Factors affecting insulin-stimulated in vitro glucose oxidation in rat epididymal adipocytes

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FACTORS AFFECTING INSULIN-STIMULATED IN VITRO GLUCOSE OXIDATION IN RAT EPIDIDYMAL ADIPOCYTES

BY

LISA C. MERRILL
B.A., University of New Hampshire, 1989

THESIS

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Animal Science

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td>PAGE</td>
</tr>
<tr>
<td>I.</td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>Glucose and Adipocytes</td>
<td>2</td>
</tr>
<tr>
<td>Regulation of Glucose Oxidation in Adipose Tissue</td>
<td>3</td>
</tr>
<tr>
<td>The Isolated Adipocyte Model</td>
<td>4</td>
</tr>
<tr>
<td>Factors Affecting Adipocyte Glucose Oxidation in Response to Insulin Stimulation</td>
<td>4</td>
</tr>
<tr>
<td>Meal-feeding and Glucose Oxidation in Adipocytes</td>
<td>17</td>
</tr>
<tr>
<td>Metabolic Adaptations of Meal-fed Rats</td>
<td>19</td>
</tr>
<tr>
<td>In Vivo Studies</td>
<td>20</td>
</tr>
<tr>
<td>In Vitro Studies</td>
<td>23</td>
</tr>
<tr>
<td>The Missing Factor</td>
<td>27</td>
</tr>
<tr>
<td>II.</td>
<td></td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>29</td>
</tr>
<tr>
<td>Glucose Metabolism Study</td>
<td>29</td>
</tr>
<tr>
<td>Reagents</td>
<td>34</td>
</tr>
<tr>
<td>Solutions</td>
<td>35</td>
</tr>
</tbody>
</table>
III. REAGENTS, ENVIRONMENTAL CONDITIONS, AND ANIMAL MODEL AS THEY AFFECT INSULIN-STIMULATED GLUCOSE OXIDATION IN RAT ADIPOCYTES ........................................... 43

IV. EFFECTS OF MEAL-FEEDING ON IN VITRO INSULIN-STIMULATED GLUCOSE OXIDATION IN RAT EPIDIDYMAL ADIPOCYTES ................................................................. 48

INTRODUCTION ........................................................................... 48

MATERIALS AND METHODS .......................................................... 52

Animals .................................................................................... 52

Tissue Preparation and Adipocyte Isolation ...................................... 52

Adipocyte Incubation .................................................................. 53

CO₂ Production ........................................................................ 53

Cell Size and Number ................................................................ 54

Data Analysis ........................................................................... 55

RESULTS .................................................................................... 56

Rat and Adipocyte Characteristics .................................................. 56

Glucose Oxidation ..................................................................... 56

Anatomical Changes in Gastrointestinal Tracts of Rats .............. 57

DISCUSSION ............................................................................... 66

V. CONCLUSION .......................................................................... 72

REFERENCES .............................................................................. 73

APPENDIX .................................................................................. 82

APPENDIX A: IACUC APPROVAL .................................................. 83
LIST OF TABLES

Table 1 – Effects of Reagent Changes on Glucose Oxidation..........................44
Table 2 – Effects of Environmental Changes on Glucose Oxidation.................45
Table 3 – Effects of Animal Changes on Glucose Oxidation..........................46
Table 4 – Fat Pad Weight, Cell Number and Size, Body Weight and Age:
Three Time Points.........................................................................................58
Table 5 – Glucose Oxidation in the Absence and Presence of Insulin..............59
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Rat Testicle and Fat Pad</td>
<td>42</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Glucose Oxidation</td>
<td>60</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Insulin-Stimulated Glucose Oxidation</td>
<td>60</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Insulin-Stimulated Glucose Oxidation</td>
<td>61</td>
</tr>
<tr>
<td>Figure 5a</td>
<td>Rat Abdominal Cavity at 0 H Fasted</td>
<td>62</td>
</tr>
<tr>
<td>Figure 5b</td>
<td>Rat Stomach at 0 H Fasted</td>
<td>63</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Rat Abdominal Cavity at 10 H Fasted</td>
<td>64</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Rat Abdominal Cavity at 20 H Fasted</td>
<td>65</td>
</tr>
</tbody>
</table>
ABSTRACT

FACTORS AFFECTING IN VITRO INSULIN-STIMULATED GLUCOSE OXIDATION IN THE RAT EPIDIDYMAL ADIPOCYTE

by

Lisa C. Merrill

University of New Hampshire, September 2008

Since Martin Rodbell published his collagenase method for isolating rat adipocytes from their stomal-vascular matrix in 1964, researchers have modified the procedure in an attempt to maximize results. These modifications are also purported to decrease the interlaboratory variability observed by researchers who utilize this procedure. Strict adherence to these modifications does not, however, necessarily result in robust, repeatable in vitro insulin-stimulated glucose oxidation. The purpose of this study was to optimize Rodbell's procedure for measuring in vitro glucose oxidation in rat adipocytes, and to measure the effect of meal feeding on this process.

For optimization experiments, adipocytes were isolated from the epididymal fat pads of 58 male rats (38 Sprague Dawley and 20 Wistar). For meal-feeding experiments, adipocytes were isolated from the epididymal fat pads of 32 male Wistar rats at one of three time points after feeding: 0 H fasting...
(n = 10 rats), 10 H fasting (n = 10 rats), 20 H fasting (n = 8 rats) and two pilot experiments at 5 H fasting (n = 4 rats). The adipose tissue from two rats was pooled for each experiment. Insulin sensitivity was measured after incubation with 6 mM D-[U-14C]-glucose and 10^-7 M insulin, by measuring $^{14}$CO$_2$ production.

Adipocytes from rats in 26 optimization experiments showed that the observed lack of insulin-stimulated glucose oxidation was not caused by reagents, environmental factors, or strain/size of animal. They also showed that the cause was likely related to an uncontrolled factor associated with the animals (ie, the pattern of food intake).

Unlike adipocytes from rats in the optimization experiments, adipocytes from rats in the meal-feeding experiments showed a robust, reliable, expected increase (two to three-fold) in insulin-stimulated glucose oxidation. The average fold response in insulin-stimulated glucose oxidation was 3.5-fold. There was no significant difference in nmol glucose oxidized to CO$_2$ between the three time points, but a trend toward decreasing nmol glucose oxidized in the presence of insulin over time was observed ($p = 0.15$). The adipocytes also showed a decrease in variability of absolute insulin stimulation over time (coefficient of variation decreased from 53% at 0 H-fasted to 29% at 20 H-fasted).

In conclusion, meal-feeding rats for approximately three weeks resulted in reproducible, robust insulin stimulation of glucose oxidation of adipocytes. This is the first study to investigate the short-term effects of two hour meal-feeding on adipocyte glucose oxidation.
CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Insulin is a protein hormone that is released by the pancreas in response to an increase in blood glucose concentration. Dysregulation of plasma insulin concentration is implicated in numerous pathologic conditions including insulin resistance, obesity, metabolic syndrome, and type 2 diabetes mellitus. Work from our laboratory showed that brominated flame retardants, which are lipophylic compounds and are sequestered in adipocytes, depress insulin stimulation of glucose oxidation by rat adipocytes, mimicking a state of insulin resistance (Hoppe and Carey, 2007). However, successful measurement of insulin-stimulated glucose oxidation in adipocytes is intermittent and not reliably reproduced following the method of Rodbell (1964). Because there are a myriad of factors that can influence this method, a careful and thorough examination of these factors, ranging from chemicals to environmental conditions to animals, is critical in order to better understand insulin-stimulated glucose oxidation of adipocytes.
Glucose and Adipocytes

Adipocytes have traditionally been regarded as passive storage depots of lipid (Flier, 1995). Research attention has focused on the lipid storage of adipocytes for two reasons. First, storage of excess calories as triacylglycerol and then mobilization of the triacylglycerol in times of need is the primary role of these cells. Second, the health consequences associated with excess lipid storage are numerous and serious, with more adults and children becoming obese each year, in both developed and developing countries (Rubinowitz, 2008; Aronne and Isoldi, 2007; Ogden et al., 2006; Saltiel and Pessin, 2002; Fruhbeck et al., 2001). Less attention has been paid to the maintenance function of adipocytes, namely cellular respiration and metabolic activities.

Glucose is an important energy source for most tissues of the body. Its uptake and metabolism is a process that adipocytes share with all mammalian cells. Adipocytes metabolize glucose to one of the following measurable end-products: carbon dioxide (CO₂), triglyceride (glycerol and fatty acids), glycogen, pyruvate, and lactate. In vitro studies have shown that in small rats, under basal conditions (i.e., in the absence of insulin), 25-30% of glucose is oxidized to CO₂ in small adipocytes, 10% is converted to pyruvate, 1-10% is metabolized to lactate (Fried et al., 1982; Crandall et al., 1983; Groff et al., 1992; DiGirolamo, 2001), 55-60% to triglyceride, and <2-3% to glycogen. The addition of insulin results in a change in the pattern of the production of each of these intermediates, although the pattern is dependent upon numerous factors. These factors include the weight and nutritional state of the animal, glucose concentration of the
incubation medium, the size and concentration of cells in the medium
(DiGirolamo and Fried, 1987). Recent work indicates that the presence of insulin
increases the amount of glucose converted to CO₂ (DiGirolamo, 2001).

**Regulation of glucose oxidation in adipose tissue**

There is both short-term and long-term regulation of glucose oxidation in
adipose tissue. Short-term regulation occurs in the form of allosteric activation or
inhibition of key enzymes (hexokinase, phosphofructokinase, and pyruvate
dehydrogenase). Long-term regulation includes hormonal control of the amount
of enzyme and glucose transport proteins synthesized. One key regulatory
hormone is the protein insulin, which is released from pancreatic β cells in
response to a surge in serum glucose concentration. Insulin stimulates the
uptake of glucose by adipocytes via the tissue-specific, facilitated diffusion
glucose-transporter 4 (GLUT4) located in the plasma membrane (James et al.,
1988; Birnbaum, 1989; Czech and Corvera, 1999; Saltiel and Pessin, 2002).
While basal serum glucose enters adipocytes via the constitutively expressed
glucose-transporter 1 (GLUT1) transporters (Wang, 1987; Oka et al., 1988;
Zorzano et al., 1989), the increased glucose load associated with a meal requires
a 20- to 40-fold stimulation of GLUT4 translocation from intracellular storage
vesicles to the plasma membrane (Hadley, 2007).
The isolated adipocyte model

In a procedure devised by Martin Rodbell (1964), adipocytes are liberated from adipose tissue during digestion with the enzyme collagenase. Since their fat content is so high, adipocytes separate from the dense matrix cells by flotation via centrifugation. Isolated adipocytes can be exposed to regulatory hormones, like insulin, under different incubation conditions, allowing for subsequent analysis of the adipocytes’ response. Specifically, using $^{14}$C-glucose as a tracer, one can quantify the amount of glucose oxidized to CO$_2$. Prior to this procedure, researchers were limited to expressing glucose metabolic responses either on the basis of adipose tissue wet weight, protein content, or deoxyribonucleic acid (DNA) content. Rodbell’s work provided an important means of examining glucose oxidation on the basis of cell number, providing a more precise means of comparison than the aforementioned means since other cells that comprise the adipose tissue are eliminated from the analysis (DiGirolamo et al., 1974; Owens et al., 1979; Lavau et al., 1979; DiGirolamo and Fried, 1987).

Factors affecting adipocyte glucose oxidation in response to insulin stimulation

Over the years, Rodbell’s procedure has been modified by researchers in an attempt to maximize results. Researchers have uncovered eight factors that influence basal glucose oxidation by the adipocyte as well as the magnitude of the insulin response observed when following Rodbell’s procedure. These
factors have also been purported to decrease the interlaboratory variability observed by researchers who utilize this method (DiGirolamo and Fried, 1987).

**Collagenase**

The first factor to influence insulin-stimulated glucose oxidation in adipocytes is the proper selection of collagenase. Collagenase is, arguably, the key component of the adipose tissue digestion process, allowing the liberation of adipocytes from their connective tissue matrix. Partially purified or crude collagenase preparations, once thought to be comprised of a single enzyme, *clostridiopeptidase A* are, in fact, a mixture of several types of collagenases plus a significant amount of a proteolytic enzyme that attacks casein (Kono, 1969). Contaminating proteolytic enzymes can cause damage to the isolated cell’s surface, resulting in lysis and a decreased cell yield (Al-Jafari et al., 1986). Use of highly purified collagenase alone (A-α and B-α) from *Clostridium histolyticum*, however, does not resolve the problem as it is ineffective in dispersing individual cells from the tissue. To disperse fresh tissue using highly purified collagenase requires the addition of trypsin and/or chymotrypsin (Kono, 1969).

To ameliorate the deleterious effects of crude collagenase preparations, the concentration of collagenase is recommended to be kept at 0.5 mg/ml and the digestion period of the minced adipose tissue should not exceed one hour (Al-Jafari et al., 1986). It has been conceded, however, that at such a low concentration of collagenase, cell yield may not be high enough for experimental needs, potentially requiring the digestion of a larger volume of tissue.
Determining the proper balance between collagenase concentration and duration of digestion through a series of studies may be necessary (Al-Jafari et al., 1986; DiGirolamo, 2001).

**Bovine Serum Albumin**

The second factor affecting adipocyte glucose oxidation in response to insulin is the proper selection of bovine serum albumin (BSA). BSA serves three important roles in the digestion and isolation processes (DiGirolamo and Fried, 1987). First, the addition of this simple protein helps to recreate the physiologic conditions of the plasma. Second, BSA neutralizes the free fatty acids that result from intracellular hydrolysis of triglycerides. BSA is a known carrier of fatty acids in the serum (Cushman and Rizack, 1970); by supplying BSA in the buffer, intracellular fatty acids are drawn to leave the cell, thereby reducing intracellular fatty acid levels and the resultant lipolysis caused by accumulated intracellular fatty acids (DiGirolamo and Fried, 1987). Third, BSA helps minimize the adherence of added hormones (particularly insulin) to glassware and plastic incubation vials. However, Cushman and Rizack (1970) report no change in the insulin-stimulated CO₂ production of adipocytes in the presence or absence of albumin, indicating that the insulin was readily available for use by the cells regardless of whether albumin was added.

Since BSA can be contaminated with materials that can interfere with metabolism of the adipocyte, and can contain variable levels of compounds such as endotoxins which can potentiate the action of insulin (personal communication
DiGirolamo recommends the purchase of three to four samples of different lots of Sigma BSA (DiGirolamo, 2001). He tests these samples in combination with small amounts of different lots of Worthington Chemicals collagenase by conducting experiments to determine which combination of collagenase and BSA gives the expected insulin response observed in his lab (approximately a two to three-fold increase). Fatty acids are a particularly deleterious contaminant in BSA; therefore, the use of ethanol extracted BSA from Sigma is beneficial since it is essentially fatty acid-free (DiGirolamo and Fried, 1987). Once the optimum combination has been determined, Dr. DiGirolamo suggests purchasing and storing 1 g of collagenase and at least 1 to 2 kg BSA to ensure an adequate supply throughout the course of the study as there is lot-to-lot variation observed with each of these compounds (DiGirolamo 2001).

**Radiolabeled Glucose**

The third factor is the type of radiolabeled glucose used as a tracer. The use of uniformly labeled glucose ([U-14C]-glucose) is best when all of the potential carbon end-products of glucose metabolism are being measured. However, only 1/6th of the label will appear as CO2 in the short term, with radioactivity distributing to carbon atoms of other products including lactate, pyruvate, fatty acids and glycogen. The use of [1-14C]-glucose, which produces [1-14C]-glyceraldehyde 3-phosphate and ultimately 14CO2, is a more efficient means of measuring glucose conversion to CO2.
Glucose Concentration

A fourth factor is glucose concentration in the incubation medium. This factor is particularly important for small cells (40-50 µl) from young rats since large adipocytes (500-600 µl) from older rats lose the ability to respond to insulin-stimulation as the glucose concentration in the medium increases (DiGirolamo et al., 1974). To obtain desired results in insulin-stimulated glucose oxidation, it is best to use a physiologic concentration of glucose (6 mM) in the incubation medium (DiGirolamo, 2001), although others have been successful with the use of an alternative glucose concentration. Hoppe & Carey (2007) obtained successful insulin-stimulation using 8mM glucose concentration while Guerre-Millo et al. (1985) and Kovanen et al. (1975) were successful using a concentration of 5 mM.

Rat Size/Age

A fifth factor is the animals used in the procedure. DiGirolamo's work has shown that older, heavier rats exhibit a minimum effective dose (MED) for an insulin effect 100x greater than younger, leaner rats (DiGirolamo and Rudman, 1968). The MED was defined as the lowest concentration of insulin which caused a statistically significant (p<0.01) increase in quantity of glucose metabolized to CO₂. The researchers examined three groups of rats ranging in weight from 130 g (five weeks old) to 385 g (18 weeks old). The younger leaner rats showed the greatest insulin sensitivity of the rats with an MED of 5 µU/ml.
and a maximum of 7 μmol glucose converted to CO\textsubscript{2}/100 mg fat-free dry weight/2h. The 340 g rats (14 weeks old) showed less insulin sensitivity than the youngest rats with an MED of 50 μU/ml and maximum of 4 μmol glucose converted to CO\textsubscript{2}/100 mg fat-free dry weight/2h. The oldest, largest rats (18 weeks of age) showed the least insulin sensitivity with an MED of 500 μU/ml and a maximum of 2 μmol glucose converted to CO\textsubscript{2}/100 mg fat-free dry weight/2h.

To determine whether the decrease in insulin sensitivity measured in the older, larger rats was due to an increase in age or an increase in body weight, the researchers examined the metabolism of epididymal adipose tissue of several groups of rats. One group of rats age one year or older and weighing 480-650 g were converted from an ad libitum diet (average food intake of 29.8 g chow/day) to a limited food intake (10 g chow/day) designed to reduce their weight. After three weeks on this restricted diet, some animals were sacrificed while the remaining rats were returned to an ad libitum diet. Animals sacrificed at the end of the three week restriction period showed greater insulin sensitivity than before food restriction and weight reduction. Once food was re-introduced, the rats showed an increase in insulin sensitivity that resembled the 150 g rats described above. This response lasted until refeeding day seven. As the ad libitum period of refeeding continued, weight gain returned to that observed before diet restriction, as did the decrease in insulin responsiveness of the adipose tissue. These findings indicated that loss of insulin sensitivity in the older, heavier rat is due to weight as opposed to age and that it is reversible by weight loss.
Adipocyte Size

Closely related to the issue of age and body weight is the size of the adipocyte, which can dramatically affect insulin stimulation. Adipocytes obtained from lean 150 g rats (30-50 pl) show a greater responsiveness to insulin than adipocytes from obese 650 g rats (500-750 pl) (DiGirolamo et al., 1974; DiGirolamo, 1981). This increased response appears to be mediated by an increased capability of the small adipocytes to respond to changes in glucose concentration by shifting utilization of glucose from glyceride synthesis to CO₂ production and fatty acid synthesis.

The use of small adipocytes not only changes the pattern of glucose utilization, it also increases the insulin sensitivity of the cells. A study conducted by DiGirolamo and Owens (1976) compared insulin-stimulated glucose oxidation to CO₂ in adipocytes ranging in size from 47 pl to 637 pl, obtained from rats sized 152 g and 687 g respectively. Using 47 pl cells, insulin-stimulated glucose conversion to CO₂ was 2.6 times basal (1.58 to 4.07 µmol glucose/10^7 cells/hr). Using 637 pl cells, insulin-stimulated glucose conversion to CO₂ was 1.3 times basal (0.9 to 1.14 µmol glucose/10^7 cells/hr).

Adipocyte Concentration and Adenosine

Low adipocyte concentration is known to foster lipolysis and negatively affect rates of adipocyte glucose oxidation (DiGirolamo et al., 1993). In the basal state, an increase in cell concentration from 0.168 to 1.252 x 10^6 cells/ml
results in a two times increase in glucose converted to CO\textsubscript{2} (1.6 versus 0.8 \(\mu\)mol glucose/\(10^7\)cells/90 min). In the presence of insulin the increase in cell concentration results in a 1.5 increase in glucose converted to CO\textsubscript{2} (4.4 versus 2.9 \(\mu\)mol glucose/\(10^7\)cells/90 min).

Adenosine is a nucleoside comprised of adenine and ribofuranose, which is involved in biochemical processes including energy transfer (e.g. ATP metabolism). It also serves as a hormone for cell signaling (via the cyclic AMP pathway). Adenosine is released from some cells upon degradation of ATP. While adenosine has many effects throughout the body, its ability to mimic the actions of insulin on adipocyte glucose oxidation is relevant to this review. The adenosine released by adipocyte suspensions is shown to augment the effects of insulin on glucose oxidation. The addition of 0.01\(\mu\)M adenosine (similar to the endogenous amount released into incubation medium by adipocyte suspensions) to the incubation medium resulted in an insulin stimulated conversion of glucose to CO\textsubscript{2} 1.4 times basal (7.24 to 9.82 nmol/10\(^5\)cells/h) (Schwabe et al., 1974).

Concentrated suspensions of adipocytes (1 \(\times\) 10\(^5\) cells/ml), have also been shown to release enough adenosine to inhibit adenylate cyclase (Schwabe et al., 1973). By inhibiting adenylate cyclase, the conversion of ATP to cAMP is not catalyzed and lipolysis is decreased.

In contrast to the findings of Schwabe et al., DiGirolamo and colleagues (1993) found that adenosine and adenosine deaminase did not affect the results of their glucose oxidation studies, although they did agree that the amount of adenosine released into the incubation medium is inversely proportional to the
cell density and cell size (Capogrossi et al., 1986). The researchers incubated varying concentrations of cell suspensions with an adenosine receptor agonist, N-6(2-phenylisopropyl) adenosine (PIA). Their results showed that addition of PIA had little to no effect on total basal and insulin-stimulated glucose metabolism, and had no effect on the pattern of metabolism to end-products. Since these results are in contrast to their findings that increasing cell concentration increases conversion of glucose to CO$_2$ and glyceride-fatty acids (see above), they concluded that adenosine release by adipocytes into the medium was not an explanation for the effects of cell concentration on glucose metabolism.

To support this conclusion, they measured metabolic end-products obtained following incubation of adipocytes with adenosine deaminase. The researchers found that in the basal state a slight decrease in total glucose metabolism occurred (from 4.2 to 3 μmol glucose/10$^7$ cells/90 min). In the insulin-stimulated state, the addition of adenosine deaminase had no effect on glucose metabolism. DiGirolamo et al. (1993) concluded that since the effect of cell concentration on total glucose metabolism was not blocked by adenosine deaminase nor mimicked by PIA, the amount of adenosine in the medium was not directly linked to the effects of varying cell concentrations.

Having ruled out adenosine and adenosine deaminase as an explanation for their findings, DiGirolamo et al. (1993) proposed the following alternative explanation. Low cell concentration is associated with an increased rate of lipolysis. This results in increased levels of intracellular free fatty acids, which is
believed to have two effects (Schwabe et al., 1973; Schwabe et al., 1974; DiGirolamo and Owens, 1976; DiGirolamo et al., 1993). First, it suppresses fatty acid synthesis and CO$_2$ production. Second, it results in diffusion of the free fatty acids down a concentration gradient and release into the extracellular medium. The free fatty acid level in the medium of low cell concentration incubations has been observed to be six to eight times higher when compared to the free fatty acid level in the medium of high cell concentration incubations (DiGirolamo et al., 1993). The high extracellular levels result in an increase in cell lysis due to the detergent-like action of the free fatty acids. New glucose entering the adipocyte would be directed toward the formation of glyceride-glycerol (as well as lactate and pyruvate) as a means of providing substrate for the intracellular free fatty acids to form triglyceride. As cell density increases and the lipolytic rate per cell decreases, there is a decrease in intracellular free fatty acid concentration and a reversal of this pattern as more glucose is converted to fatty acids and CO$_2$ and less enters the nonoxidative pathways that form glyceride-glycerol, lactate, and pyruvate.

**Additional Factors**

**Magnesium**

Other researchers have determined that factors other than the eight influence the results obtained using Rodbell's procedure. Kandeel et al. (1996) point to the need for a high enough concentration of magnesium in the incubation medium. By decreasing the magnesium ion concentration from physiologic
levels (1.24 mM) to 0.16 mM (as can occur in states of insulin-resistance), the authors showed a significant decrease in insulin-stimulated adipocyte glucose oxidation rates, while glucose transport remained unchanged. At insulin doses of 5 ng/ml and 25 ng/ml, glucose oxidation decreased by approximately 60 and 62%, respectively, when adipocytes were incubated in a low magnesium medium compared to a physiologic incubation medium.

**Diet Composition**

Lavau *et al.* (1979) investigated the effect of diet composition on glucose oxidation in rat adipocytes. Feeding rats a high fat diet for seven days had no effect on insulin-stimulated glucose transport into the cells, but did have an effect on insulin-stimulated glucose oxidation to CO₂. Feeding a high fat diet resulted in 200 nmol glucose oxidized/10⁶ cells/2 h as compared to the 800 nmol/10⁶/2 h from rats fed a low fat diet.

**Gender**

A final published variable that alters responsiveness of rat adipocyte glucose oxidation is attributed to gender. Adipocytes isolated from paraovarian fat pads of female rats were compared to adipocytes isolated from epididymal fat pads of male rats in terms of insulin effectiveness. At the highest insulin concentrations (0.36 nM to 2.5 nM), there was no difference in stimulation of glucose conversion to CO₂ between the two sexes. However, at the submaximal dose of 0.06 nM insulin, the paraovarian adipocytes converted 1.9 times the
glucose to CO2 that epididymal cells did (0.19 versus 0.1 µmol glucose/10^6 cells/2 h, p<0.01). At a slightly higher dose of insulin (0.18 nM), the paraovarian adipocytes converted 1.5 times the glucose to CO2 that the epididymal cells converted (0.21 versus 0.14 µmol glucose to CO2/10^6 cells/2 h, p<0.05) (Guerre-Millo et al., 1985).

**Recommendations**

In order to maximize the amount of glucose converted to CO2 both in the absence and presence of insulin, DiGirolamo recommends using small adipocytes (obtained from a growing 150 g rat) at an approximate density of 10% (v/v), and a physiologic 6 mM concentration of glucose. By combining these factors, in the presence of insulin, the relative utilization of glucose by the adipocytes shifts from primarily glyceride-glycerol to CO2 and fatty acids (DiGirolamo and Fried, 1987). Finally, Dr. DiGirolamo uses the Wistar strain of rat (DiGirolamo and Owens, 1976; Owens et al., 1979; Francendese and DiGirolamo, 1980; Crandall et al., 1983; Newby et al., 1988; Newby et al., 1989; Groff et al., 1992), although others have obtained good results with the use of alternative strains of rat, including the Sprague-Dawley (Romsos and Leveille, 1974; Schwabe et al., 1974; Olefsky, 1976; Kandeel and Balon, 1996; Fine and DiGirolamo, 1997; Hoppe and Carey, 2007; Shih et al., 2007), MRC Hooded (Al-Jafari et al., 1984), and Zucker rats (DiGirolamo and Fried, 1987). By following these recommendations, one can expect a two to three-fold insulin response in adipocyte glucose oxidation to CO2.
Elusive Factor

Accounting for each of the above-described factors may provide a best case scenario under which one could obtain expected levels of insulin-stimulated glucose oxidation, but it does not guarantee robust, repeatable results (see Chapter 3). Researchers who have utilized this adipocyte isolation procedure for decades, concede that the insulin-stimulation effect can disappear for a period of time \(^1\) and that Rodbell's cell isolation procedure results in cells that are unpredictable in their physiologic behavior (Honnor et al., 1985). Unfortunately, the loss in insulin sensitivity has not been documented in published literature.

One potential source of variability is season. While this has not been examined as a cause of variability in adipocyte glucose oxidation measurements, it has been observed to affect the precision of serum measurements of non-essential fatty acids, triglycerides, and phospholipids (Ahlers et al., 1982). Season has also been shown to affect the antilipolytic effects on adipocytes of adenosine and insulin (Honnor et al., 1985; Londos et al., 1985). Additionally, there are numerous diurnal rhythms in the body, creating normal variability on a daily or monthly basis of countless enzymes, hormones, and metabolites (Hara and Saito, 1980; Scheving et al., 1983; Whitmore et al., 2000; reviewed in Hastings et al., 2007; Kohsaka and Bass, 2006).

Another factor that is often not controlled in the life of the experimental rodent model is feeding. By tradition, this animal model is fed *ad libitum*. Being

\(^1\) personal communication between Gale Carey and Mario DiGirolamo December 21, 2007; email communication between Gale Carey and Susan Fried January 4, 2008
a nocturnal animal, experimental, *ad libitum*-fed rats are known to consume 85 to 90% of their food during the 12 hour dark cycle (Wiley and Leveille, 1970; Romsos and Leveille, 1974; Limal *et al*., 1981; Sugden *et al*., 1999). What is not known is precisely when and how much of that food is consumed: is a large portion consumed at the start of the dark period and then smaller amounts consumed throughout the night? Is a small amount eaten slowly over the entire 12 hour period? Are larger portions consumed at the start and end of the dark cycle? Is there no pattern? Or is the pattern different for each animal? Florence and Quarterman (1972) found that the quantity and timing of food consumption dramatically affects the magnitude and shape of intraperitoneal glucose tolerance test curves. An overnight fast is often employed when measuring glucose tolerance in order to decrease the influence of food intake on measured results. These authors examined the effect on test results of the amount and pattern of food consumed prior to the overnight fast. Their results showed that the height of the curve and the glucose concentration (particularly at 30 minutes after dosing) were inversely proportional to the amount of food consumed in the 24 hour period preceding the starvation period.

Meal Feeding and Glucose Oxidation in Adipocytes

Meal Feeding: The Basics

To answer the questions posed above would require monitoring the animals and measuring their food consumption throughout the 12 hour dark
cycle. In lieu of this, a researcher can choose to meal-feed the rats. Meal-feeding is a process by which animals are fed during a short time span each day. The length of time can vary with the experiment, the researcher, and age of the animal; it can be set at 2 or 4 hours/day for smaller rats or 8 hours/48 hours for larger rats (Carey et al., 1993). The amount of the food presented to the animal during the specified time frame can vary as well, although most researchers impose no limit, preferring instead to allow the animal to regulate its own volume of food intake. Intake by rats fed a two hour meal has been shown to be 75% of the amount consumed by ad libitum-fed rats (Stevenson et al., 1964; Leveille, 1970).

This technique is employed primarily as an important synchronizer of many behaviors and biological rhythms, ranging from the aging process and associated ailments (Weindruch and Walford, 1982; Masoro, 1985; Nelson and Halberg, 1986; Holehan and Merry, 1986; reviewed in Masoro, 1988) to mitotic activity to the activity of numerous hepatic and digestive enzymes (Tepperman and Tepperman, 1958; Cohn and Joseph, 1960; Hollifield and Parson, 1962; Saito et al., 1976; Phillipens, 1980; reviewed in Boulos and Terman, 1980; Kohsaka and Bass, 2006). Meal-feeding is also an important method used to restrict weight gain in experimental rats since feeding in the traditional ad libitum fashion is known to cause rapid weight gain, a trend that continues throughout the life of the animal (DiGirolamo and Rudman, 1968, Salans and Dougherty, 1971; Reiser and Hallfrisch, 1977; Cleary et al., 1987). While meal-feeding has not yet been employed as a short-term synchronizer of insulin-stimulated glucose
oxidation in the isolated adipocyte, its effects on other aspects of glucose metabolism as well as plasma glucose and insulin levels in rats have been studied.

**Metabolic Adaptations of Meal-Fed Rats**

The meal-fed rat must adapt metabolically to the stress that this feeding regimen creates. The large amounts of food that must be ingested in a short time frame require that the digestive tract make adaptive changes. The stomach of the meal-fed rat has been shown to enlarge by nearly 50% (Holeckova and Fabry, 1959; Phillipens et al., 1977) and gastric emptying has been shown to be delayed (Lima et al., 1981). Thereafter, the small intestine has been shown to increase in size by 40%, thereby increasing its capacity and absorptive area and resulting in a 40% increase in glucose absorption. These changes are believed to occur over the course of days (Fabry and Kujalova, 1960; Tepperman and Tepperman, 1964; Fabry and Braun, 1967; Leveille and Chakrabarty, 1968; Leveille, 1970). In order to utilize the increase in glucose presented to the tissues of these animals, and to provide a continual source of oxidative fuel during the hours of fasting, many enzymes in the tissues of several organs must increase their activity, particularly in the liver, muscle, and adipose tissue (Fabry and Kujalova, 1960; Fabry and Braun, 1967; Leveille and Chakrabarty, 1968; Leveille, 1970).
In Vivo Studies

Sources of Oxidative Fuel

Because meal-fed rats do not have a constant supply of food, yet they still require a constant supply of oxidative fuel, researchers surmised that these animals rely on stored glycogen and lipid to a greater extent than ad libitum-fed animals. The respiratory quotient (RQ) provides a way of determining the source of energy being utilized by an animal at any given time. It is a measure of the volume of oxygen consumed and carbon dioxide produced by a particular tissue and is expressed as a ratio: VCO₂/VO₂. If glucose is the primary source of energy being utilized, the RQ = 1. Whole body RQ measurements on meal-fed versus ad libitum-fed rats have been conducted. Leveille and O’Hea (1967) showed that for 8 hours after beginning their daily meal, meal-fed rats have a RQ value greater than 1.0, indicating that glucose is the major source of oxidative fuel and that lipogenesis is occurring. Twelve hours after beginning their meal, the RQ value was 0.87, which was close to the average RQ values obtained from ad libitum-fed rats during the day. The 0.87 value indicates that carbohydrate (glucose and glycogen) is providing approximately 50% of the calories for these animals. By 22 to 24 hours post-prandial, the RQ value in the meal-fed rats had dropped to 0.73, suggesting that lipid was providing 90% of the calories being utilized by these rats.
Plasma Glucose Levels

An investigation of the effects of meal-feeding showed that throughout most of the day, meal-fed animals maintain plasma glucose concentrations approximately 17 mg/dl below their ad libitum-fed counterparts (p<0.001) (Masoro et al., 1992). The only time this was not the case was after the meal-fed animals were provided with food, at which time, their plasma glucose levels approached those of the ad libitum-fed rats. In other studies, serum glucose levels were measured in ad libitum and meal-fed rats after being given a bolus of glucose either orally, intraperitoneally, or intravenously. In each case, the spike in plasma glucose following the glucose load was lower in the meal-fed rats, and the values returned to basal more quickly compared to the ad libitum-fed rats (Leveille and Chakrabarty, 1968; Wiley and Leveille, 1970; Romsos and Leveille, 1974) indicating a greater ability on the part of peripheral tissues (ie adipose and muscle) to assimilate glucose.

Plasma Insulin Levels

Given the observed changes in plasma glucose levels described above, accompanying changes in the plasma insulin levels of meal-fed rats would be expected as well. Sugden et al. (1999) have shown that four weeks of meal-feeding resulted in a plasma insulin concentration that was significantly elevated from 15 µU/ml (basal level) to 30 µU/ml by two hours after provision of food (p<0.5). Four hours after the removal of food, the concentration returned to basal level (approximately 19 µU/ml). The maximum and minimum plasma insulin
concentration in *ad libitum*-fed rats were shown to be larger than those of meal-fed rats, at 44 μU/ml and 12 μU/ml, respectively.

The effect of 12 weeks of meal-feeding on serum insulin levels in rats was examined by Reiser and Hallfrisch (1977). Animals were sacrificed 12 to 14 hours after their food was removed and blood was collected. The single time-point serum insulin level of the meal-fed rats was 1.5 times that in *ad libitum*-fed rats (18.1 versus 28.0 μunits/ml, p<0.05). This finding was confirmed by a separate longitudinal study, in which plasma insulin levels were found to be markedly lower in meal-fed rats than *ad libitum*-fed rats, a finding that remained consistent throughout the entire 20 month study (Masoro *et al.*, 1992).

Alternatively, another examination of serum insulin levels in three week, meal-fed versus *ad libitum*-fed rats found higher serum levels of this hormone regardless of whether the animals were fed or fasted (Wiley and Leveille, 1970). Measurements were taken on animals fasted for 22 hours and then force-fed either a 40% glucose solution or 2 g of diet contained in 10 ml water. While both groups of rats responded with a comparable rise in serum insulin (approximately 20 μU/ml) within 15 minutes of intubation with the 40% glucose solution, the absolute levels were different between the two groups of animals. At time zero, the insulin level of the meal-fed rats was 42 μU/ml and in the *ad libitum*-fed rats, it was 17 μU/ml. Measurements were taken every 30 minutes until 120 minutes. By 120 minutes, the insulin level of the meal-fed rats was still rising, while that of the *ad libitum*-fed rats was still dropping. A different pattern emerged following intubation with 2 g of diet. At time zero, the approximate insulin level of the meal-
fed rats was 24 μU/ml versus 14 μU/ml in the ad libitum-fed rats. Fifteen minutes after intubation both groups had spiked to their maximum point of 58 μU/ml in the meal-fed animals and 42 μU/ml in the ad libitum-fed rats. From 15 to 60 minutes post-intubation, the plasma levels decreased in both groups to final values of approximately 36 and 32 μU/ml in the meal-fed and ad libitum-fed rats, respectively.

Two possible explanations exist for the different plasma insulin level results obtained in the Wiley and Leveille study compared to the Sugden et al. and Reiser et al. studies. First, the rats in the Wiley and Leveille study were meal-fed for three weeks only. It is possible that the animals required more time to adapt physiologically to the meal-feeding process. Second, force-feeding and diet composition just prior to the time of sacrifice were different from a) the meal-feeding regimen and diet composition during the study and b) from the feeding regimen and diet composition used with the rats in the other two studies. Indeed, when the Wiley and Leveille study rats were intubated with their normal chow (diluted in water), the pattern of changes in absolute serum insulin levels more closely resembled those seen in the Sugden et al. study.

**In Vitro Studies**

**Insulin Sensitivity of Adipose Tissue and Isolated Adipocytes**

Since plasma glucose and insulin levels are different in meal-fed rats compared to ad libitum-fed rats, it is reasonable to investigate the insulin
sensitivity of adipose tissue, a key organ involved in the physiologic adaptations observed in these animals. Insulin sensitivity has been assessed in four ways. First, glucose uptake and phosphorylation by adipose tissue has been estimated in meal-fed animals and expressed as a glucose utilization index (GUI). This value reflects the rates of uptake and phosphorylation of 2-deoxy[3H]D-glucose, as measured by the accumulation of the phosphorylated form of this radiolabeled compound. The GUI values in the parametrial fat depot of ad libitum control rats remain relatively constant throughout the day at 3 ng/min/mg (Sugden et al., 1999). The GUI values in meal-fed rats just prior to the provision of food are also 3 ng/min/mg. However, by the end of the two hour meal, in the meal-fed rats, GUI values increase significantly to 28 ng/min/mg (p<0.5). This value returned to basal concentration seven hours after the removal of food. The results indicate an overall increase in insulin sensitivity and glucose utilization by adipose tissue associated with meal-feeding.

In a second method, the CO2 production from glucose oxidation by epididymal adipose tissue has been compared in the presence and absence of insulin to determine the degree of insulin-sensitivity of that tissue. Meal-feeding of weanling rats for 12 weeks results in a significant increase in insulin-stimulated oxidation of [1-14C]glucose to 14CO2 compared to ad libitum-fed control rats (Reiser and Hallfrisch, 1977). The insulin sensitivity, expressed as the mean difference between CO2 production from glucose in the absence and the presence of insulin, was 219 nmol CO2/100mg for meal-fed rats, and
66 nmol CO₂/100mg for *ad libitum*-fed rats (*p*<0.05). The increased insulin-sensitivity remained statistically significant and was similar in magnitude in the groups of rats examined after seventeen weeks of feeding.

In another study examining adipose tissue oxidation of [U-¹⁴C]glucose to ¹⁴CO₂ in the presence of insulin, tissue from meal-fed rats converted 3.9 times the glucose to CO₂ than tissue from *ad libitum*-fed rats (1.959 versus 0.497 μmoles of ¹⁴C-glucose to CO₂/100mg tissue/3 hours; *p*<0.001) (Leveille and Hanson, 1965). Both of these studies support the notion that meal feeding increases the insulin sensitivity of adipose tissue.

A third method for assessing insulin sensitivity in meal-fed rats involves examining the EC₅₀ value of insulin required to stimulate uptake of [³H]-2-deoxyglucose by isolated adipocytes. Lima *et al.* (1994) meal-fed rats for two hours a day for four weeks. At the end of the four weeks, the rats were sacrificed and the researchers measured the amount of insulin-stimulated [³H]-2-deoxyglucose uptake by isolated adipocytes at 8:00 a.m. (the start of the meal feeding period) and at 4:00 p.m. Adipocytes from the meal-fed rats sacrificed at 8:00 a.m. had an insulin EC₅₀ value of 0.175 ng/ml, which was significantly lower than the value from the adipocytes of *ad libitum*-fed rats at the same time point (0.678 ng/ml). The lower EC₅₀ value indicated an enhanced ability of the adipocytes of meal-fed rats to respond to insulin. By 4:00 p.m. the values were no longer different statistically.

Finally, a fourth method of assessing insulin sensitivity utilizes the method developed by Rodbell (1964) described above in which isolated adipocytes are
incubated with $^{14}$C-glucose in the absence and presence of insulin. While mealfeeding has not yet been employed as a short-term synchronizer of insulin-stimulated glucose oxidation in the isolated adipocyte, it has been examined in a year-long study that utilized the obese Zucker rat. These rats are often used as a model of early-onset obesity as they are hyperlipidemic, hyperinsulinemic, and insulin resistant (Cleary et al., 1987). These researchers used the lean Zucker rat model as a control and provided each group with food in an *ad libitum* or meal-fed fashion for a total of four groups: *ad libitum* obese, meal-fed obese, *ad libitum* lean, meal-fed lean. Basal nmol of glucose oxidized to CO$_2$/10$^6$ cells/hr was similar among all four groups (approximately 8-9 nmol/10$^6$ cells/hr); however, significant differences were observed when insulin was added. Adipocytes from *ad libitum* and meal-fed lean rats converted 14 and 25 nmol glucose to CO$_2$/10$^6$ cells/hr, respectively, versus adipocytes from the *ad libitum* and meal-fed obese rats, which converted 11 and 9 nmol/10$^6$ cells/hr, respectively. Adipocytes from lean Zucker rats maintained their insulin sensitivity long-term, particularly those that were meal-fed exhibiting an insulin response 2.7 times that of basal.

**Glycolytic Enzymes**

In order to handle the increased glucose load and insulin sensitivity detailed above, enzymes in the adipocytes of meal-fed rats likely become upregulated. The activity of the three, regulatory glycolytic enzymes (hexokinase, phosphofructokinase, and pyruvate kinase) in the epididymal adipose tissue of
meal-fed versus *ad libitum*-fed rats has been compared by measuring the transformation of 1 nanomole of substrate per minute (Leveille, 1970). Of the three, two are more active in meal-fed animals. These two enzymes are hexokinase and pyruvate kinase, indicating an increased phosphorylation of glucose entering the adipocyte and an increased conversion of phosphoenolpyruvate to pyruvate in the adipose tissue of meal-fed rats.

Phosphofructokinase (PFK) was the third enzyme examined. The PFK activity in *ad libitum*-fed rats was 5 nmol/min and in meal-fed rats it was 6 nmol/min. These numbers are not statistically different.

**The Missing Factor**

Much of the work concerning adipocyte metabolism has been conducted on isolated adipocytes and has utilized Rodbell's procedure (1964). There is an extensive body of published work detailing known experimental and animal-related factors that yield reliable, repeatable, interlaboratory results. Anecdotal information suggests that strict adherence to the aforementioned factors does not guarantee success. Although Honnor et al. (1985) have shown that overnight starvation played an important role in reducing lipolytic variability of adipocytes, the role of the timing of food intake on insulin-stimulated glucose oxidation in adipocytes has not been explored.

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2 personal communication between Gale Carey and Mario DiGirolamo December 21, 2007; email between Gale Carey and Susan K. Fried January 4, 2008; personal communication between Gale Carey and Michael Spurlock August 2007; personal communication between Gale Carey and Alan Greene January 2008; Chapter 3
The ability of meal feeding to synchronize a large number of physiologic parameters is well-known. By making food available to the rats during one concise time period each day, the variability caused by unknown amounts and time of food intake is eliminated. The work presented in this thesis determined whether meal feeding synchronizes processes associated with nutrition to produce a robust, reliable, and repeatable insulin-stimulation of rat adipocyte glucose oxidation.
CHAPTER II

MATERIALS AND METHODS

I. Glucose Metabolism Study:

Animals

This study was conducted using 32 unrelated, male, weanling (51-75 g) Wistar rats bred at Charles River Laboratories (Wilmington, MA). The rats were housed two to one plastic tub until the second week of their arrival; at that time they were switched to individual tubs. The rats were maintained at ambient temperature and humidity and fed standard rat diet (Purina LabDiet 5P00, Brentwood, MO) and water ad libitum for two days after arrival. On the third day, the rats were switched to an inverted 12-h light/12-h dark cycle (dark from 7:00 a.m.-7:00 p.m.) and the rat chow was available during the dark cycle only. On the fifth day after arrival, the rat chow availability was reduced to a two hour window from 9:00 a.m. to 11:00 a.m. Water was provided ad libitum. Rats were maintained on this feeding schedule until they reached a body weight of 175-220 g. Two to four weeks are required for the rats to reach this weight range and for the physiologic and metabolic adaptations to occur (ie gastric and small
intestinal hypertrophy, upregulation of hepatic and digestive enzymes, increased secretion of hormones, etc) (Holeckova and Fabry, 1959; Fabry and Kujalova, 1960; Tepperman and Tepperman, 1964; Fabry and Braun, 1967). All procedures were approved by the University of New Hampshire Animal Care and Use Committee, #070601 and #080303.

**Preparation of Tissue Samples**

Rats were euthanized via carbon dioxide inhalation, and the right and left epididymal adipose tissues (Figure 1) were removed at three time points after meal-feeding: immediately, 10 hours, and 20 hours (n = 10 rats/time point, except 20 hours after feeding where n = 8 rats/time point) plus two pilot experiments at 5 H fasting (n = 4 rats). Tissue samples were weighed and placed in a plastic container of warm (approximately 37°C) saline to remove excess blood. The adipose tissue from two rats was pooled for each experiment. The samples were transported to the laboratory in the plastic container inside an insulated box containing 37°C tap water. Tissue samples were transported to the laboratory for processing within 15 minutes.

**Preparation of Isolated Adipocytes**

Tissue samples were incubated with collagenase to dissociate adipocytes in a modification of Rodbell's method (1964). The adipose tissue was minced with scissors, rinsed with warm saline (0.9% NaCl) on 1000 μm mesh (pre-rinsed with double-distilled H₂O prior to the start of the experiment), and blotted dry on
one piece of 90 mm diameter circular Whatman filter paper. The minced adipose tissue was then weighed into two 1 g portions in small weigh boats. Each 1 g portion was rinsed into a polypropylene flask with 3 mL warm working Kreb’s-Ringer-Bicarbonate buffer (KRBw) containing 2% bovine serum albumin (BSA). One mL of KRBw was added to a small glass test tube containing previously weighed and refrigerated 4 mg of type 1 collagenase (w/v). This mixture was vortexed and added to each flask containing the adipose tissue. The two flasks were placed in a shaking water bath (Precision Scientific, Chicago, IL) at 37°C and 80 oscillations per minute, gassed with 95%O2:5%CO2 for six seconds, sealed with a cork, and incubated for 42 minutes. Adipocytes were isolated as described by Hoppe and Carey (2007) with the following three modifications: the flasks incubated at 37°C for 42 minutes at 80 oscillations/min, the infranate from washed adipocytes was removed by puncturing the bottom of the 50 mL polypropylene centrifugation vial with a 22 g needle attached to a 60 CC syringe, and the final glucose concentration of the incubation medium was 6 mM.

**Adipocyte Incubation**

The ability of adipocytes to convert 14C-glucose to 14CO2 in the absence and presence of insulin was measured in vitro in 7 mL polypropylene vials. The vials were pre-rinsed with double-distilled H2O prior to the start of each experiment and placed in an inverted position on a tube rack to dry. Each experiment utilized nine incubation vials and during centrifugation of the isolated adipocytes, the following volumes of KRBw were added to the appropriate
incubation vials in triplicate: 112 uL (background), 82 uL (basal), and 62 uL (insulin-stimulated). The vials were then placed in the shaking water bath set to 37°C and 50 oscillations per minute, and 600 uL of the final cell suspension (316,166 to 397,600 cells/750 µl) was added to each vial using a cut 1000 uL pipette tip to minimize damage to the cells. At 30 second intervals, 37.5 µL of 6 mM D-[U-14C]-glucose solution (approximately 0.4 µCi/vial) and 30 µL of ADA solution were added to each vial. Twenty µL of porcine insulin was added to each insulin-stimulated vial. The basal and insulin-stimulated vials were gassed for five seconds with 95%O2:5%CO2. The vials were sealed with a rubber serum stopper fitted with a hanging center well with a trimmed stem and containing a 0.5 x 1 cm piece of filter paper folded accordion-style. For the background vials, 0.1 mL 60% perchloric acid (HClO4) was added, followed by 37.5 µL of D-[U-14C]-glucose solution and immediately sealed with serum stoppers. All vials were returned to the shaking water bath for an additional 60 minutes at 37°C and 80 oscillations per minute to allow for complete 14CO2 collection.

**CO2 Production**

CO2 production over the 90 minute incubation was measured by following the conversion of D-[U-14C]-glucose to 14CO2 in a method described by Rodbell (1964). 14CO2 was trapped in a CO2 trapping solution of phenethylamine:methanol:toluene (1:1:2, v/v/v). Ten minutes prior to the end of the 90 minute incubation period, two separate 1 cc tuberculin syringes fitted with 18 gauge, 1.5 inch needles were filled with either 60% HClO4 or CO2 trapping
solution. Five minutes prior to the end of the 90 incubation, 0.3 mL of CO₂ trapping solution was injected through the serum stopper into the hanging center well of the background vials. These vials were transferred from the shaking water bath to the bench top where they remained for 60 minutes. After the 90 minute incubation, at 30 second intervals, 0.3 mL of the CO₂ trapping solution was injected into the hanging center wells of the remaining vials followed by 0.1 mL of 60% HClO₄ injected through the serum stopper directly into the incubation medium to terminate the incubation. Samples were returned to the shaking water bath for an additional 60 minutes at 37°C and 80 oscillations per minute.

After 60 minutes, all vials were transferred to a ventilated hood. The serum stoppers were carefully removed from each vial and using tweezers, and the hanging center wells were separated from the serum stoppers. The outside of each center well was wiped with a Kimwipe to remove any radioactive contamination, and transferred to a 20 mL glass scintillation vial. Ten mL of aqueous scintillation cocktail was added to each scintillation vial and the vials were vigorously shaken. Vials were kept in the dark for at least 15 minutes to minimize chemiluminescence, and then counted in a Beckman LS 6000IC liquid scintillation counter (Beckman Instruments, Fullerton, CA).

One scintillation vial, containing 10 mL of scintillation cocktail only was used to determine background radioactivity. Three scintillation vials, each containing 20 uL of the 6 mM unlabeled D-glucose with 100 μCi/mL D-[U-¹⁴C]-glucose solution plus 10 mL scintillation cocktail were counted to determine the
specific activity of the D-[U-14C]-glucose. The specific activity was used to calculate glucose conversion to CO2. Results were expressed as nmol glucose converted to CO2 by 10^5 adipocytes/90 minutes.

**Determination of Cell Number and Size**

In a ventilated hood, duplicate 600 µL aliquots of cell suspension were added to 20 mL plastic scintillation vials containing four volumes (2400 µL) 1% (w/v) osmium tetroxide-50mM collidine buffer, pH 7.4, to fix the adipocytes following a method previously described (Carey and Sidmore, 1994). The vials were placed in a water bath (Precision Scientific, Chicago, IL) maintained at approximately 37°C. Maintaining the water level at the neck of the vials was necessary to preserve adipocyte integrity. After 48 hours in the water bath, vial contents were filtered, washed with 0.01% Saline-Triton X-100, and osmium-fixed cells were resuspended in 6 to 7 mL of resuspension solution. Cell number was determined by manually counting 12 fields/experiment of 6 µL aliquots of cells on a hemacytometer at the 10X objective on a Leitz microscope (Wetzlar, Germany). Cell size was measured using computerized image analysis (Meservey and Carey, 1994).

**II. Reagents**

Adenosine deaminase, from calf intestine (10 mg) was purchased from Roche Diagnostics (Indianapolis, IN). Collagenase Type 1(Lot # X6C8693,
19 u/mg) was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Glycerol, anhydrous was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Toluene and perchloric acid were purchased from Fischer Scientific (Fairlawn, NJ). D-[U-\textsuperscript{14}C]-glucose (250μCi, Lot #3589538) and Ultima Gold Scintillation Cocktail were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). Osmium tetroxide was purchased form Colonial Metals (Elkton, MD). Albumin from bovine serum (BSA, Lot # 057K0737 and insulin (porcine pancreas, 28.7 USP units/mg) were purchased from Sigma Chemicals (St. Louis, MO). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

III. Solutions

Glucose Oxidation Solutions:

Stock Solutions for Krebs Ringer –Bicarbonate Buffer (KRB):

Solutions for stock KRB are stable for several months at 4°C. The solutions were prepared as follows: 2 M NaCl was prepared by dissolving 58.44 g NaCl (MW 58.44) in double-distilled H\textsubscript{2}O; the volume was brought to 500 mL in a graduated cylinder. 1 M KCl was prepared by dissolving 7.46 g KCl (MW 74.6) in double-distilled H\textsubscript{2}O; the volume was brought to 100 mL in a graduated cylinder. 1 M CaCl\textsubscript{2}-2H\textsubscript{2}O was prepared by dissolving 14.702 g CaCl\textsubscript{2}-2H\textsubscript{2}O (FW 147.02) in double-distilled H\textsubscript{2}O; the volume was brought to 100 mL in a graduated cylinder. 1 M MgCl\textsubscript{2}-6H\textsubscript{2}O was prepared by dissolving 20.33 g MgCl\textsubscript{2}-6H\textsubscript{2}O (FW 203.30) in double-distilled H\textsubscript{2}O; volume was brought to 100 mL.
in a graduated cylinder. 1 M KH$_2$PO$_4$ was prepared by dissolving 13.609 g KH$_2$PO$_4$ (MW 136.09) in double-distilled H$_2$O; the volume was brought to 100 mL in a graduated cylinder. 1 M HEPES was prepared by dissolving 119.15 g HEPES (MW 283.3) in double-distilled H$_2$O; the volume was brought to 500 mL in a graduated cylinder and the pH was adjusted to 7.76. 1 M NaHCO$_3$ was prepared by dissolving 42.005 g NaHCO$_3$ (MW 84.01) in double-distilled H$_2$O; the volume was brought to 500 mL in a graduated cylinder.

**Stock Krebs Ringer-Bicarbonate Buffer (pH 7.4):**

A 2 L volume of Stock Kreb's Ringer-Bicarbonate Buffer (KRBs) was prepared fresh at the beginning of a week's experiments. The stock buffer was stable for one week at 4°C and was prepared by adding 1 L double-distilled H$_2$O to a large beaker; then, the following solutions were added in order while stirring: 50 mL 1M HEPES (pH 7.76), 125 mL 2M NaCl, 10 mL 1M KCl, 2 mL 1M KH$_2$PO$_4$, 2.5 mL 1M MgCl$_2$-6H$_2$O, 2.5 mL 1M CaCl$_2$-2H$_2$O, and 50 mL 1M NaHCO$_3$. Working pH was adjusted to 7.4 and the final volume was brought to 2 L in a graduated cylinder with double-distilled H$_2$O.

**Working Krebs Ringer-Bicarbonate Buffer, 2% BSA (pH 7.4):**

To ensure the integrity of the components of the Working Kreb's Ringer-Bicarbonate Buffer 2% BSA (KRB'2), it was prepared fresh two hours prior to the start of each experiment by adding 200 mL KRBs to a beaker to which 22 mg L-ascorbic acid (FW 176.1), 0.5 mL 0.5M glucose solution, and 2.5 mL 100 μM adenosine solution were added, while stirring. The beaker was then covered with parafilm and the buffer was equilibrated by bubbling with
95%O₂:5%CO₂ for 10 minutes. Five g BSA was dissolved in the buffer by vigorously mixing it in the still-covered beaker for approximately five minutes. The pH was adjusted to 7.4 and the final volume was brought to 250 mL in a graduated cylinder with KRBs. The covered beaker was then transferred to the shaking water bath and allowed to warm to 37°C.

**Working Krebs Ringer-Bicarbonate Buffer, 4% BSA (pH 7.4):**

As with the KRB'2, the integrity of the components of the Working Kreb's Ringer-Bicarbonate Buffer 4% BSA (KRB'4) was ensured by preparing it two hours prior to the start of each experiment by adding 40 mL KRBs to a beaker. Ascorbic acid (4.4 mg), 0.1 mL 0.5M glucose, and 0.5 mL 100 µM adenosine were added to the KRBs, while stirring. The beaker was then covered with parafilm and the buffer was equilibrated by bubbling with 95%O₂:5%CO₂ for 10 minutes. Two g BSA was dissolved in the buffer by vigorously mixing it in the still-covered beaker for approximately five minutes. The pH was adjusted to 7.4 and the final volume was brought to 50 mL in a graduated cylinder with KRBs. The covered beaker was then transferred to the shaking water bath and allowed to warm to 37°C.

**0.5 M Glucose:**

A stock solution of 0.5 M glucose was prepared at the start of the study and stored in 3 mL aliquots at -20°C. The stock was prepared by dissolving 18.02 g D-(+)-glucose (FW 180.2) in double-distilled H₂O; the volume was brought to 200 mL in a graduated cylinder. Prior to the start of each experiment,
one aliquot was removed from the freezer and allowed to thaw to room temperature on the bench top.

100 µM Adenosine:

A stock solution of 100 µM adenosine was prepared at the start of the study and stored in 4 mL aliquots at -20°C. The stock was prepared by dissolving 6.75 mg adenosine (MW 267.2) in double-distilled H₂O; the volume was brought to 250 mL in a graduated cylinder. Prior to the start of each experiment, one aliquot was removed from the freezer and allowed to thaw to room temperature on the bench top.

0.9% Sodium Chloride:

The saline solution was prepared fresh at the beginning of a week's experiments and stored at 4°C. The solution was prepared by dissolving 9 g NaCl (FW 58.44) in double-distilled water; the volume was brought to 1 L in a graduated cylinder.

10⁻⁷ M Insulin:

A stock solution of 10⁻⁶ M porcine insulin was prepared at the start of the study and stored in 3 mL aliquots at -20°C. The solution was prepared by dissolving 4.3 mg insulin in 1 mL of 0.9% saline. Four drops of 0.01N HCl were added to solubilize the insulin. A 10-fold dilution with saline was performed to yield a stock solution of 10⁻⁶ M. During the digestion of the adipose tissue, one aliquot of the stock solution was removed from the freezer and kept on ice until the end of the cell centrifugation. At that time, another 10-fold dilution with 0.9% saline was carried out and the insulin was kept on ice until one minute before the
incubation was to begin. At that time, the vial was transferred to a tube rack and
guarded to warm to room temperature.

**Adenosine Deaminase:**

The adenosine deaminase (ADA) solution was prepared fresh for each
experiment while the isolated adipocytes were undergoing centrifugation. To
prepare the ADA solution, 7.2 μL of ADA (1560 units/mL) was added to 202.8 μL
of 0.9% saline, for a final volume of 210 μL and final concentration of 1 unit per
30 μL of solution. The vial was vortexed to mix the contents and kept on ice until
one minute before the incubation was to begin. At that time, the vial was
transferred to a tube rack and allowed to warm to room temperature.

**133.33 mM Unlabeled D-Glucose:**

A stock solution of 133.33 mM D-(+)-glucose was prepared at the start of
the study and stored in 4 mL aliquots at -20°C. The solution was prepared by
dissolving 2.402 g D-(+)-glucose FW (180.2) in double-distilled H₂O; the volume
was brought to 100 mL in a graduated cylinder. During adipocyte digestion, one
aliquot was removed from the freezer and allowed to thaw on the bench top.

**D-[U-¹⁴C]-Glucose:**

The D-[U-¹⁴C]-Glucose was prepared fresh for each experiment. During
adipocyte digestion, 75 μL of D-[U-¹⁴C]-glucose in ethanol 1:9 was added to a 20
ml glass vial, placed on ice, and transferred to a ventilated hood. Ethanol was
evaporated by attaching a 5 mL pipet tip to rubber tubing to deliver nitrogen gas.
The gas was turned on at a rate just high enough to cause slight movement of
the D-[U-¹⁴C]-glucose in the vial. The procedure was completed once the volume
in the vial appeared to be just 10% of its original amount (approximately two to five minutes). A 116 mM solution was prepared by adding the following to the vial: 675 μL of 133.3 mM unlabeled D-glucose and 92.5 μL double-distilled H₂O. The vial remained on ice until one minute before the incubation was to begin. At that time, the vial was transferred to a tube rack specifically labeled for radioactive use. To achieve a final concentration of 6 mM in each incubation vial, 37.5 μL of the 116 mM solution was added to the vials.

CO₂ Trapping Solution:

The CO₂ trapping solution was prepared at the beginning of the study and was stable indefinitely when stored in a dark bottle. The solution was prepared by mixing 12.5 mL phenethylamine, 12.5 mL methanol, and 25 mL toluene in a ventilated hood, yielding a 1:1:2 v/v/v solution.

Osmium Fixing Solutions:

Stock Collidine Buffer (0.2 M):

The stock collidine buffer was prepared fresh at the beginning of the study and stored at 4° C. The stock buffer was prepared by dissolving 26.4 mL of 2,4,6 trimethylpyridine in double-distilled H₂O in a ventilated hood and brought to a final volume of 1 L. The solution was allowed to mix for 24 hours.

Working Collidine Buffer (0.05 M):

The working collidine buffer was prepared fresh at the beginning of the study and stored at 4° C for three months. The working buffer was prepared by
mixing 250 mL of stock collidine buffer and 225 mL of 0.01 N HCl in a ventilated hood. The final volume was brought to 1 L in a graduated cylinder with 0.9% saline.

1% Osmium Tetroxide Solution:

The 1% (w/v) osmium tetroxide solution was prepared 12 hours prior to the start of an experiment and stored in a dark bottle at room temperature for one week. The solution was prepared by dissolving 1 g osmium tetroxide in 100 mL of working collidine buffer (warmed to 37° C) while stirring. The pH of the working collidine buffer was adjusted to 7.4 at the time of solution preparation.

Saline-Triton Solution (0.01%):

The 0.01% Saline-Triton solution was prepared fresh at the beginning of the study and stored at 4° C. To prepare this solution, 10 mL of 1% (v/v) Triton X-100 was dissolved in saline. The final volume was brought to 1 L in a graduated cylinder with 0.9% saline.

Resuspension Solution:

The resuspension solution was prepared fresh at the beginning of the study and stored at 4° C. The solution was prepared by dissolving 19.25 g NaCl (FW 58.44) in 200 mL double-distilled H2O. Five mL 1% Triton X-100 and 277.5 mL glycerol (FW 92.09) were added and mixed thoroughly. The final volume was brought to 500 mL in a graduated cylinder with double-distilled H2O.
Figure 1: Rat testicle and attached epididymal fat pad (arrow).
CHAPTER III

Reagents, Environmental Conditions, and Animal Model as They Affect Insulin-Simulated Glucose Oxidation in Rat Adipocytes

To determine the cause of the lack of reproducible insulin response in pilot glucose oxidation experiments, a series of 26 trouble-shooting experiments was conducted utilizing ad libitum-fed rats. In these experiments, rat adipocytes were isolated from the left and right epididymal fat pads of male rats and incubated under varying conditions related to reagents, environmental experiment conditions, and animal model. Adipocyte glucose conversion to $^{14}$CO$_2$ was measured both in the presence and absence (control) of insulin. Calculation of insulin stimulation as a percent of control was determined.

The first set of 14 experiments evaluated whether the lack of insulin-stimulated glucose oxidation was due to reagents and procedures. The results suggest that purchasing new reagents, preparing the solutions with new double-distilled water, using different combinations of BSA and collagenase, preparing the insulin solution with 0.1 N HCl, searching for the optimal radiolabeled glucose concentration, removing and replacing adenosine to the buffer, and devising a new method of washing the cells did not restore insulin sensitivity.
<table>
<thead>
<tr>
<th>Factor</th>
<th>% Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal D-[U-14C]-glucose concentration (done twice)</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; time: 8 &amp; 12mM: 0%</td>
</tr>
<tr>
<td></td>
<td>4mM: 73%</td>
</tr>
<tr>
<td></td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; time: 0%</td>
</tr>
<tr>
<td>Insulin preparation with 0.1N HCl &amp; Optimal glucose concentration of final buffer</td>
<td>0%</td>
</tr>
<tr>
<td>New bottle of insulin &amp; 3 different lots of collagenase (done twice)</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; time: Collagenase A &amp; C: 0%</td>
</tr>
<tr>
<td></td>
<td>Collagenase B: 10%</td>
</tr>
<tr>
<td></td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; time: 0%</td>
</tr>
<tr>
<td>Different manufacturer and type of BSA</td>
<td>0%</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt; trapping solution (done twice)</td>
<td>Oxidation not measured; trapping solution is 95-100% effective</td>
</tr>
<tr>
<td>Increased length of collagenase digestion (performed twice)</td>
<td>0%</td>
</tr>
<tr>
<td>New plastic ware &amp; reagents, D-[U-14C]-glucose concentration, insulin concentration, newly purchased reagents</td>
<td>1mM glucose &amp; 10&lt;sup&gt;-5&lt;/sup&gt;M insulin: 36%</td>
</tr>
<tr>
<td></td>
<td>8mM glucose &amp; 10&lt;sup&gt;-5&lt;/sup&gt;M insulin: 10%</td>
</tr>
<tr>
<td>Removal of adenosine from buffers; varied insulin and glucose concentrations</td>
<td>1mM glucose &amp; 10&lt;sup&gt;-5&lt;/sup&gt;M insulin: 48%</td>
</tr>
<tr>
<td></td>
<td>1mM glucose &amp;10&lt;sup&gt;-7&lt;/sup&gt; insulin: 36%</td>
</tr>
<tr>
<td></td>
<td>8mM glucose &amp; 10&lt;sup&gt;-5&lt;/sup&gt;M insulin: 37%</td>
</tr>
<tr>
<td></td>
<td>8mM glucose &amp;10&lt;sup&gt;-7&lt;/sup&gt; insulin: 44%</td>
</tr>
<tr>
<td>Method of washing cells during isolation</td>
<td>0%</td>
</tr>
<tr>
<td>Adenosine added back to buffers</td>
<td>20%</td>
</tr>
<tr>
<td>Increased BSA in isolation buffer from 1 to 2% and new vials vs. old vials</td>
<td>New vials: 63%</td>
</tr>
<tr>
<td></td>
<td>Old vials: 68%</td>
</tr>
<tr>
<td>New double-distilled water to make reagents</td>
<td>16%</td>
</tr>
</tbody>
</table>
The second set of seven experiments evaluated whether the lack of insulin-stimulated glucose oxidation was due to environmental factors. The results suggest that, with the exception of one set of experimental vials (unrinsed polypropylene centrifugation tubes) none of the environmental factors examined restored insulin sensitivity.

Table 2 – Effects of Environmental Changes on Glucose Oxidation

<table>
<thead>
<tr>
<th>Factor</th>
<th>% Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Removed PBDE contaminated glass and plastic ware</td>
<td>0%</td>
</tr>
<tr>
<td>Contamination of polypropylene centrifugation tubes</td>
<td>Ethanol rinsed: 94%; water rinsed: 83%; no rinse: 210%</td>
</tr>
<tr>
<td>Effect of light/dark on plastic ware due to presence of Tinuvin 770</td>
<td>Light: 57%</td>
</tr>
<tr>
<td>Laboratory setting and ADA manufacturer</td>
<td>Dark: 53%</td>
</tr>
<tr>
<td>Incubation time course (including pH measurement, incubation vials, switch to porcine insulin)</td>
<td>30': 39%; 60': 67%; 90': 63%</td>
</tr>
<tr>
<td>Contamination of incubation vials</td>
<td>Unrinsed vials: 12%; Rinsed vials: 11%</td>
</tr>
<tr>
<td>Effect of light in the lab and preparation of insulin with buffer vs. saline</td>
<td>Dark lab + saline prep: 45%; Dark lab + buffer prep: 38%; Light lab + saline prep: 0%; Light lab + buffer prep: 25%</td>
</tr>
</tbody>
</table>
The final set of five experiments evaluated whether the lack of insulin-stimulated glucose oxidation was due to animal factors.

### Table 3 – Effects of Animal Changes on Glucose Oxidation

<table>
<thead>
<tr>
<th>Factor</th>
<th>% Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague-Dawley rats from different supplier, collagenase B vs. C, use of plastic tubing during cell washing</td>
<td>0%</td>
</tr>
</tbody>
</table>
| Wistar rats, collagenase B vs. C, removed plastic tubing during cell washing | Collagenase B: 40%  
Collagenase C: 0%               |
| Mario DiGirolamo’s recommendations (90 g rats)                         | 489%            |
| Mario DiGirolamo’s recommendations (177 g rats)                        | 264%            |
| Mario DiGirolamo’s recommendations (176 g rats)                        | 18%             |

The final two experiments utilizing 177 g and 176 g rats were performed 24 hours apart from one another. These experiments were conducted using the same reagents; the buffers were made from the same bottles of stock; the animals were from the same breeding facility, arrived to the University in the same shipment, were handled the same, weighed the same, were the same age, and were euthanized at the same time of day. However, the first experiment yielded a 264% insulin stimulation of adipocyte glucose oxidation over basal, while the second experiment yielded 18% stimulation. Since all experimental conditions had been held constant, we considered any remaining animal factors that may have varied between the two pairs of rats. Because the animals were fed in the traditional *ad libitum* fashion, we realized that the pattern of food intake
was a variable that had not yet been held constant. This led to the design of a study in which \textit{in vitro} insulin-stimulated glucose oxidation of adipocytes was examined in rats whose food intake was synchronized via meal-feeding.
CHAPTER IV

Effects of Meal-Feeding on In Vitro Insulin-Stimulated Glucose Oxidation in Rat Epididymal Adipocytes

INTRODUCTION

With the incidence of obesity on the rise both in developed as well as underdeveloped countries, and in children as well as adults (Fruhbeck and Gomez-Ambrosi, 2001; Saltiel and Pessin, 2002; Ogden et al., 2006; Aronne and Isoldi, 2007; Rabinowitz, 2008), much of the attention regarding fat cells surrounds their physiologic roles of lipid storage and breakdown and how they contribute to numerous chronic diseases. Less attention is focused on the maintenance functions of adipocytes, including cellular respiration and metabolic activities. The primary source of energy utilized by the adipocyte to perform these maintenance functions is glucose, the uptake and metabolism of which is regulated by insulin, among other regulatory hormones.

In order to study metabolism in isolated adipocytes under different experimental conditions, researchers have implemented the method developed by Martin Rodbell (1964) in which $^{14}$C is used as a radiolabeled tracer to follow
the insulin-stimulated oxidation of glucose to CO₂. However, since the time of Rodbell’s published work, several factors have been revealed that are found to increase the magnitude, reliability, and repeatability of results. Strict adherence to these experimental and animal model factors does not guarantee success, as shown by the results of trouble-shooting experiments conducted by this laboratory in an attempt to obtain consistently reliable insulin-stimulated glucose oxidation results.

One factor that is often overlooked in the life of the experimental rodent animal model is feeding. By tradition, the experimental rat is fed ad libitum. In addition to creating obese animals, ad libitum feeding leads to rats that develop early endocrine hypersecretion, hypertrophy (especially in the liver, kidney and endocrine organs), hyperplasia and metabolic disruption before endocrine tumors can be observed (reviewed in Keenan et al., 1999). The most common endocrine tumors observed in these animals are those of the pituitary, pancreas and target tissues such as the mammary gland (Holehan and Merry, 1986). Overnutrition associated with ad libitum feeding results in an unhealthy animal that is not physiologically "normal" and represents a variable that could explain the increased variability and lack of reproducibility observed in rodent bioassays (Keenan et al., 1997). An alternative feeding method that not only synchronizes food intake but also results in leaner, healthier animals, is meal-feeding.

Meal-feeding is a process by which animals are fed during a short time span each day. The length of time can vary, ranging from 2 to 4 hours/24 hours for smaller rats to 8 hours/48 hours for larger rats (Carey et al., 1993). While the
amount of food presented in that time span can be fixed, most researchers impose no limitation, with intake in a two hour period averaging 75% of the amount that an ad libitum-fed rat would consume in a day (Wiley and Leveille, 1970; Romsos and Leveille, 1974; Lima et al., 1981; Sugden et al., 1999).

Numerous metabolic and physiologic adaptations occur in the meal-fed rat. These include a lower body weight, longer life span (Weindruch and Walford, 1982; Masoro, 1985; Nelson and Halberg, 1986; Holehan and Merry, 1986; reviewed in Masoro, 1988), decreased mitotic activity, hypertrophy of the stomach and small intestine and synchronization of numerous hepatic and digestive enzymes (Tepperman and Tepperman, 1958; Holeckova and Fabry, 1959; Cohn and Jospeh, 1960; Hollifield and Parson, 1962; Saito et al., 1976; Phillipens, 1980; reviewed in Boulos and Terman, 1980; Kohsaka and Bass, 2006). While meal-feeding has not yet been implemented as a short-term synchronizer of insulin-stimulated glucose oxidation in the isolated rat adipocyte, its effects on other aspects of glucose metabolism as well as plasma glucose and insulin concentrations in rats have been studied.

Using the young male rat and adhering to conditions recommended as optimal for glucose oxidation (DiGirolamo 2001; personal communication between Gale Carey and Mario DiGirolamo December 21, 2007), the current study examined the effects of meal-feeding on the magnitude and variability of insulin-stimulated glucose oxidation in the adipocyte in vitro at three time points throughout the day. The first time point was immediately after removal of food.
(11:00 a.m.), the second was 10 hours post-prandial (9:00 p.m.), and the third was 20 hours post-prandial (7:00 a.m.).
MATERIALS AND METHODS

Animals

Thirty-two male Wistar rats weighing 51-75 g, purchased from Charles River Lab, Wilmington, MA, were used for this experiment. Rats were offered rat chow (ProLab HMR 3000) and water ad libitum for two days. Their light/dark cycle was inverted on the third day (dark 7:00 am-7:00 pm) and their food intake was restricted to the dark cycle only. On the fifth day after arrival, the food was available from 9:00 am-11:00 am, and the rats were maintained on this feeding schedule until they reached a body weight of 175-220 g, approximately 3 weeks. Two pilot experiments were performed five hours after feeding, using two rats per experiment. Four to five experiments (using 2 rats per experiment) were performed immediately after feeding, 10 hours after feeding, and 20 hours after feeding. All procedures were approved by the University of New Hampshire Animal Care and Use Committee, #070601 and #080303.

Tissue Preparation and Adipocyte Isolation

Following euthanasia via CO₂ gas inhalation, right and left epididymal fat pads were removed from two rats. Fat pads were minced and dissociated in a polypropylene flask with 1 mg/ml collagenase type 1 (Worthington, code #CLS-1,
lot #X6C8693) and 4 mL of Kreb’s-Ringer-Bicarbonate (KRB) buffer containing 2% bovine serum albumin (Sigma, catalog #A7030, lot #057K0737) in a modification of Rodbell’s method (1964). Adipocytes were isolated as described by Hoppe and Carey (2007) with the following three modifications: the flasks incubated at 37°C for 42 minutes at 80 oscillations/min, the infranate from washed adipocytes was removed by puncturing the bottom of the 50 mL polypropylene centrifugation vial with a 22 g needle attached to a 60 CC syringe, and the final glucose concentration of the incubation medium was 6 mM.

**Adipocyte Incubation**

The ability of adipocytes to convert $^{14}$C-glucose to $^{14}$CO$_2$ in the absence and presence of insulin was measured *in vitro* in 7 mL polypropylene vials, each containing 600 µL of the adipocyte resuspension. At 30 second intervals, 37.5 µL of 6mM D-[U-$^{14}$C]-glucose solution (0.4 µCi), 30 µL of adenosine deaminase (1 unit/vial), and 20 µL of buffer or porcine insulin ($1\times10^{-7}$M) was added to each vial in triplicate. The vials were gassed for six seconds with 95%O$_2$:5%CO$_2$ and immediately sealed with a rubber serum stopper fitted with a hanging center well containing filter paper. All vials were incubated for 90 minutes at 37°C and 80 oscillations per minute.

**CO$_2$ Production**

The conversion of D-[U-$^{14}$C]-glucose to $^{14}$CO$_2$ was measured over the 90 minute incubation following the method described by Rodbell (1964). $^{14}$CO$_2$ was
trapped in 0.3 mL of a CO$_2$ trapping solution (phenethylamine:methanol:toluene, 1:1:2, v/v/v), added to each hanging center well just prior to the end of the 90 minute incubation. The incubation was terminated by adding 0.1 mL of 60% HClO$_4$ to the adipocyte solution. Triplicate vials provided background values by adding 60% HClO$_4$ just before D-[U-$^{14}$C]-glucose. Background vials were transferred from the shaking water bath to the bench top where they remained for 60 minutes. All other vials were returned to the shaking water bath for an additional 60 minutes at 37°C and 80 oscillations per minute to allow for complete $^{14}$CO$_2$ collection.

After 60 minutes, the serum stoppers were removed from each vial. Hanging center wells were separated from the serum stoppers, the outside was wiped, the wells were transferred to scintillation vials, and 10 mL of aqueous scintillation cocktail was added to each vial. Vials were kept in the dark for at least 15 minutes to minimize chemiluminescence, and then counted in a Beckman LS 6000IC liquid scintillation counter. Results were expressed as nmol glucose converted to CO$_2$/10$^5$ adipocytes/90 minutes.

**Cell Number and Size**

Duplicate 600 µL aliquots of cell suspension were added to 20 mL vials containing 2400 µL 1% (w/v) osmium tetroxide solution to fix the cells (Carey and Sidmore, 1994). Cell number was determined by manually counting 12 fields/experiment of 6 µL aliquots of cells on a hemacytometer at the 10X
objective on a Leitz microscope (Wetzlar, Germany). Cell size was measured using computerized image analysis (Meservey and Carey, 1994).

**Data Analysis**

Results are presented as means ± SD. Data were analyzed by one-way analysis of variance (ANOVA) using InStat Graph Pad software.
RESULTS

Rat and Adipocyte Characteristics

Body weight of rats in this study averaged 199 g and was not significantly different among experimental time points (Table 4). Similarly, rat age averaged 55 days, and was not significantly different among time points. Number of cells/750μl, cell size [both diameter (μm) and volume (pl)], and epididymal fat pad weight were not significantly different among the three time points. While the mean age of the rats was 55 days, there was nearly a two week range in age that may have afforded the older rats more time to accumulate fat, thereby increasing the cell volume of adipocytes. Indeed, cell volume was significantly correlated with increasing age (p=0.002, r=0.614).

Glucose Oxidation

Basal glucose oxidation rates averaged 9.0 nmol/10^5 cells/90 min (Table 5), but tended to decrease with hours fasted (p=0.16). The nmol of glucose oxidized at 0 H were 30% and 53% greater than at 10 H and 20 H, respectively. Likewise, glucose oxidation rates in the presence of insulin averaged 32 nmol/10^5 cells/90 min (Table 5) and tended to decrease with hours fasted (p=0.15). The nmol glucose oxidized in the presence of insulin at 0 H were 57% and 71% greater than at 10 H and 20 H, respectively. Insulin-stimulated glucose
oxidation was statistically greater than basal glucose oxidation at each of the three time points (p<0.05) (Figure 2). When each experiment was normalized for basal glucose oxidation, absolute insulin stimulation at 0 H fasted was 69% greater than at 10 H fasted and 78% greater than at 20 H fasted (Figure 3). Absolute glucose oxidation tended to decrease with hours fasted (p = 0.17). Additionally, the coefficient of variation at 0 H was 53% and fell to 45% and 29% at 10 H and 20 H, respectively. Fold response of insulin stimulated over basal was similar (p = 0.75) among the three time points and averaged 3.5 (Figure 4). The coefficient of variation at 0 H was 27% and decreased to 17% by 20 H.

**Anatomical Changes in the Gastrointestinal Tracts of the Rats**

At 0 H the stomachs of the rats were very large (Figure 5a). With the rats placed in dorsal recumbency, it was easy to appreciate the size of the stomach protruding out from under the liver, extending well beyond the margins of the liver within the abdominal cavity. The size of the small intestine was relatively unenlarged (Figure 5a). The fact that the stomach was filled with partially digested food (as opposed to air) was confirmed by observing the translucent portion of the stomach which was visibly filled with partially digested food (Figure 5b). At 10 H the size of the stomach had diminished, but lead to an increase in the size of the large intestine (Figure 6). By 20 H the stomach had returned to the normal position under the liver (Figure 7). In order to see the stomach, the liver had to be retracted. The large intestine was much bigger in size at this time point.
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0 H</td>
<td>1.0 ± 0.2</td>
<td>336840 ± 19641</td>
<td>49.4 ± 2.1</td>
<td>63.1 ± 7.3</td>
<td>207 ± 17</td>
<td>53 ± 5.6 (range 48-65)</td>
</tr>
<tr>
<td>10 H</td>
<td>1.0 ± 0.09</td>
<td>338369 ± 34301</td>
<td>50.0 ± 2.7</td>
<td>66.3 ± 10</td>
<td>192 ± 16</td>
<td>54 ± 3.9 (range 47-62)</td>
</tr>
<tr>
<td>20 H</td>
<td>1.1 ± 0.1</td>
<td>369542 ± 26084</td>
<td>49.7 ± 1.5</td>
<td>64.1 ± 1.5</td>
<td>197 ± 5.2</td>
<td>58 ± 2.2 (range 54-63)</td>
</tr>
</tbody>
</table>

Table 4: Effect of hours fasted on pooled (n=2) epididymal fat pad weight, cell number and size, rat body weight and age. Average pooled fat weight, cell number and size, rat body weight and age did not differ among the three time points. Results are expressed as means ± SD, n=4-5 experiments per time point.
Table 5 - Glucose Oxidation in the Absence and Presence of Insulin

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>Basal</th>
<th>Insulin Stimulated</th>
<th>Absolute Insulin Stimulation</th>
<th>Fold Increase with Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 H</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.2±2.7</td>
<td>41.5</td>
<td>30.3</td>
<td></td>
<td>3.7</td>
</tr>
<tr>
<td>12.8</td>
<td>58.9</td>
<td>46.1</td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>8.6</td>
<td>19.5</td>
<td>11.0</td>
<td></td>
<td>2.3</td>
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<tr>
<td>14.8</td>
<td>67.3</td>
<td>52.5</td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>8.7</td>
<td>28.9</td>
<td>20.2</td>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>Average¹</td>
<td>11.2±2.7</td>
<td>43.2±20</td>
<td>32.0±17 (53%)</td>
<td>3.7±1.0 (27%)</td>
</tr>
<tr>
<td>10 H</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>19.6</td>
<td>13.5</td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>4.3</td>
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<td>13.2</td>
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<tr>
<td>Average¹</td>
<td>8.6±3.7</td>
<td>27.5±11</td>
<td>18.9±8.5 (45%)</td>
<td>3.3±0.9 (27%)</td>
</tr>
<tr>
<td>20 H</td>
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<tr>
<td>7.4</td>
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<td>12.6</td>
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<td>34.6</td>
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<td>22.7</td>
<td>17.0</td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>Average¹</td>
<td>7.3±1.6</td>
<td>25.3±6.4</td>
<td>18.0±5.3 (29%)</td>
<td>3.5±0.6 (17%)</td>
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Table 5: Effect of hours fasted on glucose oxidation in the basal (absence of insulin) and insulin-stimulated states.
¹ Averages are means ± SD. Values in parentheses are coefficient of variation (C.V.) around the mean. P = 0.15 for insulin-stimulated glucose oxidation among the time points. P = 0.17 for absolute insulin stimulation among the time points.
Figure 2: Glucose Oxidation

**Glucose Oxidation in the Absence and Presence of Insulin**

![Bar chart showing glucose oxidation in basal and insulin-stimulated states for 0H, 10H, and 20H fasted conditions.](image)

**Figure 2:** Effect of hours fasted on glucose oxidation in the basal (absence of insulin) and insulin-stimulated states. Results are expressed as means ± SD. *p <0.05 compared to absence of insulin at the same hours fasted.

Figure 3: Insulin-Stimulated Glucose Oxidation

**Glucose Oxidation: Absolute Insulin Stimulation**

![Bar chart showing calculated absolute insulin-stimulated glucose oxidation for 0H, 10H, and 20H fasted conditions.](image)

**Figure 3:** Effect of hours fasted on calculated absolute insulin-stimulated glucose oxidation. Results are expressed as means ± SD. Glucose oxidation in the presence of insulin tended to decrease with hours fasted (p = 0.17).
Figure 4: Insulin-Stimulated Glucose Oxidation

Glucose Oxidation: Fold Response to Insulin

Figure 4: Effect of hours fasted on fold increase of insulin-stimulated glucose oxidation over basal. No differences were noted among the time points (p=0.75). Results are expressed as means ± SD.
Figure 5a: Rat abdominal cavity at 0 H-fasted (immediately after 2-hour meal). Stomach (arrows) is markedly enlarged in both the translucent and opaque portions and extends beyond the margins of the liver.
Figure 5b: Rat stomach at 0 H-fasted. While both portions of the stomach are filled with food, it is visible only in the translucent portion.
Figure 6: Rat abdominal cavity at 10 H-fasted. Stomach was still large (red arrow), and extended beyond the margins of the liver, although to a lesser extent than the stomach of the 0 H-fasted rat. The colon (black arrow) was enlarged.
Figure 7: Rat abdominal cavity at 20 H-fasted. Stomach (red arrow) had returned to normal size and position in the abdominal cavity (under the liver). Colon (black arrows) was markedly enlarged.
DISCUSSION

Ad libitum feeding has been described as the "most poorly controlled variable [in rodent bioassays] and it adversely affects every physiological process and anatomical structure to the molecular level" (Keenan et al., 1999). By controlling this variable using a meal-feeding regimen, our findings demonstrate that adipocytes from meal-fed rats show robust and reliable glucose oxidation in the presence and absence of insulin. In contrast to 26 experiments yielding from zero to marginal insulin response of glucose oxidation in adipocytes of ad libitum-fed rats, 16 experiments conducted with adipocytes from meal-fed rats demonstrated insulin stimulation of glucose oxidation that was consistent with expected results based on the literature (DiGirolamo, 2001; Hoppe and Carey, 2007).

The two to three-fold insulin response of adipocyte glucose oxidation expected from rats at this body weight (DiGirolamo, 2001) was obtained in a consistent manner immediately, 10 hours, and 20 hours after feeding. The variability of the response was greatest immediately after eating, but tended to decline within 20 hours of additional fasting. Leveille and Hanson (1965) refed rats after a 22 hour fast and also found that the greatest variability in their adipose tissue glucose oxidation results occurred at the end of a two hour meal (similar to the 0 H-fasted time point in the present study).

A second finding of this study was the trend toward a decrease in insulin-stimulated glucose oxidation with length of fast (p = 0.15), consistent with
published literature (Olefsky, 1976; Newby et al., 1990). Despite this trend toward a decrease in nmol of glucose oxidized, the fold insulin-stimulation persisted across all time periods (average of 3.5-fold). The fold insulin response was accompanied by a decrease in variability at 20 H fasted. Close examination of the fold increase with insulin results at 10 H reveals the existence of one value (4.8) well out of the range of the remaining values (2.5 to 3.2). Given the small number of replicates at each time point, this one extreme value increases the coefficient of variation at 10 H fasted, potentially masking an increase in reliability by this length of time fasted.

Published literature using adipocytes from ad libitum-fed rats demonstrates that glucose oxidation is inversely related to cell size. Indeed, a comparison of the amount of glucose oxidized to CO₂ from adipocytes in the present work to adipocytes from ad libitum-fed rats supports this observation. Using adipocytes from ad libitum-fed rats (body weight of 150 g and cell volume of 47 pl), DiGirolamo and Owens (1976) report basal glucose conversion to CO₂ at 1.58 μmol/CO₂/10⁷ cells/hr and insulin-stimulated conversion was 4.07 μmol/CO₂/10⁷ cells/hr, representing a 2.6-fold insulin-stimulation. In adipocytes from meal-fed rats in the present study (body weight of 199 g and cell volume of 65 pl), basal glucose conversion to CO₂ was 0.61 μmol glucose/10⁷ cells/hr and insulin-stimulated conversion was 2.1 μmol glucose/10⁷ cells/hr, representing a 3.4-fold insulin stimulation. Although the magnitude of insulin stimulation was similar in the two studies, the larger cell volume of the meal-fed
rats as compared to the ad libitum-fed rats supports the lower glucose oxidation results (DiGirolamo, 1981).

Consistent adipocyte concentration in the incubation vials is necessary for obtaining reproducible and successful glucose oxidation. DiGirolamo et al. (1993) found that increasing cell density from $0.17 \times 10^6$ to $1.25 \times 10^6$ cells/ml increased the amount of glucose converted to CO$_2$ from 0.8 to 1.6 µmol glucose/10$^7$ cells/90 min in the basal state and from 2.9 to 4.2 µmol glucose/10$^7$ cells/90 min in the presence of insulin, respectively. The average cell number in the present study was $0.46 \times 10^6$ cells/ml (range = $0.45 \times 10^6$ – $0.49 \times 10^6$ cells/ml) and was found to be consistent among the experiments (Table 1). At this cell density, 0.9 µmol glucose/10$^7$ cells/90 min were converted to CO$_2$ in the basal state and 3.2 µmol glucose/10$^7$ cells/90 min were converted to CO$_2$ in the presence of insulin. Therefore, although the cell density in this study was on the lower end of the recommended range, the glucose oxidation results compare favorably to those of DiGirolamo et al. (1993).

The results from this study are in agreement with published reports concerning the body weight differences between meal-fed and ad libitum-fed rats. The rats in this study gained an average of 50 g per week as opposed to the 70 g weekly weight gain expected in similar aged, ad libitum-fed rats (Hoppe and Carey, 2007). Also in keeping with the literature are the slightly larger average fat pad weight and cell diameter of the meal-fed rats in this study in comparison to ad libitum-fed rats. Average epididymal fat pad weight from the rats in this study (1.03 g) was 12% greater than from ad libitum-fed rats (0.92 g).
weighing an average of 200 g (Jamdar et al., 1986). And average cell diameter of the adipocytes from the rats in this study (49.7 μm) was 7% greater than the diameter of adipocytes from ad libitum-fed rats (46.6). While average cell size was larger, average cell volume of the adipocytes from the meal-fed rats (64.5 pl) was dramatically smaller when compared to adipocytes from ad libitum-fed rats weighing 214 g (120 pl) (Thacker et al., 1987). The adipocyte mass and morphology profile of the meal-fed rat is in keeping with the findings of Fabry and Braun (1967). The increased lipogenesis and feed efficiency known to occur in meal-fed animals (Tepperman and Tepperman, 1964) is not the result of increased lipid deposition within individual cells. A 27% increase in adipose tissue DNA (p<0.01) from rats meal-fed for seven weeks versus ad libitum-fed rats is supported by smaller fat cells observed via histological examination (Braun et al., 1965). No statistical analysis was conducted on cell size/100g of tissue examined. It is possible that the signal for adipocyte hyperplasia described in these rats is via peroxisome proliferator-activated receptor γ2 (PPARγ2), which is a lipid-activated transcription factor (Flier, 1995).

Of interest was the finding in this study that increasing age of the rats was positively correlated with increasing cell volume (p=0.002). This finding could be a result of normal growth, but is also likely a consequence of the meal-feeding regimen. As the meal-fed rat ages, physiologic responses to this pattern of food intake improve (enlargement of stomach and small intestine, delayed gastric emptying, increased glucose absorption, increase in hepatic and adipocyte
metabolic enzymes), potentially allowing for the production and storage of more fat in cells.

Anatomical differences between the 0 H- and 10 H-fasted rats were dramatic when compared to the 20 H-fasted and ad libitum-fed rats. The striking size of the stomach after the two hour meal was expected based on the work of Tepperman and Tepperman (1964), who described gastric hypertrophy in three hour meal-fed rats. The stomachs of these meal-fed rats contained 20-22 ml of material, vs. the 5 or 6 ml found in the stomachs of ad libitum-fed rats. The large size of the stomach 10 hours later was surprising. Delayed gastric emptying has been reported as a change that accompanies meal-feeding (Lima et al., 1981), but the degree to which the size of the stomach had not changed since the 0 H-fasted time point was unexpected. The return of the stomach to its usual position within the abdominal cavity after the 20 H-fast was expected and similar to the size observed in ad libitum-fed rats.

This study demonstrates that meal-feeding rats results in repeatable, robust adipocyte insulin-stimulated glucose oxidation. There are two potential mechanisms to explain this observed meal-feeding response. One mechanism is synchronization of peripheral molecular clocks that organize metabolism and feeding behavior (Damiola et al., 2000; Kohsaka and Bass, 2006). The expression of several genes known to be circadian controlled have been identified in fat depots of mice (Zvoncic et al., 2006). Two of these genes, Bmal1 and Clock, play a role in the regulation of glucose homeostasis in mice (Rudic et al., 2004). Additionally, Kreier and colleagues (2006) have demonstrated a
neuronal connection originating in the visceral adipose tissue, projecting to numerous parts of the central nervous system, including the suprachiasmatic nucleus in the hypothalamus, an area of the brain very close in proximity to satiety centers.

The second potential mechanism is synchronization of daily variations observed in serum insulin and glucose levels. Meal-feeding controls for the variability that ad libitum feeding can cause in the timing and amount of food consumed. Indeed, while the nmol of glucose oxidized per 10^5 cells per 90 minutes in the basal and insulin-stimulated states were higher just after eating, the variability was also greatest at that time point. Fasting the animals resulted in a trend toward a decrease in the nmol of glucose oxidized, but it also resulted in a decrease in the variability of the results obtained, thereby increasing the reliability of Rodbell's isolation procedure, as predicted by Keenan et al., (1998 and 1999).

Future work should include an expansion of this study to include more animals and pair-weighted, ad libitum-fed animals as controls. Another factor to investigate in future research is the effect on glucose oxidation of an overnight fast in an ad libitum-fed rat. Honnor et al. (1985) utilized an overnight fast to eliminate the tremendous variability of lipolysis results obtained from ad libitum-fed rats. Whether or not overnight fasting, in contrast to meal-feeding, is sufficient to eliminate variability of insulin-stimulation of glucose oxidation remains to be determined.
CHAPTER V

CONCLUSION

Meal-feeding is a pattern of food intake utilized by researchers as a method of synchronizing numerous metabolic processes. Meal-feeding was employed in this study as a means of synchronizing food intake after 26 experiments conducted on adipocytes from ad libitum-fed rats yielded unpredictable and variable insulin-stimulated glucose oxidation results. Results from two pilot experiments and fourteen study experiments show that adipocytes from meal-fed rats exhibited an expected 3-fold response to insulin stimulation. The results also show that the variability in this response decreased with hours fasted. Thus, this feeding regimen resulted in robust, reliable, in vitro, insulin-stimulated glucose oxidation of rat epididymal adipocytes. Future experiments should include an increase in the number of animals examined at each time point and weight-paired, ad libitum-fed control animals. Also of interest would be a measurement of insulin-stimulated glucose oxidation from adipocytes of ad libitum-fed animals following an overnight fast.
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APPENDIX A

University of New Hampshire

Research Conduct and Compliance Services, Office of Sponsored Research
Service Building, 51 College Road, Durham, NH 03824-3585
Fax: 603-862-3564

26-Jun-2007

Carey, Gale B
Animal & Nutritional Sciences, Kendall Hall
Durham, NH 03824

IACUC #: 070601
Project: PBDEs and Adipocyte Metabolism
Category: B
Approval Date: 18-Jun-2007

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the protocol submitted for this study under Category B on Page 5 of the Application for Review of Vertebrate Animal Use in Research or Instruction - the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Roger Wells at 862-2726 or Julie Simpson at 862-2003.

For the IACUC,

Jessica A. Bolker, Ph.D.
Chair

cc: File
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