance the reduction of ROS (figure 4d). Therefore, concentrations of ascorbic acid treatments used from this point forward are 60 μ M, while the concentration of α -tocopherol is 40 μ M. As seen in figure 4c and 4d, we used antioxidant concentrations that were used previously on differentiating adipocytes.

Antioxidant Treatment Decreases ROS Levels

To determine the effectiveness of the antioxidants in adipocytes, the levels of ROS on Days 10 and 15 were determined in the presence and absence of antioxidant concentrations optimized previously. Ascorbic acid treatment significantly decreased ROS levels in both Day 10 and 15 adipocytes to levels significantly lower than those seen during the flux period (figure 5). A concentration of 60 μ M ascorbic acid showed a significant decrease in ROS levels compared to untreated adipocytes at both Day 10 ($p = 0.040$) and at Day 15 ($p = 0.023$) (figure 5). Similarly, α -tocopherol significantly decreased ROS levels to a greater extent than ascorbic acid at Day 10 ($p = 0.004$) and at Day 15 ($p = 0.023$) when compared to untreated cells (figure 5). These findings indicate that since the levels of ROS are significantly lowered when compared to untreated cells, Day 10 and 15 adipocytes are not resistant to the effects of ascorbic acid and α -tocopherol (figure 5).

Decreased ROS Levels do not Effect Lipid Accumulation

While some studies have shown that ROS play a role in insulin resistance, other studies have implicated lipid droplet size as a factor in the development of insulin resistance. As adipocytes are constantly maintained in high glucose conditions they intake glucose and gain lipid storage. Day 10 adipocytes have numerous small lipid droplets and very few large droplets whereas Day 15 adipocytes have far more larger

droplets (figure 6). As lipid is accumulated in adipocytes the lipid droplets coalesce into larger droplets and render the cell resistant to insulin signaling¹³. As the lipid droplets increase in size and decrease in number inside adipocytes, the levels of ROS increase¹³. As expected, the majority of lipid droplets in Day 10 and 15 adipocytes are smaller than 6.25 μm in diameter (figure 7). As droplets size increases the number of droplets diminishes, and the majority of droplets should be relatively small (<6.25 μm). As adipocytes gain lipid the ROS levels increase; they also become insulin resistant^{9,13}. What is unknown is the relationship between the size of lipid droplets in adipocytes and levels of ROS. To determine this relationship we treated Day 7 adipocytes with antioxidants and measured lipid droplet diameter and distribution after 3 and 8 days of treatment (figure 7, table 1). Of the cells counted, the number of cells containing each lipid droplet size range does not significantly differ between control and treatment groups on Day 10 or 15 (table 1). Decreasing ROS using antioxidants should allow increased storage of lipid in adipocytes compared to untreated adipocytes. Both ascorbic acid and α -tocopherol treatment did not change lipid accumulation on Day 10 or 15 compared to untreated cells (figure 6). Based on the distribution of lipid droplets as well as the number of large droplets per cell, neither ascorbic acid nor α -tocopherol treatment altered lipid parameters.

ROS Levels Alter GLUT4 Protein Expression

As adipocytes become larger and insulin resistant the expression of GLUT4, a key component in the insulin signaling pathway, is decreased. Studies have shown that decreased GLUT4 expression is coupled with decreased insulin stimulated glucose uptake⁴⁴. To verify that Day 10 adipocytes are insulin sensitive and Day 15 adipocytes are insulin resistant we assayed the levels of GLUT4. A decrease in GLUT4 expression

would indicate that the cells have a decreased ability to take-up glucose. Total protein was extracted and subject to a 10% SDS-PAGE; and subsequently immunoblotted with antibodies to GLUT4 and actin. The expression of GLUT4 decreased from Day 10 to 15 in untreated adipocytes as is seen in figure 8a. The decrease in GLUT4 expression is indicative of decreased insulin sensitivity. Adipocytes treated with antioxidants displayed increased expression of GLUT4 at Day 15 compared those that were untreated (figure 8b-c). Ascorbic acid treatment allows for equal GLUT4 expression on Days 10 and 15 (figure 8b). Similarly, α -tocopherol treatment allowed for equal or increased GLUT4 expression (figure 8c). Our results indicate that decreased ROS level allows for increased GLUT4 expression, and thus increased insulin sensitivity at Day 15.

IRS-1 Activation

GLUT4 mediated glucose up-take requires activation of the insulin signaling pathway. The primary substrate of the insulin receptor is IRS-1. Using antioxidants to decrease ROS levels, IRS-1 phosphorylation status was evaluated by extracting and subjecting total proteins to a 6% SDS-PAGE, and performing an immunoblot with antibodies to phosphorylated IRS-1, or with antibodies to total IRS-1. As seen in figure 9a, Day 10 adipocytes exhibit about a 6 fold increase in IRS-1 phosphorylation and a 5 fold increase at Day 15, however this difference is not significant indicating that the stimulation of IRS-1 activation by insulin signaling is not affected by adipocyte age. When ROS levels were decreased, via treatment with antioxidants ascorbic acid or α -tocopherol, the phosphorylation of IRS-1 increased both on Day 10 and 15 when normalized to the phosphorylation levels on each respective day without antioxidant treatment (figure 9b). IRS-1 phosphorylation exhibited a higher fold increase on Days 10 and 15 when treated with α -tocopherol compared with ascorbic acid. Phosphorylation

of IRS-1 was not affected by adipocyte age as its status was increased equally on both days when treated with ascorbic acid or α -tocopherol. Thus, our findings indicate that IRS-1 phosphorylation is not suppressed by high levels of ROS in Day 10 or 15 adipocytes in contrast to studies by Lin *et al.* which used Day 8 and 12 adipocytes and saw decreased phosphorylation of IRS-1 as a result of increased ROS. This may indicate that another signaling molecule in the insulin signaling cascade is potentially affected by ROS and hindering insulin sensitivity⁷.

AKT Activation

Insulin activated IRS-1 leads to the activation of AKT. AKT is a key component of insulin stimulated glucose uptake; phosphorylation of AKT is a major component in mediating GLUT4 translocation⁴⁵. At high glucose concentrations when adipocytes become insulin resistant the phosphorylation of AKT decreases⁷. Previous work has shown that when H₂O₂ is applied to insulin-sensitive adipocytes, the activation of AKT is inhibited⁴⁵. AKT phosphorylation status was evaluated in the presence and absence of antioxidants by extracting and subjecting total proteins to a 10% SDS-PAGE, and immunoblot analysis with antibodies to phosphorylated AKT or total AKT. As seen in figure 10a, Day 15 adipocytes have about a 10 fold increase in AKT activation compared to Day 10 adipocytes. This may indicate that at Day 15 there is an underlying mechanism that functions to respond to insulin signaling as the cells become resistant. When ROS were decreased in adipocytes, via ascorbic acid or α -tocopherol treatment, the phosphorylation of AKT increases both on Day 10 and 15, but follows the trend seen in untreated cells of increasing phosphorylation of AKT at Day 15 compared to Day 10 (figure 10). AKT phosphorylation levels in the presence of antioxidants was normalized to AKT phosphorylation levels in insulin stimulated untreated adipocytes. Our studies

indicate that AKT is not a primary target of ROS in adipocytes during the development of insulin resistance.

CHAPTER IV

DISCUSSION

Insulin resistance is a precursor of type II diabetes mellitus. Hyperglycemic conditions induce high insulin concentrations in the blood and stimulate the insulin signaling cascade in adipose tissue initiating free radical formation through increased ATP production via glucose oxidation. These high glucose conditions also lead to the onset of insulin resistance. The role which ROS play in the development of insulin resistance may be protective, functioning to alleviate excess radical formation via glucose oxidation; it may also be a byproduct of increased glucose uptake and oxidation, adversely leading to insulin resistance.

The majority of studies examine changes in insulin signaling in differentiating adipocytes, which encompasses Day 0 through Day 7, while adipocytes are extremely insulin sensitive. However, examining insulin sensitivity after Day 7 and its natural decline can provide insight into the development of disease states, like insulin resistance. Once adipocytes are fully differentiated (Day 7) they respond to insulin signaling and grow in size but, over time, they lose insulin sensitivity approximately around Day 12. Typically, most studies examining insulin sensitive events use Day 7 to 10 adipocytes⁴⁶. Very few studies have examined the insulin response in adipocytes after Day 10. It is important to study adipocytes as they become insulin resistant to determine the factors, like ROS, that influence insulin signaling.

Adipocytes are insulin responsive and thus perform glucose uptake from Day 7 to Day 10^{7,15,28}. After Day 10, insulin sensitivity and glucose uptake decline rapidly. In insulin sensitive adipocytes (Day 7-10) GLUT4 expression is high allowing for good glucose uptake, however GLUT4 expression decreases by Day 15 indicating decreased glucose uptake and insulin resistance (figure 8a). Day 10 and 15 adipocytes have equal phosphorylation of IRS-1 suggesting that the targets involved in insulin resistance are downstream of IRS-1 (figure 9a). Surprisingly, AKT phosphorylation increases from Day 10 to 15 indicating that an insulin independent mechanism is initiating phosphorylation of AKT at Day 15 when the adipocytes are insulin resistant (figure 10a). This suggests that there is a complex regulatory mechanism. It may be that while adipocytes are sensitive to insulin signaling, ROS regulate AKT phosphorylation, but after the destabilization of ROS levels, adipocytes become insulin resistant and there is a response mechanism in place to increase AKT activation.

We also observed a novel trend in ROS levels from Day 7 to 21 as adipocytes lose insulin sensitivity. The oscillating trend of ROS (figure 3) production after terminal differentiation was unexpected. We are the first to show this pattern of ROS levels. Okada *et al.* 2010 observed lipid accumulation and ROS levels out to Day 28, but only looked periodically, at Day 8, 18, and 28; Okada *et al.* observed a significant decrease in ROS at each time point, but only looked at days where we observed a decline as well; they did not observe the intermediate increase in ROS every 4 days⁴⁷. Because we monitored ROS levels every other day we were able to document the fluctuating levels of ROS. This trend could be due to the onset of insulin resistance; as adipocytes become insulin resistant they may have a feedback mechanism to decrease both glucose uptake and ROS levels to alleviate stress on cells¹³. Also, the fluctuation in

ROS levels could be a result of the antioxidant response system in the adipocyte; when oxidative stress is experienced there is an increase in expression of antioxidant enzymes to compensate for the stress. Alteration in the antioxidant response mechanism activating Nrf2 responsive gene transcription could account for this flux and should be investigated further. The pattern of ROS levels suggests that there is a cycling of oxidative stress responses every few days in response to high glucose concentrations. To determine that the high ROS levels exhibited on Days 7-11, 15, and 19 are a function of high glucose concentrations in the media, ROS levels should be determined in adipocytes treated with low glucose media (5mM) (plasma homeostatic glucose concentrations).

As adipocytes age they accumulate ROS⁷. In this study the source of free radical formation was designed to be endogenous, stemming from the oxidation of glucose from the high glucose medium (25mM), and not as an exogenous source utilized in previous studies^{7,15}. Typically, 3T3-L1 cells are differentiated in a high glucose medium, mimicking hyperglycemic conditions similar to what is seen in pre-diabetic subjects. Previous research has shown that preadipocytes differentiated in either high or low glucose medium display differences in insulin sensitivity. Lin *et al.* (2005) showed that differentiation in a high glucose medium induced severe insulin resistance when compared to cells differentiated in a low glucose medium⁷. Moreover, an increased level of ROS in adipose tissue is associated with the onset of diabetes¹³. As ROS are produced endogenously, the free radicals take the form of the superoxide anion, hydrogen peroxide, or the peroxy radical. Endogenously there are multiple point sources of these free radicals; to understand which source is contributing to the level of

ROS generation would lead to a better understanding of how ROS are involved in insulin resistance onset.

The insulin signaling cascade promotes glucose uptake in adipocytes and stores it as triacylglycerol. The lipid content within the adipocyte is indicative of insulin sensitivity. As adipocytes increase their lipid deposits, multiple smaller lipid droplets indicate sensitivity to insulin signaling, while larger lipid droplets are indicative of insulin resistance. Day 10 adipocytes have small lipid droplets and very few large droplets; conversely Day 15 adipocytes have amassed more small lipid droplets, but also have more large lipid droplets (figure 6, table 1). The effect of ROS on lipid droplet formation has not been observed previously. Insulin sensitive adipocytes have smaller lipid droplets, and high levels of ROS (figure 3). As adipocytes become insulin resistant the level of ROS fluctuate; at Day 15 the ROS levels are relatively high (figure 3).

Antioxidants ascorbic acid and α -tocopherol were employed to suppress ROS levels and determine the role of ROS in the development of insulin resistance. The addition of ascorbic acid or α -tocopherol significantly decreased ROS levels at Day 10 and 15 (figure 5). Though the adipocytes are becoming insulin resistant, they are not resistant to the antioxidant effects of ascorbic acid or α -tocopherol (figure 5). As insulin resistance occurs there is a difference in the activation and suppression of crucial insulin signaling proteins (table 2). Previously, as indicated

by Lin *et al.*, IRS-1 is activated when phosphorylated on Tyr608/632⁷. However, decreased ROS levels greatly increased phosphorylation of IRS-1 and AKT on both Day 10 and 15 equally (figure 9, 10). Once quantified it was evident that decreased ROS levels do not have a significant effect on lipid accumulation (figure 7, table 1).

Since ascorbic acid is a polar molecule, it is taken up into the cell through passive, facilitative, and active diffusion. In adipocytes it is transported into the cell via dehydroascorbic acid-facilitative diffusion via the GLUT1, 3, and 4 transporters and has been found to be competitive with glucose²³. As we see in figure 4, as the concentrations of ascorbic acid treatment increase, the level of ROS plateau until Day 15 with 500 μ M treatment. The spike in ROS levels at this day may be attributed to the pro-oxidant qualities of ascorbic acid at high concentrations (figure 4c)⁴⁸. However, the lower concentrations were sufficient to induce a significant reduction of ROS levels and allowed us to examine how lowered levels of ROS affect adipocytes.

With antioxidant treatment GLUT4 expression was observed not to decrease but remained consistent from Day 10 to 15 (figure 8). This finding indicates that ROS are a major player in the regulation of GLUT4 expression, and that high levels of ROS are detrimental to the insulin sensitivity of adipocytes. ROS have been identified as playing a role in the development of insulin resistance; yet it is difficult to determine the exact mechanism it works through. Here we have demonstrated that ROS do play a role in suppression the activation of IRS-1 and AKT (table 2). We also have identified that ROS do not have an integral role in the accumulation and storage of lipid. By decreasing ROS levels we observed consistent expression of GLUT4 in adipocytes that otherwise would have developed insulin resistance.

When studying the effects of ROS it is also important to study antioxidant enzyme activity. Future studies that concentrate on the activity of antioxidant enzymes as ROS levels increase (catalase, glutathione peroxidase, SOD) could clarify when oxidative stress is occurring in adipocytes as insulin resistance is developing. Also, better methods of measuring ROS levels and source points of ROS in cells are needed

to understand a more accurate picture of the molecular mechanism occurring at the onset of insulin resistance. Using antioxidants to decrease ROS levels has been useful for this study, however clarification of how ascorbic acid and α -tocopherol are transported into cells would result in a better understanding of how much is transported into adipocytes and if increased application of these antioxidants results in increased antioxidant function. Moreover, N-acetyl cysteine (NAC) or another strong antioxidant may give more defined results as to how decreased ROS levels affect insulin signaling. While most clinical studies have been inclusive when treating insulin resistant individuals with antioxidants, this may have been due to the fact that treatment was administered after the onset of insulin resistance. As we have seen here antioxidant treatment allowed maintenance of insulin sensitivity in high glucose conditions. Though we did not look at recovery of insulin sensitivity in untreated Day 15 adipocytes, we did observe the maintenance of insulin sensitivity with antioxidant treatment when comparing the analysis of Day 15 to untreated Day 15 adipocytes.

In conclusion, the regulation and development of insulin resistance in 3T3-L1 adipocytes is much more complex and influenced by more than increased levels of ROS, however ROS do play a major role in its development through GLUT4 suppression. Here we have demonstrated that exogenous and endogenous ROS influence insulin resistance via different mechanisms (table 3). The addition of exogenous ROS inhibits phosphorylation of IRS-1 whereas when looking at endogenous ROS levels this is unaffected (table 3). The development of insulin resistance is better thought of as a continuing process, not a state, as the signaling molecules and ROS levels are in constant flux from the trend seen in the levels of ROS. Further examination of how endogenous ROS effect insulin signaling will allow for a more comprehensive

understanding on the mechanism behind insulin resistance development. When diagnosed with insulin resistance or pre-diabetes, exercise and a healthy diet can help to combat/reverse this diagnosis. This continuous process that is insulin resistance is best recognized and responded to quickly.

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APPENDIX A

TABLES

Table 1. Frequency of lipid droplets sized by diameter. Lipid droplet diameter was measured and categorized in 20 adipocytes per treatment and imaged at 100X using light microscopy.

Diameter	Day 10				Day 15			
	<6.25µm	6.25-12.5µm	12.5-25µm	>25µm	<6.25µm	6.25-12.5µm	12.5-25µm	>25µm
Vehicle	20	18	4	0	20	17	9	0
Ascorbic Acid	20	14	10	1	20	13	4	0
α-tocopherol	20	15	1	0	20	18	9	1

Table 2. Characteristics of insulin sensitive and insulin resistant adipocytes. Comparative depiction representing the differences of adipocyte physiology and how endogenous, exogenous, and scavenged ROS influence insulin signaling.

Day	Day 10		Day 15	
	-	+	-	+
Antioxidant Treatment	-	+	-	+
Glucose Uptake	Good	Good	Poor	Good
GLUT4	High expression	High expression	Low expression	High expression
IRS-1	Phosphorylated	Increased phosphorylation	Phosphorylated	Increased phosphorylation
AKT	Phosphorylated	Increased phosphorylation	Increased phosphorylation	Increased phosphorylation
Lipid Droplet Size	Small and large droplets	Small and large droplets	Increased lipid storage	Increased lipid storage
ROS Levels	High	Low	High	Low

Table 3. Comparison of endogenously produced and exogenously added ROS. Summary of differences between previous studies examining the effects of added exogenous, H₂O₂ and our study examining endogenously produced ROS on insulin signaling and sensitivity^{5,7,15,18}.

Source of ROS	Exogenous	Endogenous
Glucose Uptake	Poor	Poor
GLUT4	Low expression	Low expression
IRS-1	Suppressed Activation	No change in activation
AKT Phosphorylation	Decreased	Increased
ROS Levels	High	High

APPENDIX B

FIGURES

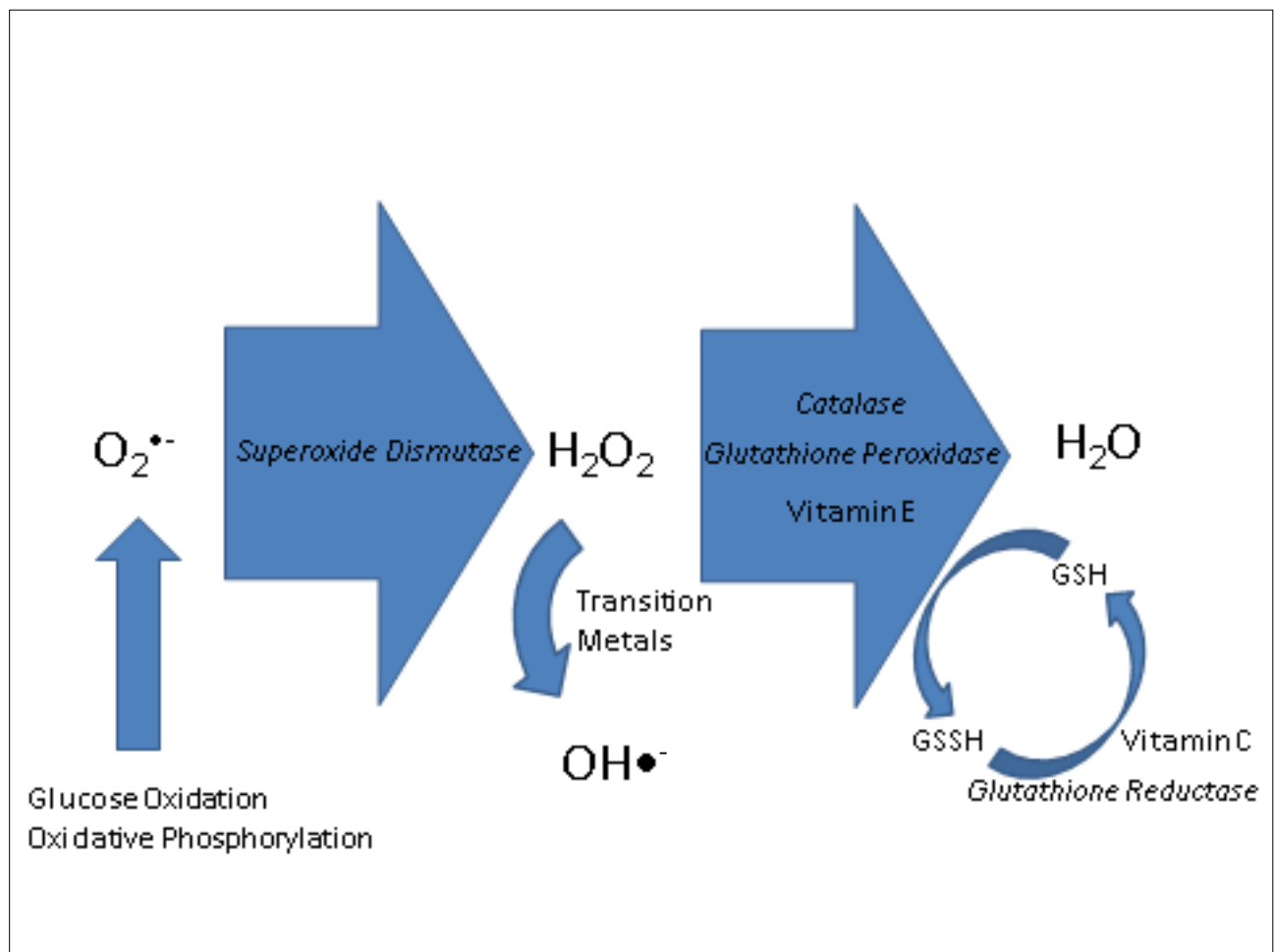


Figure 1. Antioxidant mechanism. Schematic representation of free radical formation and the antioxidant response mechanism. Vitamin E and C mimic antioxidant enzymes glutathione peroxidase and reductase respectively. Superoxide anion ($O_2^{\bullet-}$), Hydrogen peroxide (H_2O_2), Hydroxyl Radical (OH^{\bullet}), Glutathione reduced form (GSH), and Glutathione oxidized form (GSSH).

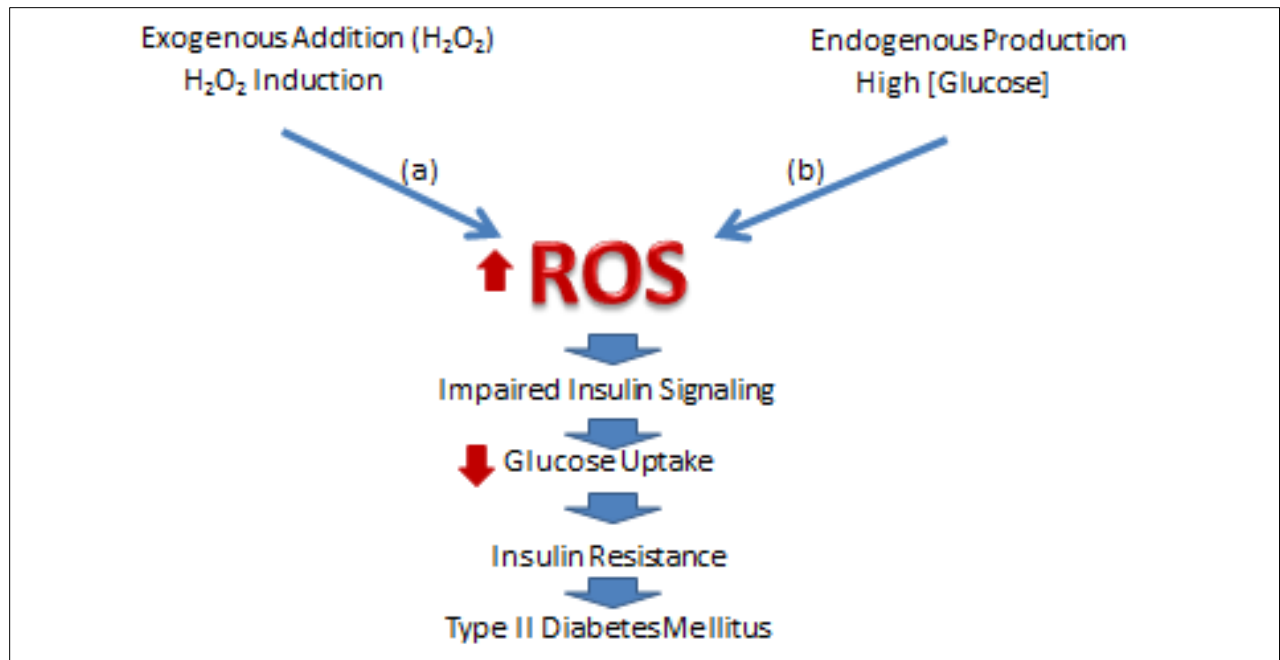


Figure 2. Production of ROS in adipocytes. Schematic representation of the effects of exogenous ROS and the proposed effects of increased endogenous ROS on the development of insulin resistance.

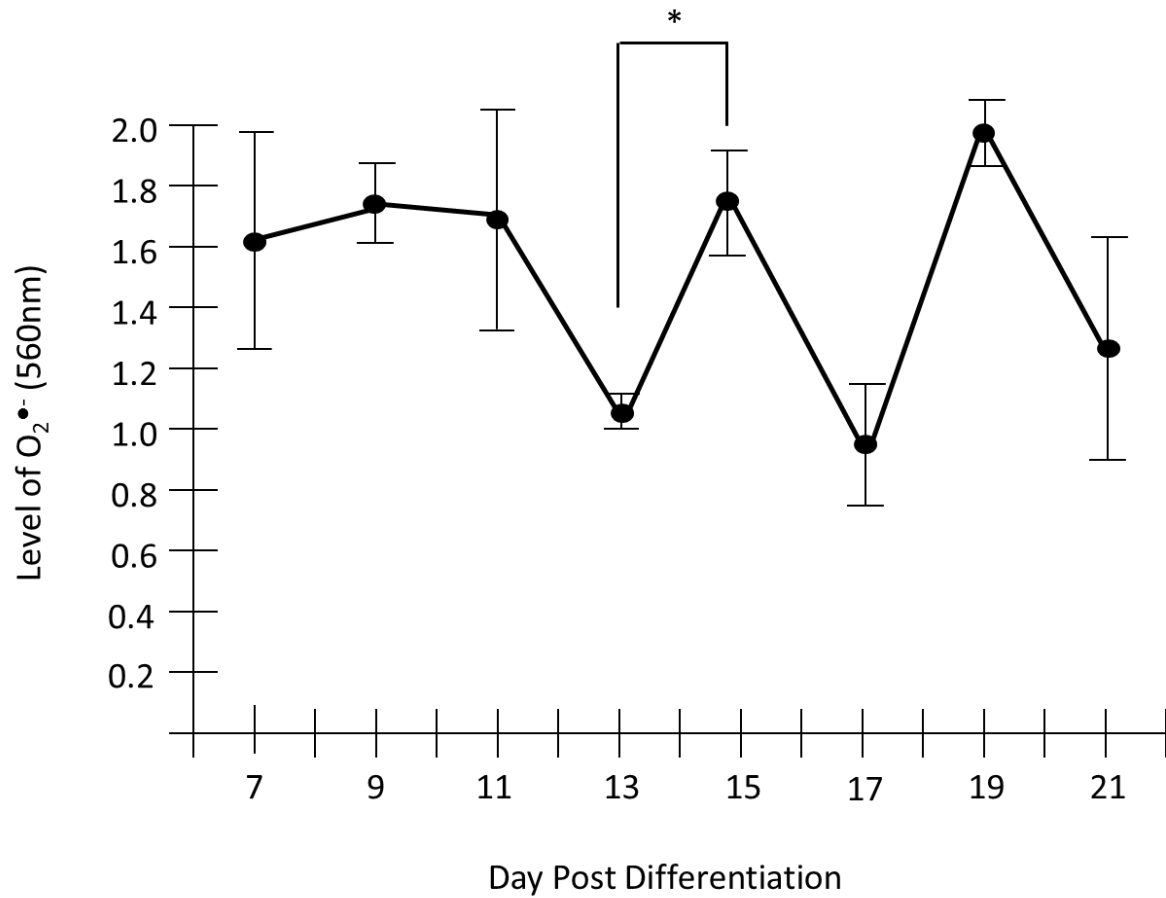


Figure 3. Production of ROS in mature 3T3-L1 adipocytes. Mature adipocytes were assayed for ROS (O₂^{•-}) production by NBT reduction every other day from Day 7 to 21. Cells were treated with 0.2% NBT for 90 min in the dark. NBT was reduced to formazan by ROS and dissolved in 50% acetic acid. The absorbance was determined at 560 nm. Results are representative of 3 independent experiments. (* $p=0.032$)

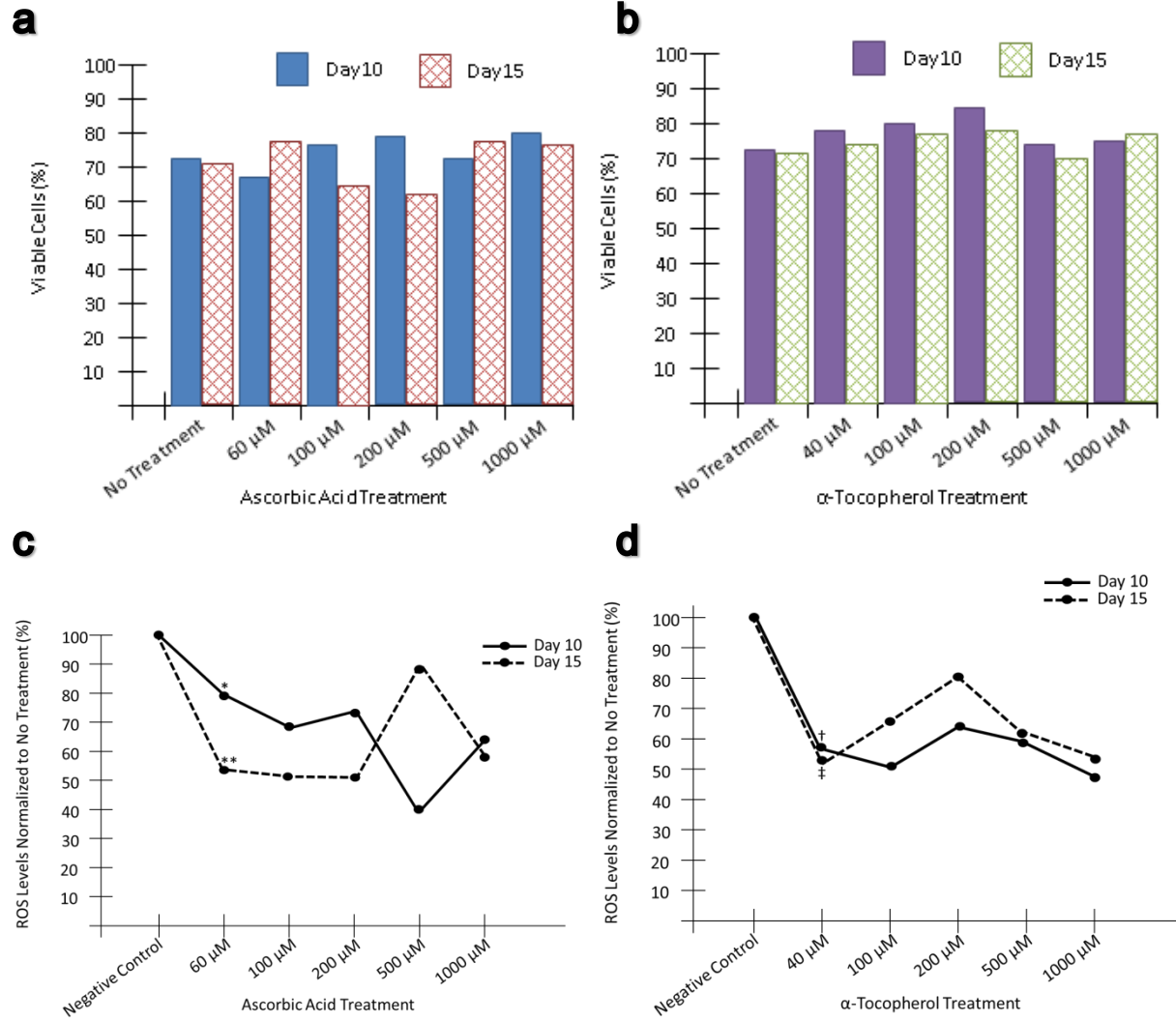


Figure 4. Determination of the effective concentration of ascorbic acid and α -tocopherol for treatment of mature 3T3-L1 adipocytes. Mature 3T3-L1 adipocytes were treated with ascorbic acid (0 to 1000 μ M), α -tocopherol (0 to 1000 μ M). Viability was assessed by FLOW Cytometry with increasing concentrations of (a) ascorbic acid and (b) α -tocopherol. Levels of ROS were measured via the NBT assay in the presence or absence of ascorbic acid (c), or α -tocopherol (d). (* $p=0.04$, ** $p=0.017$, † $p=0.004$, ‡ $p=0.023$)

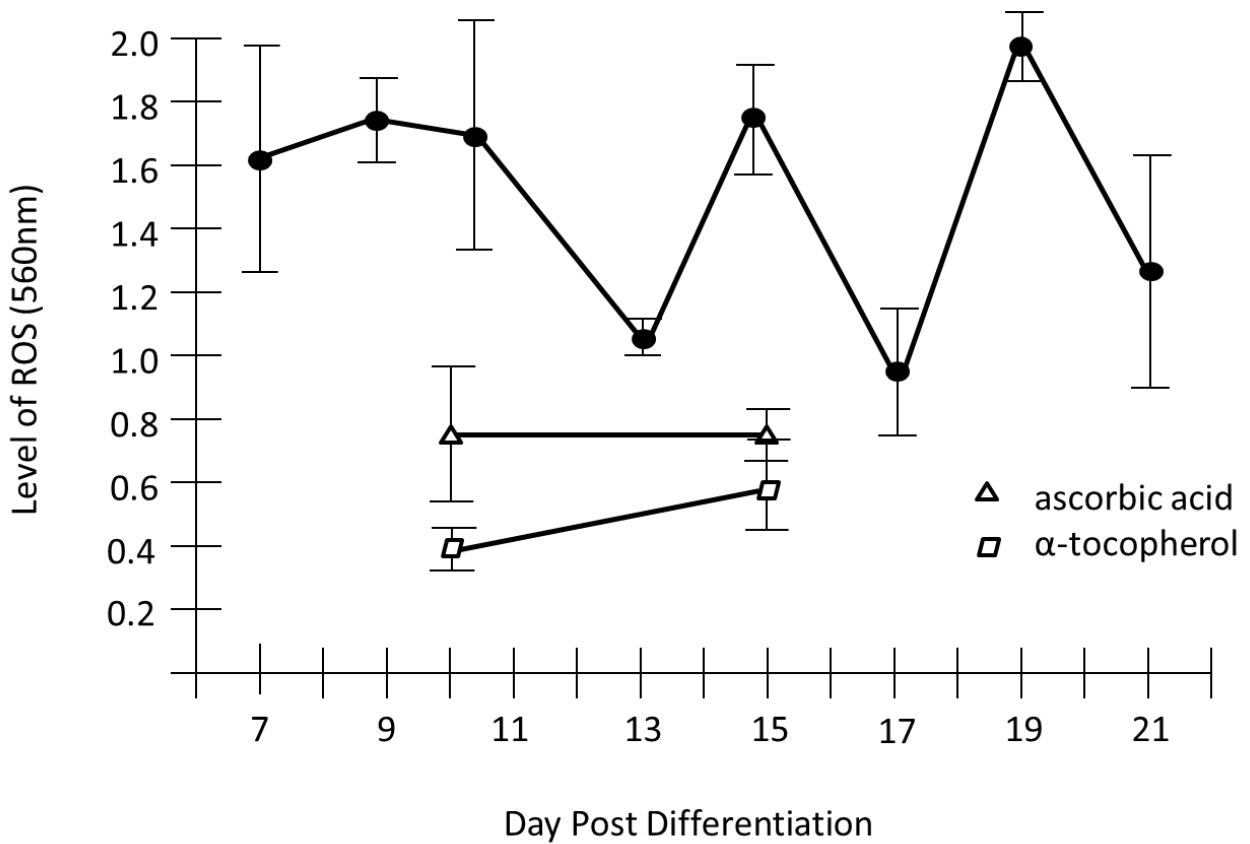


Figure 5. Antioxidant treatment of 3T3-L1 adipocytes. Mature adipocytes treated with 60 μ M ascorbic acid or 40 μ M α -tocopherol were measured for ROS levels using the NBT assay on D10 and D15. Results are representative of 3 independent experiments.

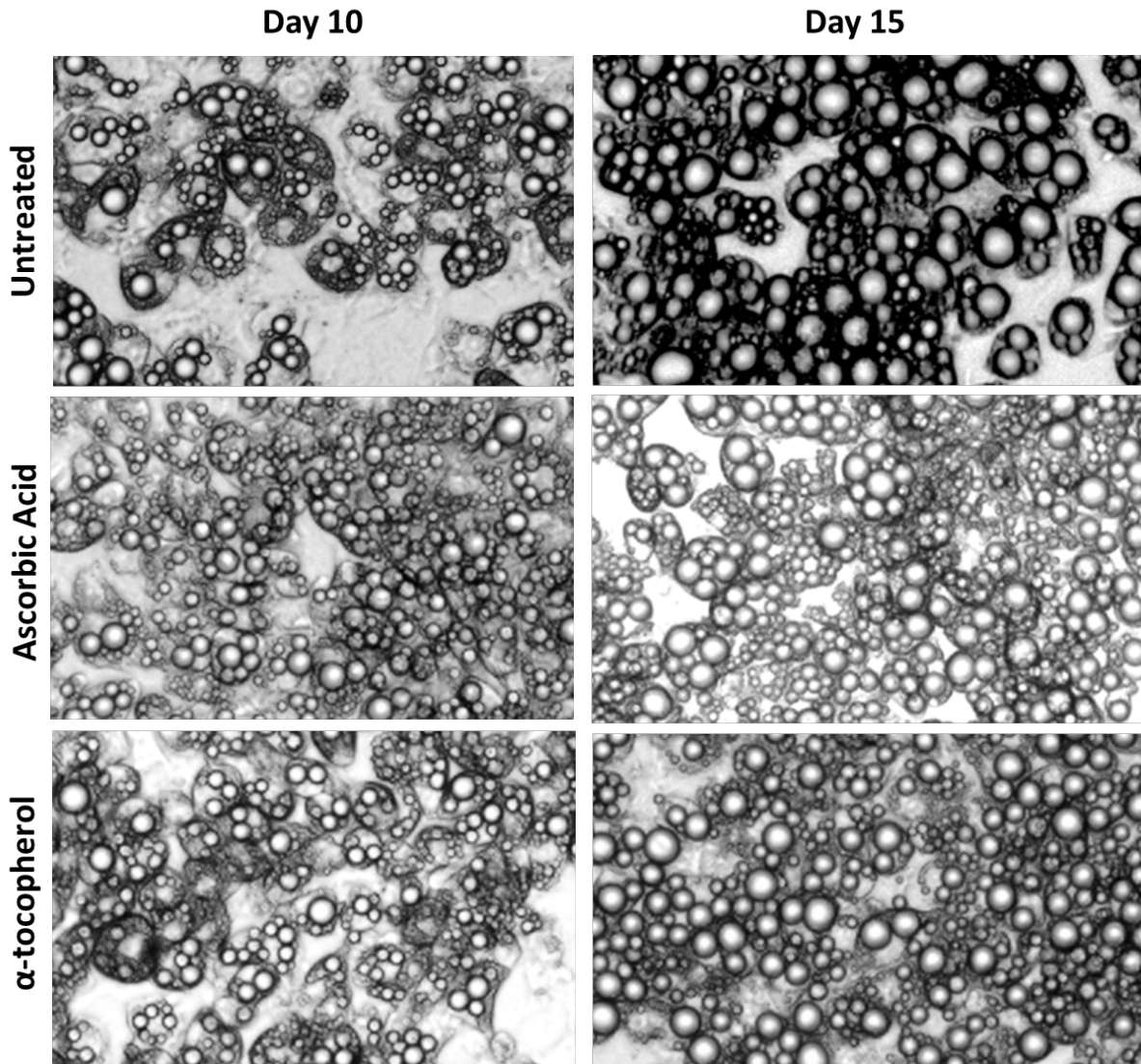


Figure 6. Lipid accumulation in 3T3-L1 adipocytes. Day 7 adipocytes were either untreated or treated for 3 or 8 days with 60 μ M ascorbic acid or 40 μ M α -tocopherol. Unstained adipocytes were imaged on Days 10 and 15 on an AMG EVOSxl imaging station using phase contrast microscopy at 40X during differentiation.

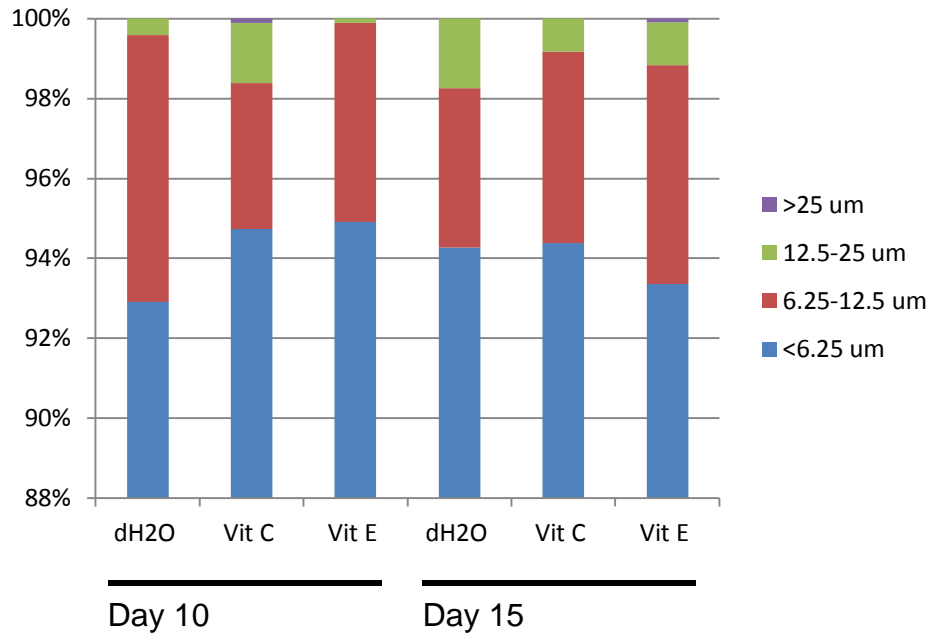


Figure 7. Lipid distribution by size. Adipocytes were treated with vehicle (dH₂O), 60 μM ascorbic acid, or 40 μM α-tocopherol beginning at Day 7. At Day 10 and 15 they were imaged on the AMG EVOSxl at 100X. Lipid droplets from 20 adipocytes per day and treatment were counted and categorized by size at 100X. The percent distribution of lipid droplets by size was calculated and is displayed above.

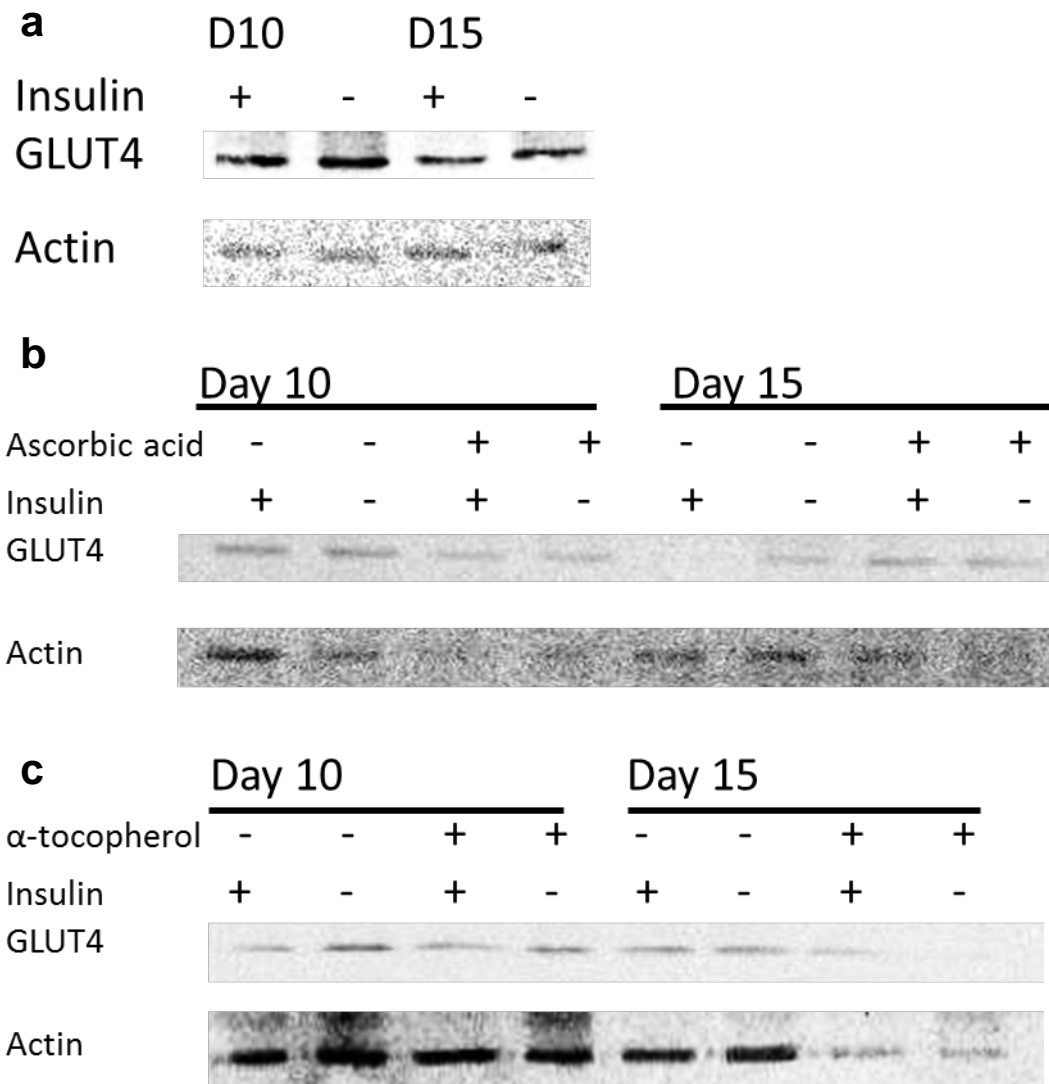


Figure 8. Expression of GLUT4 with ascorbic acid or α -tocopherol treatment. Day 7 3T3-L1 adipocytes were untreated (a) or treated for 3 to 8 days with either 60 μ M ascorbic acid (b), or 40 μ M α -tocopherol (c). On day 10 or 15, cells were serum starvation for 4 hrs, and then either left untreated or treated with insulin (100 nM) for 20 min. Cell lysates were analyzed by SDS-PAGE and then immunoblotted with antibodies to GLUT4 or pan-actin. Results are representative of 3 independent experiments.

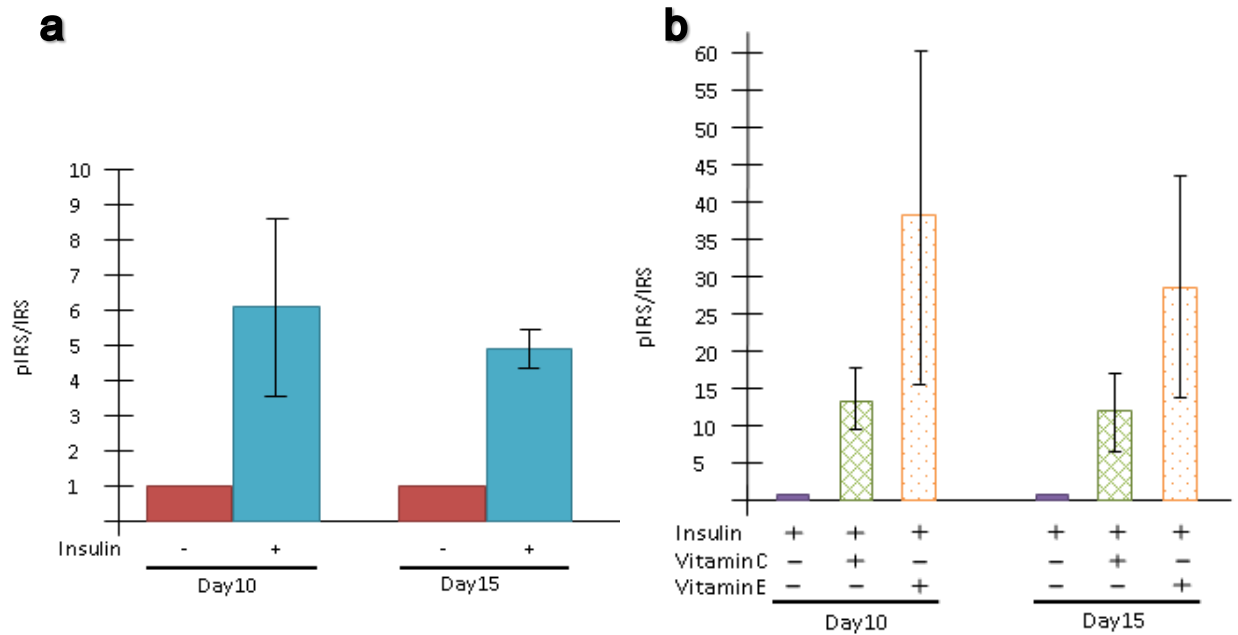


Figure 9. Activation of IRS-1 with ascorbic acid or α -tocopherol treatment. 3T3-L1 adipocytes were pretreated with either 60 μ M ascorbic acid, 40 μ M α -tocopherol, DMSO, or dH₂O. On day 10 or 15, cells were serum starvation for 4 hrs, and then either left untreated or treated with insulin (100 nM) for 20 min. Cell lysates were analyzed by SDS-PAGE and then immunoblotted with antibodies to IRS-1 pTYR⁶⁰⁸ or IRS-1. (b) Antioxidant treatment pIRS levels were normalized to corresponding Day and insulin stimulation levels shown in (a). Results are representative of 3 independent experiments.

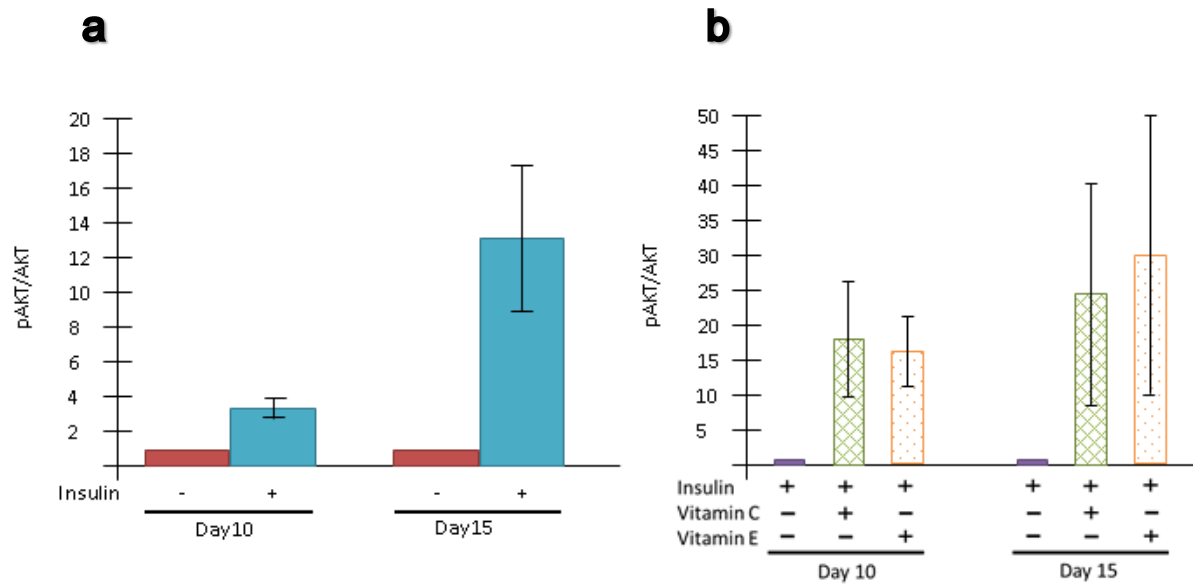


Figure 10. Activation of AKT with ascorbic acid and α -tocopherol treatment. 3T3-L1 adipocytes were pretreated with either 60 μ M ascorbic acid, 40 μ M α -tocopherol, DMSO or dH₂O. On day 10 or 15, adipocytes were serum starved for 4 hrs and either left untreated or treated with insulin (100 nM) for 20 min. Cell lysates were analyzed by SDS-PAGE and then immunoblotted with pAKT or AKT. (b) Antioxidant treatment pAKT levels were normalized to corresponding Day and insulin stimulation levels shown in (a). Results are representative of 3 independent experiments.