Design and Testing of an Infrared Temperature System to Test the Effects of Chemical Stimulation on Heat Production in Cell Cultures

Nicholas Gerbo
Louisiana State University and Agricultural and Mechanical College

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DESIGN AND TESTING OF AN INFRARED TEMPERATURE SYSTEM TO TEST THE EFFECTS OF CHEMICAL STIMULATION ON HEAT PRODUCTION IN CELL CULTURES

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College In partial fulfillment of the Requirements for the degree of Master of Science in Biological and Agricultural Engineering

In

The Department of Biological and Agricultural Engineering

By
Nicholas M. Gerbo
B.S., Louisiana State University, 2005
December 2008
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<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenine Diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenine Monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine Triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown Adipose Tissue</td>
</tr>
<tr>
<td>BRL</td>
<td>BRL-37344</td>
</tr>
<tr>
<td>CL</td>
<td>CL 316-243</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FPR</td>
<td>Fluorescent Plate Reader</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanine Diphosphate</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanine Monophosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine Triphosphate</td>
</tr>
<tr>
<td>MDI</td>
<td>0.5 mmol/l 3-isobutyl-methylxanine, 1 µmol/l dexamethasone, and 1.7 µmol/l insulin</td>
</tr>
<tr>
<td>MIR</td>
<td>Mid Infrared</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>PPARγ coactivator-1α</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptor</td>
</tr>
<tr>
<td>PPRE</td>
<td>PPAR Response Element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>Re</td>
<td>Equivalent Radius</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory binding protein</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling Protein</td>
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<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
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</table>
Abstract

Temperature is an important physiological parameter of cell biology. The thermal energy output of cells in vitro can offer insight into cellular processes. Standard methodology of measuring cellular thermal output has been through microcalorimetry. In this work, infrared thermography is presented as an alternative to microcalorimetry and other measurement techniques. An enclosure that controls the temperature of cells and allows for injection of temperature control of chemical agents and subsequent measurement of cell temperature using an infrared camera has been designed and tested. The enclosure was tested with 3T3-L1 adipocytes treated with the β3 adrenergic agonist CL 316-243 for up to seven days in order to stimulate heat generation.

The optimization of the system was successful in ensuring that large temperature fluctuations in the design were minimized. It was shown that there was no statistical difference (p>0.5) in the temperature of empty cell wells treated with vehicle, therefore significant differences observed in cell studies are due to differences in cellular metabolism brought about by chemical stimulation. A statistical power test based on the initial characterization using a 95% confidence interval shows that 3 replicates should be adequate to detect an average temperature increase of approximately 0.3°C assuming the treatment standard deviation remained the same as the control blanks.

The enclosure was tested with 3T3-L1 adipocytes treated with the synthetic agonist CL 316-243 for 0, 4 and 7 days prior to being measured using infrared thermography and are shown to heat up significantly in some cases. Standard deviations increased slightly in the cell treatment tests and therefore larger temperature increases were necessary to establish a significant increase. It was hypothesized that the 7-day
treatment would have the largest response, however some of the 4-day treatments were shown to have statistically significant increases over controls whereas the seven-day treatments only indicated a tendency of temperature increase over the control. This is likely due to the desensitization of the cells.

Advances in IR techniques and decreasing cost of IR technology makes it likely that IR technology use in biotechnology will increase in the future.
Chapter 1: Introduction

1.1 Background

The United States of America and the world in general have seen a marked increase in the average weight of their populations (World Health Organization 2000; Bays 2004; Eckel RH 2004). Currently, 64% of the U.S. adult population is considered to be some form of obese, from overweight to morbidly obese (Pressler 2005). It is believed that the main causes of obesity are prolonged periods of decreased physical activity and high fat diets (Schrauwen and Westerterp 2000), but other theories, such as the effect of dietary intake of monosodium glutamate (Hermanussen, Garcia et al. 2006) are being investigated. The term “metabolic syndrome” has been coined to describe the problems that are associated with obesity. The metabolic syndrome is a group of disorders that are often observed together, including obesity, insulin resistance, glucose intolerance, hypertension and dyslipidemia (Moller and Kaufman 2005). Soon, the metabolic syndrome will push cigarette smoke out of its’ place as the number one risk factor for cardiovascular disease in the US (Gogia and Agarwal 2006) unless treatment and prevention measures are employed. It is important to maintain a healthy weight to avoid the negative health aspects of obesity and the possible development of insulin resistance (Gogia and Agarwal 2006), causing an increase in blood glucose, and ultimately leading to Type-two diabetes.

In light of the prevalence of these circumstances, investigations into the molecular causes and effects of obesity have received a tremendous amount of attention in the last several decades. One item that was thoroughly investigated was the seeming diversity of
metabolic efficiency of people. Some people simply use more and store less of the energy they consume. Researchers therefore have begun searching for a “magic bullet” to tune the metabolic efficiency in order to control weight and other health factors.

1.2 Metabolic Efficiency

One mechanism suspected to account for the inconsistent metabolic efficiency of different individuals was based on the plasticity of specific adipose tissue’s energy consumption. Brown adipose tissue (BAT), which is present in small rodents and infants of larger mammals, is very rich in mitochondria when compared to normal white adipose. This buildup of mitochondria and the associated elevated vascularization gives these cells a distinctive reddish brown color, hence their name (Bengtsson, Redegren et al. 1996). Brown adipocytes tend to have multiple small pockets of stored triacylglycerols (termed multilocular adipocytes), rather than traditional white adipocytes, which at maturity are typically unilocal. The additional mitochondria allow brown adipose to use more free fatty acids (FFAs) rather than releasing them into the blood stream to be used by other tissue. One of the functions of brown adipose tissue is to facilitate nonshivering thermogenesis, a phenomenon of thermoregulation in small mammals (Carlson and Cottle 1956; Depocas, Hart et al. 1956; Bukowiecki, Collet et al. 1982; Ashwell, Jennings et al. 1983; Jacobsson, Stadler et al. 1985; Milner and Trayhurn 1989).

The feature that gives brown adipose its high degree of metabolic flexibility is a specific protein that is present in the mitochondria of the cells. This protein, initially termed Thermogenin and later Uncoupling Protein I (UCP1), has a unique function to uncouple (hence the name) oxidative phosphorylation from mitochondrial proton transport (Matthias, Ohlson et al. 2000). The proton gradient that is generated from the
reduction of nicotinamide adenine dinucleotide (NAD\(^+\)) and flavin adenine dinucleotide (FAD\(^{2+}\)) via the electron transport chain is used to generate heat, instead of facilitating the ATP Synthase reaction (Figure 1). In addition to this protein, BAT also expresses a high level of other proteins involved in the transport and beta oxidation of fatty acids, which contribute to the elevated oxidative capacity of the cells (Cannon and Nedergaard 2004).

![Figure 1. The mitochondrial Electron Transport Chain with UCP1 Proton transport chain. UCP1 bypasses ATP Synthase and creates heat (Nelson 2005).](image)

The idea of some cells having variable degrees of metabolic efficiency led scientists to hypothesize that if an obese person could have their fat cells altered to be more like brown adipose tissue, which can produce heat instead of ATP, he/she would become less metabolically efficient. Less metabolic efficiency would mean that a fewer number of the consumed calories would be converted into a useable form, as some energy would be lost to heat. Concordantly, there was a belief that thermoregulation was
dysfunctional in obese due to under active sympathetic nervous system (the system that controls fat storage and breakdown and other important metabolic processes, (Trayhurn 2005), but this belief has recently been challenged (Eikelis and Esler 2005), and it is well established that energy expenditure is higher in obese subjects (Prentice, Black et al. 1986; Bandini, Schoeller et al. 1990; Prentice, Black et al. 1996). Recent studies have shown a correlation between polymorphisms in UCP2 and UCP3 and obesity onset (Kawaguchi, Masuo et al. 2006; Masuo, Katsuya et al. 2006), supporting the case of deficient uncoupling capacity as a causative agent. It has been shown that mitochondrial dysfunction can cause triglyceride accumulation (Vankoningsloo, Piens et al. 2005) but it is unclear if this is a causative agent or logical clinical manifestation, as triglyceride accumulation is a likely outcome of reduced beta oxidative capacity. The potential ability to pharmacologically control metabolic efficiency led to investigations of brown adipose tissue, with hopes of controlling differentiation from white to brown adipocytes (as BAT is rare in adult humans, (Bengtsson, Redegren et al. 1996), as well as BAT activity after differentiation.

It is presently considered unlikely that brown adipocytes play a significant role in adult human thermogenesis (Bengtsson, Redegren et al. 1996) because of their small numbers in adult humans (one brown adipocyte for every 100-200 white adipocytes, (Oberkofler, Dallinger et al. 1997)). There has been recent evidence of BAT in adult humans (Nedergaard, Bengtsson et al. 2007), suggesting that obesity treatment using brown adipose tissue could be viable (Cadenas, Buckingham et al. 1999) but research with murine models has so far been unsuccessful in producing a safe and effective therapy for humans (Weyer, Gautier et al. 1999). Other research areas that show promise
in producing an effective anti-obesity treatment are the examination of the effects of peroxisome proliferator-activated receptors (PPARs), sterol regulatory binding proteins (SREBPs), and leptin and other secreted hormones involved in the neuro-hormonal control of energy use.

Logically, white adipocytes were the next target for research, as less was known about the cells themselves, and increases in their size and number is the defining characteristic of obesity. It had been long assumed based on the physiology of white adipose tissue that the function of white adipocytes was limited to storage and breakdown of triacylglycerols (TAGs) (Cannon and Nedergaard 2004), while providing thermal and mechanical insulation for the whole organism. The lipid containing vacuole can comprise 95% of a mature white adipocytes’ volume (Carmen and Victor 2006). It was believed that white adipose was the end point of a signal pathway; WAT simply stored and released FFAs when signaled to do so (Krug and Ehrhart-Bornstein 2005). It is now known that white adipose tissue (WAT) is a complicated endocrine organ that is involved in a wide variety of vital processes (Krug and Ehrhart-Bornstein 2005).

White adipose tissue is generally not believed to have a significant thermogenic capacity (Bengtsson, Redegren et al. 1996) and basal adipose energy consumption is relatively low compared to the brain and other major organs (Heymsfield 2003). This idea has been challenged (Granneman 2003) based on the observation that restoring $\beta_3$ adrenergic receptors expression only in BAT of $\beta_3$ knockout mice does not restore normal thermogenesis, but restoring $\beta_3$ adrenoceptors expression in BAT and WAT does (Grujic, Susulic et al. 1997; Ito 1998). It has also been shown that mice lacking UCP1 still demonstrate 20-40% of wild-type thermogenesis in response to acute $\beta_3$ adrenergic
stimulation (Da-Wei Gong 2000). The idea that white adipose contributes significantly to the metabolic output of an organism is unlikely, but has persisted due to the aforementioned studies, and because only BAT and WAT posses β3 receptors and therefore the effects of stimulation were limited to those organs. It has been shown however, that there are peripheral effects of acute β3 adrenergic stimulation in muscle tissue too, likely due to elevated level of plasma free fatty acid (Nakamura, Nagase et al. 2001; Garcia-Roves, Huss et al. 2007).

These observations do not preclude the possibility of WAT adopting features of BAT upon specific external stimulation. In fact, this has been reported in several instances including cold exposure and acute β3 adrenergic treatment (Cinti, Cancello et al. 2002), a diet rich in n-3 polyunsaturated fatty acids of marine origin (Flachs, Horakova et al. 2005), PPARα activators (Cabrero, Alegret et al. 2001), PPARδ activators (Wang, Lee et al. 2003), adenovirus mediated expression of PPARγ coactivator 1α (PGC-1α) (Tiraby, Tavernier et al. 2003), hormonal signaling using leptin (Commins, Watson et al. 1999), and retinoic acid treatment (Tomas, Jimenez-Jimenez et al. 2004; Mercader, Ribot et al. 2006). It is noteworthy that several of the above examples are merely stimulation of the same pathway at different points (Nedergaard, Petrovic et al. 2005) and that increased expression of important genes did not necessarily translate into increased metabolic activity (Thurlby, Wilson et al. 1987).

1.2.1 β Adrenergic Receptors and Agonists

Heat production from BAT and FFA release from WAT is mediated by the sympathetic nervous system via adrenergic signals (Cannon and Nedergaard 2004). Adrenergic receptors are broken into two groups; alpha and beta that are both G-(7TM)
coupled proteins (Kobilka, Matsui et al. 1987). Beta-receptors are classified as such because they stimulate adenylyl cyclase while alpha-receptors inhibit it. Alpha 2 adrenergic receptors play a role in modulating neurotransmitter release from sympathetic and adrenergic neurons (Hein, Altman et al. 1999). Beta 3 receptors lack the functional groups that are typically associated with desensitization (Emorine, Marullo et al. 1989; Nahmias, Blin et al. 1991). This form follows the function of adrenergic stimulation in that thermogenesis is often a prolonged requirement for animals. Desensitization would therefore not be advantageous for the animal, although the expression level of the mRNA that code for the β3 receptor is transiently down regulated during stimulation (Granneman and Lahners 1995; Klaus, Muzzin et al. 1995; Bengtsson, Redegren et al. 1996). One of the reasons that stimulation of these nerves has remained an attractive prospect for obesity therapy is because the receptors are almost exclusively found in white and brown adipose (Cannon and Nedergaard 2004) although some undesirable peripheral effects in other tissues in response to an elevated FFA level in the plasma have been observed (Nakamura, Nagase et al. 2001).

The three most frequently used β3 agonists are Norepinephrine (NE), CL 316243 (CL) and BRL- 37344 (BRL). NE is frequently used in studies because it bears the most physiological significance since it is the naturally occurring signal. BRL is a selective β3 agonist; at higher concentrations it will stimulate β2 receptors too. CL is the most acute β3 agonist known so it has been useful in studies aimed at distinguishing the functions of the β3 receptors (Cannon and Nedergaard 2004). Norepinephrine activates α1 and α2 receptors as well as β receptors. This is an important distinction and studies using one or the other should be carefully compared. As it was mentioned earlier, α2 receptors mediate the
effects of β stimulation by inhibiting adenyl cyclase. One study that compared the
effects of chronic stimulation of NE and CL found the effects of CL to be much greater
than the effects of NE on total energy expenditure and resting metabolic rate in male
Sprague-Dawley Rats (Atgie, Faintrenie et al. 1998). The studies that have seen the most
pronounced effect on WAT have used CL as their agonist of choice (Langin, Portillo et
al. 1991; Carpene, Galitzky et al. 1993; Galitzky, Reverte et al. 1993; Simard, Atgie et al.
and the expression of UCP1 has been observed in WAT after chronic CL treatment
(Nagase, Yoshida et al. 1996; Ghorbani and Himms-Hagen 1997). Chronic stimulation (7
or more days) of white adipocytes with CL has caused a significant portion of the
adipocytes (33%) to express UCP1 (Cinti 2005).

Chronic (several days) β3 adrenergic stimulation with CL in white adipose tissue
has been shown to induce a pro-inflammatory response (Li, Zhu et al. 2005). This
inflammatory response was characterized by the fractioning of the large unilocular TAG
depots into multilocular depots, the biogenesis of mitochondria, and the up regulation of
genes involved in beta oxidation (Granneman, Li et al. 2005). Previously, it has been
shown that differences in mitochondrial content of white adipocytes modulate the
oxidative capacity (Deveaud, Beauvoit et al. 2004). This increase in the number of
mitochondria and expression of beta oxidation proteins should result in an increase in the
amount of free fatty acids (FFAs) the cells can metabolize rather than exporting the FFAs
into the blood stream. These physiological changes in WAT are very characteristic of
BAT (Bengtsson, Redegren et al. 1996) and thus far have only been classified using
histological, morphological (Granneman, Li et al. 2005), and DNA microarray analysis
(Oana, Homma et al. 2005). Studying this phenomenon using new measurement techniques such as infrared thermography in vitro will provide some insight to the degree of inducible heat generation over periods of chronic stimulation in white adipose in real time. This would be an indication of the contribution of white adipose to $\beta_3$ agonist mediated thermogenesis.

1.2.2 Uncoupling Proteins

The uncoupling proteins are inner mitochondrial membrane proteins that all bear a close sequence homology to UCP1, and were therefore believed to perform a similar thermogenic role (Boss, Samec et al. 1997; Fleury, Neverova et al. 1997; Gimeno, Dembski et al. 1997; Gong, He et al. 1997; Solanes, Vidal-Puig et al. 1997). UCP2 and UCP3 are now believed to enable mild uncoupling to prevent the buildup of reactive oxygen species (ROS) in the event of depleted electron acceptors and therefore do not contribute significantly to thermogenesis (Negre-Salvayre, Hirtz et al. 1997; Arsenijevic, Onuma et al. 2000; Duval, Negre-Salvayre et al. 2002; Ruzicka, Skobisova et al. 2005), however this role has been disputed (Duval, Camara et al. 2007). There is conflicting evidence for the role of UCP3 in controlling ROS production. UCP3 deficient studies show an increase in ROS production and damage (Vidal-Puig, Grujic et al. 2000; Brand, Pamplona et al. 2002), while UCP3 over expression does not show a decrease in ROS damage (Brand, Pamplona et al. 2002; Mozo, Ferry et al. 2006). Regardless of the specific mechanisms of UCP3 action, there are several desirable effects of increased UCP3 in skeletal muscle such as increased cellular longevity, an increase in metabolic flexibility and increased insulin sensitivity (Bezaire, Seifert et al. 2007).
In contrast to UCP1, which is primarily expressed in BAT (Nicholls and Locke 1984; Nicholls and Rial 1999; Nedergaard, Golozoubova et al. 2001), UCP2 is expressed in a wide range of tissues including WAT and skeletal muscle (Fleury, Neverova et al. 1997), and UCP3 is expressed in skeletal muscle and the female reproductive tract (Vidal-Puig, Solanes et al. 1997; Rousset, Alves-Guerra et al. 2003). UCP2 mRNA expression levels are not accurate predictors of protein levels, as some tissues that transcribe the UCP2 gene show no trace of the protein using antibody detection techniques (Sivitz, Fink et al. 1999; Pecqueur, Alves-Guerra et al. 2001).

UCP3 was initially viewed with a great deal of interest because of its expression in skeletal muscle, and the high estimates of energy usage that was attributed to uncoupling (Stuart, Cadenas et al. 2001). UCP3 was thought to facilitate basal proton leak, a previously discovered phenomenon in muscle (Brand 1990) however this was disproved and it is now believed that proton leak is enabled by the adenine nucleotide transporter (Cadenas, Buckingham et al. 1999; Bezaire, Hofmann et al. 2001; Brand, Pakay et al. 2005).

It has been assumed thus far that the main action of the uncoupling proteins is to transport protons across the inner mitochondrial membrane, but other mechanisms such as fatty acid cycling/buffering have been suggested (Nicholls and Lindberg 1973; Winkler and Klingenberg 1994; Garlid, Orosz et al. 1996; Klingenberg and Huang 1999) and controversy remains over UCP mechanisms under physiological conditions (Garlid, Orosz et al. 1996; Gonzalez-Barroso, Fleury et al. 1998; Jezek, Engstova et al. 1998). The proton-buffering model postulates that protons are transported through UCP1 with the help of proton buffering amino acids, and fatty acids would function to donate protons to
the proton channel (Winkler and Klingenberg 1994; Klingenberg and Huang 1999). The fatty acid cycling model states that UCP1 facilitates transport of deprotonated fatty acids out of the mitochondrial membrane so that they may be protonated and freely transported back into the inner mitochondrial space (Skulachev 1991; Jezek, Orosz et al. 1994; Garlid, Orosz et al. 1996; Garlid, Jaburek et al. 1998; Jaburek, Varecha et al. 2001). Both mechanisms are congruent with the general model of fatty acid activation of UCP1 activity, but controversy remains as strong arguments have been made for and against both models (Gonzalez-Barroso, Fleury et al. 1998; Garlid, Jaburek et al. 2000; Garlid, Jaburek et al. 2001; Rial, Aguirregoitia et al. 2004).

UCPs have been shown to be inhibited by phosphorylated purine nucleotides such as ATP, ADP, GTP and GDP but not purine nucleotide monophosphates (AMP, GMP) (Heaton, Wagenvoord et al. 1978; Nicholls 2001). This mechanism is only hypothesized to exist in vivo, as only a limited amount of evidence for in vivo activity has been reported (Hagen, Zhang et al. 2000). Regardless of the mechanisms, UCP2 and UCP3 do not under normal circumstances contribute to nonshivering thermogenesis but UCP3 may be thermogenic under certain circumstances and could therefore be a target of pharmaceutical modulation (Echtay 2007). It is worth noting the two other UCPs, UCP4 and UCP5 that have a lower sequence homology to UCP1 and are hypothesized to also modulate ROS generation (Echtay 2007).

1.2.3 PPARs

The Peroxisome Proliferator Activated Receptors (PPARs) are members of the nuclear receptor superfamily of ligand inducible transcription factors (Desvergne and Wahli 1999; Kersten, Desvergne et al. 2000; Evans, Barish et al. 2004). When the
external ligand binds the receptor, it then binds with the retinoid X receptor (RXR) to form a heterodimer. This heterodimer, with the bound ligand, acts as a transcription factor, controlling the expression of a set of genes; it does this by binding directly to the PPAR response element (PPRE), a specific DNA sequence composed of direct repeats separated by one base pair. Gene target specificity of transcription factors is achieved by differences in the recognition sequence, the receptor configuration (i.e. heterodimer, a homodimer or a monomer), and the number of base pairs that separate the repeats (Aranda and Pascual 2001). The order of the response elements also contributes to specificity, as they can be arranged as palindromes, direct repeats, or inverted palindromes (Aranda and Pascual 2001). In the absence of a ligand, the PPAR-RXR heterodimer engage in active gene silencing by recruiting co-repressors, histone deacetylases and chromatin modifying enzymes (Ordentlich, Downes et al. 2001; Jepsen and Rosenfeld 2002; Privalsky 2004). When PPARs bind to their ligands, which are dietary lipids, the heterodimer goes through a conformational change and then binds with transcriptional activators, encouraging gene transcription (Smith 1997; Bocher, Pineda-Torra et al. 2002). The genes that are regulated by the PPARs are related to lipid metabolism, storage and transport and together are thought to coordinate lipid and carbohydrate metabolism (Semple, Chatterjee et al. 2006).

There are currently three known PPARs: PPARα, PPARγ, and PPARδ. PPARγ is expressed predominantly in adipose tissue and is important for regulating adipocyte differentiation and lipid accumulation (Sandouk, Reda et al. 1993; Tontonoz, Hu et al. 1994; Rosen and Spiegelman 2000; Ren, Collingwood et al. 2002; Rosen, Hsu et al. 2002). PPARγ is the target of a class of drugs called the thiazolidinediones (TZDs),
which are insulin sensitizing anti-diabetic drugs (Forman, Tontonoz et al. 1995; Lehmann, Moore et al. 1995). These drugs have also effected brown adipose recruitment, differentiation and increased UCP1 expression (Mercer and Trayhurn 1986; Rothwell, Stock et al. 1987), but β stimulation was required to produce changes in metabolism (Thurlby, Wilson et al. 1987). Alternatively, proteins inhibiting PPARγ degradation rather than stimulating expression or activity has been described (Floyd and Stephens 2004).

PPARα is expressed mostly in the liver, kidney, BAT, muscle and heart tissue and has been shown to coordinate the metabolic response to fasting by activating β-oxidation (Semple, Chatterjee et al. 2006). PPARα also controls gluconeogenesis (Patsouris, Mandard et al. 2004), biotransformation (Barbier, Villeneuve et al. 2003), and cholesterol metabolism (Lefebvre, Chinetti et al. 2006). PPARα receptors respond to synthetic fibrates as well as unsaturated fatty acids and eicosanoids. While genetic knockout studies implicate PPARα in a lipid regulating, anti-obesity role (Costet, Legendre et al. 1998; Guerre-Millo, Gervois et al. 2000; Mancini, Lanni et al. 2001; Vazquez, Roglans et al. 2001), it is unclear how this is facilitated (Stienstra, Duval et al. 2007). Regardless of the method of action, drugs that target these two receptors are in use and attempts to create new ones are ongoing, including a new set that target different combinations of the PPARs (Sharma 2008).

Until recently, little was known about PPARδ (Fredenrich and Grimaldi 2005), despite the fact that it was cloned in 1992 (Dreyer, Krey et al. 1992; Schmidt, Endo et al. 1992). It is poorly understood because it was not expediently investigated as it was shown to be widely expressed and therefore assumed to be an inferior pharmaceutical
target. What has been discovered is that with the increased expression profile came an increased functional profile. PPARδ is expressed in BAT, WAT, skeletal and cardiac muscle and acts to upregulate fatty acid transport and oxidation, thermogenesis (including UCP1 expression in WAT), and oxidative metabolism proteins in these tissues (Holst, Luquet et al. 2003; Tanaka, Yamamoto et al. 2003; Wang, Lee et al. 2003; Wang, Zhang et al. 2004; Abbot, McCormack et al. 2005; Barish, Narkar et al. 2006; Garcia-Roves, Huss et al. 2007). Murine receptor knockout models have produced several side effects including embryonic lethality, reduced adipose stores, myelination defects, modulated inflammation responses in skin, and impaired wound healing (Peters, Lee et al. 2000; Tan, Michalik et al. 2001; Barak, Liao et al. 2002). PPARδ is activated by a variety of saturated and polyunsaturated fatty acids in addition to several synthetic and natural eicosanoids (Yu, Bayona et al. 1995; Forman, Chen et al. 1997; Krey, Braissant et al. 1997; Xu, Lambert et al. 1999). The synthetic agonist GW501516 is the most widely tested and has been shown to induce β oxidation in skeletal muscle and adipose and UCP1 expression in WAT (Wang, Lee et al. 2003). This combination is useful as an anti-obesity drug, as shifts toward lipid metabolism in a significant portion of the body is necessary to ensure that a healthy amount of circulating lipid is maintained. It also acts to couple lipid mobilization and lipid usage and avoids side effects of other hormonal treatments such as loss of lean body mass and cardiac toxicity or excessive plasma FFAs levels (Wang, Lee et al. 2003).

There is presently an incomplete understanding of the PPARs and the above description of the PPARs is an extreme simplification of the existing knowledge. It is intended to give a brief overview into the general effects and activities of the molecular
mechanisms involved in cellular heat generation in order to illustrate that there is an abundance of molecular targets for adaptive thermogenesis.

1.2.4 PGC-1α/β

The PPARγ coactivator 1 α/β are transcription regulators that were shown to have an influence over transcription in a wide range of tissues (Giguere 2002; Wang, Lee et al. 2003; Lin, Handschin et al. 2005; Finck and Kelly 2006). PGC-1α and PGC-1β have homologous sequences and are suggested to have a degree of overlapping or redundant functionality (Kressler, Schreiber et al. 2002; Lin, Puigserver et al. 2002; Uldry, Yang et al. 2006), however this redundancy has been recently challenged (Sonoda, Mehl et al. 2007). PGC-1α has been shown to activate adaptive thermogenesis by regulating genes related to mitochondrial biogenesis and oxidative respiration (Puigserver, Wu et al. 1998; Wu, Puigserver et al. 1999; Lehman, Barger et al. 2000; Vega, Huss et al. 2000; Kelly and Scarpulla 2004; Lin, Handschin et al. 2005). It seems that PGC-1α regulates energy homeostasis in cells as when it is over expressed it causes an increased mitochondrial biogenesis and improves efficiency of the existing mitochondria through gene regulation (Wu, Puigserver et al. 1999; St-Pierre, Lin et al. 2003). Conversely, knockout models produce metabolic and performance deficiencies (Lin, Wu et al. 2004; Leone, Lehman et al. 2005; Sonoda, Mehl et al. 2007). It has been shown that chemical uncoupling of oxidative phosphorylation induces PGC-1α genes and subsequently other genes required for oxidative respiration (Rohas, St-Pierre et al. 2007).

1.2.5 Retionic Acid

Retinoic acid is a form of vitamin A that has been shown to have anti-obesity effects in mice, including UCP1 expression (Puigserver, Vazquez et al. 1996; Bonet,
Oliver et al. 2000; Ribot, Felipe et al. 2001; Felipe, Bonet et al. 2004; Mercader, Ribot et al. 2006), however, expression has not manifested in cultured models, implying an indirect signal (Mercader, Madsen et al. 2007). Retinoic acid regulates gene expression by targeting the Retinoid X Receptors (RXRs) and the retinoic acid receptors (RARs) (Bastien and Rochette-Egly 2004). There have also been reports of retinoic acid directly effecting protein activity (Bost, Caron et al. 2002; Teruel, Hernandez et al. 2003). The anti-obesity effects have been similar to the ones reported in PPARδ, i.e. increase in expression of genes involved in fatty acid oxidation and transport in WAT, BAT and skeletal muscle (Mercader, Ribot et al. 2006; Amengual, Ribot et al. 2008).

1.2.6 Adipokines

In addition to the previously described cell surface receptor pathways and transcriptional activators, there is another class of biological molecules that play a large role in energy balance and lipid accumulation called adipokines. Adipokines tend to coordinate hormonal signals of appetite, satiation and overall metabolism and they originate from adipose tissue. As these therapies do not aim to induce thermogenesis, they will only be partially reviewed here.

Leptin was one of the first known adipokines, and is predominantly secreted by WAT (Zhang, Proenca et al. 1994). Circulating levels of leptin correlated with total body fat and it was shown that pharmaceutical modulation of leptin could reduce body fat in mice (Halaas, Gajiwala et al. 1995; Maffei, Halaas et al. 1995; Halaas, Boozer et al. 1997). This model of energy regulation turned out to be incomplete, as tests on obese subjects revealed little change when administered leptin as subjects tended to develop a resistance to elevated leptin levels (Considine, Sinha et al. 1996).
The aforementioned molecular targets are all of interest for the pharmaceutical modulation of metabolic efficiency and their effects on specific tissue heat generation are of interest. The $\beta_3$ agonist CL 316243 is expected to have the largest effect on cultured white adipocytes and was therefore chosen for this initial study.

1.3 Cellular Heat Measurement Techniques

1.3.1 Microcalorimetry and Oxygen Consumption

The metabolic output of living cells in the form of dissipated heat is an important physiological factor that has been studied extensively using several different techniques. The study of thermal output of cells has the prospect of yielding several practical applications in medicine; a recent example is using microcalorimetry to more rapidly diagnose meningitis (Trampuz, Steinhuber et al. 2007). Cell heating data has also been useful in optimizing cell culture media formulation (Kemp 2001) and can be used to monitor cell culture health as it has been shown that cell heat output is a leading indicator of cell growth (Kemp 1997). The most common method to measure this parameter has been the use of microcalorimetry. Microcalorimeters have very high thermal sensitivity and have been used to quantify the total metabolic output of a variety of cells under various conditions (Kemp 1991; Kemp 1996; Kemp 2000). Additionally, microcalorimeters have been useful in the fields of biocompatibility, ecology, and other areas of science (Wadso 1995). The evolution of commercial microcalorimeters has been important for the adoption of the technology for new applications.

Isothermal titration calorimetry uses two insulated cell culture chambers surrounded by thermopiles that both measure and maintain the temperature in the vessel (Wadso 2001). The system monitors the energy consumption required to keep the
treatment and control vessel at the same temperature during the introduction of some reagent. Microcalorimetry has a very high thermal resolution (Wadso 2001), but a low spatial resolution. Smaller vessels have allowed for reduced sample sizes in order to improve temporal response, but these systems are less than ideal for recording highly accurate kinetic data during fast temperature fluctuations as any temperature changes must be transferred through conduction to the measurement thermopiles (Wadso 1995). Microcalorimetric studies tend to require lengthy and complicated setups and frequent calibration, which can be even more complicated when used simultaneously with O₂ measurement. Other disadvantages of microcalorimeters include errors due to gas permeability of tubing, damage to the cells during pumping, vessel biocompatibility, inappropriate stir rates for batch type devices and inappropriate flow rates for flow through calorimeters (Kemp 1997).

Despite these challenges, a large volume of information about the specific heat generation per cell has been reported for a wide variety of biological materials. The heat flux values range from 0.01 pW/cell for human erythrocytes to 329 pW/cell for rat hepatocytes (Kemp 1997). While this basal heat flux data is important, what is often more useful for practical application is the heat flux response due to some pharmacologic stimulation or during a relative scarcity of some important metabolite.

1.3.2 Thermal Lens Microscopy

Methods have been described to indirectly measure the temperature of single intact cells using a thermal lens technique. This procedure involves irradiating a cell with a pulsed laser to heat cellular components, followed by measurement of thermal relaxation times. Relaxation time is measured optically as cells change their refractive
index, which is detected by an additional probe laser beam (Zharov 2005). This technique has been used to measure the redox state of single cells (Lapenko, Romanovskaya et al. 2002a) and the response of cells in the presence of a drug (Lapenko, Romanovskaya et al. 2002b). The underlying assumptions in those reports are that the relationship between laser absorbance and temperature increase is linear and that no laser light is absorbed in other processes such as protein coagulation/denaturation, bubble formation, or other chemical phenomena. This technique is able to avoid using fluorescent dyes or chromophores, but relies instead on measuring laser-induced changes of the cell’s geometry due to thermal expansion rather than native induced changes in cells.

1.3.3 Thermal-Sensitive Dyes and Other Methods

Techniques to measure the temperature of cells using various thermally sensitive agents have been reported. Europium (III) thenoyltrifluoro-acetonate has been used to image cell surface heating responses (Zohar, Ikeda et al. 1998; Suzuki, Tseeb et al. 2007), while other studies have attempted to use other thermally sensitive fluorophores to detect temperature changes (Chapman, Liu et al. 1995). The use of thermo-sensitive fluorophores can produce three-dimensional images using two-photon imaging (Van Keuren 2004). These methodologies require the addition of a thermo-sensitive dye to the cell culture which could have unintended effects on cell metabolism as they require excitation with a laser or UV flash. These methods are also subject to photobleaching and thermobleaching of the signal that must be accounted for and calibrated into the system. Additionally, there may be heating effects from the excitation source that should be accounted for.
The use of a miniature thermocouple has also been reported to directly measure
the temperature of a single cell (Watanabe, Kakuta et al. 2005). The thermocouple was
fabricated directly onto a glass micropipette, which was used to both inject a cell with a
chemical substance and to measure the subsequent thermal effect. This measurement
method is prone to the thermal conductivity errors in which the thermocouple and pipette
material transports heat (Holman 2000). One of the more sophisticated techniques for
measuring the temperature of a small group of cells has involved thin film
nanofabrication and silicon micromachining of a small thermoelectric chip (Johannessen,
Weaver et al. 2002). The chip has a small well for cells that is surrounded by a reference
heater keeping the cells at physiological temperature. The reference heater is also
surrounded by an array of thermocouples to measure outward heat flux. This device was
used to measure the heat output of a small mass of cells (Johannessen, Weaver et al.
2002).

1.4 Infrared Thermography

Infrared thermography is a technique of temperature measurement that relies on
the natural emission of electromagnetic radiation of objects, which is usually maximum
in the IR range of the spectrum. All physical objects emit electromagnetic waves in the
mid-infrared (MIR) region of the spectrum and the emitted energy intensity is directly
correlated with the temperature of the material and the emissivity of the surface of the
material. Max Planck first described the blackbody emission and the equation that
describes this phenomenon (Holman 2000):

\[
W_{\lambda,b} = \frac{2\pi hc^2}{\lambda^5} \left( \frac{hc}{\lambda kT} - 1 \right)
\]
Where: \( W_{b,\lambda} \) - blackbody spectral radiant emittance at wavelength \( \lambda \); c - speed of light (m/s); h - Planck’s constant (6.6 x 10^{-34} \text{ Joule seconds}); k - Boltzmann constant (1.4 x 10^{-23} \text{ Joule/K}); T is the absolute temperature of a blackbody, and \( \lambda \) is wavelength.

The formula specifies that as temperature decreases, the peak spectral radiant emittance occurs at a higher wavelength. This explains how we can only “see” the temperature of very hot things (e.g. the coil on an electric stove), the maximum emittance of cooler objects have wavelengths which are above the visual range. By integrating the Planck equation, Stefan and Boltzman yielded a formula that establishes a relationship between radiant emittance and temperature of an object that also depends on the emissive properties of the material.

\[
W_b = \varepsilon \sigma T^4
\]

Where: \( W_b \) - total radiant emittance of a blackbody, \( \sigma \) - Stefan-Boltzmann constant (5.669 x 10^{-8} \text{ W/m}^2 \text{ K}^4), T - absolute temperature; and \( \varepsilon \) - emissivity of the object surface (the ratio of the emissive power of the object to the emissive power of a blackbody at the same temperature).

This correlation, along with highly sensitive photodetectors in the IR spectrum, allow for very good spatial, thermal and temporal resolution without interfering with the sample, as the measurement is performed remotely. The high spatial resolution allows for the measurement of many samples at once and therefore can be used in high throughput screening of the effects of pharmaceuticals on a cell population. This methodology was initially demonstrated by Paulik et. al., using transgenic yeast and brown adipocytes (Paulik, Buckholz et al. 1998). The yeast cells tested were transformed to express uncoupling protein 1 which uncouples oxidative phosphorylation from ATP generation.
making the yeast cell biochemically similar to brown adipose tissue (BAT). Brown adipose tissue generate heat in small mammals through non-shivering thermogenesis and is known to be one of the most proficient heat producing tissues (Kemp 1997). For infrared thermography to be a viable technique for receptor-activated heat detection, it would need to detect heating from cells that do not produce as much heat as brown adipose, as BAT is uncommon in most research models and is not present in significant quantities in adult humans. A similar technique has been used for substrate specificity screening in biochemical reactions (Millot 2002). The use of infrared thermography as a cell measurement tool in cellular biology could play an important role in high throughput screening of various biochemical derivates of anti obesity compounds and their effects on cellular functions and metabolism.

White adipose cells, in contrast to brown, are at the lower end of the heat producing power (49 pW/cell as opposed to 110 pW/cell for brown adipose) and the cultured murine model (3T3 L1), often used as a white adipose tissue (WAT) substitute in research, is even lower (17 pW/cell) (Kemp 1997). The pharmaceutical influence on the 3T3-L1 adipocytes has been well classified and the cells have been shown to be metabolically adaptive. In microcalorimetry studies, WAT harvested from humans displayed a heat production increase of 39.9% above basal upon insulin stimulation (Bottcher and Furst 1996) and a 55% increase under beta adrenergic stimulation (isoprenaline, a selective β₁, β₂ agonist) (Bottcher and Furst 1997a). Other microcalorimetry studies performed on white adipocytes have observed appreciable heat generation. Sorbris et. al., (Sorbris, Monti et al. 1982) and Bottcher et. al., (Bottcher and Furst 1997b) both observed that heat production from adipose tissue in lean people was
higher than observed in adipocytes harvested from obese people. In murine models, Nilsson-Ehle et. al. (Nilsson-Ehle and Nordin 1985) observed that isolated adipocytes displayed an increase in heat production upon the introduction of insulin and glucose. The attenuation of metabolic output based on temperature measurement could be therefore used as another tool for screening novel drugs created as a treatment for obesity. This information could be useful in identifying lead compounds from an assortment of chemical derivatives.

In addition to microcalorimetry, measurement of oxygen consumption has been commonly analyzed to directly determine metabolic activity occurring during oxidative phosphorylation. In one study it was observed that WAT had volumetric O$_2$ consumption rates 7 to 8 times lower than BAT when treated with insulin and triiodothyronine in cells cultured from the same animal under the same circumstances (Klaus, Ely et al. 1995). If these results are then linearly correlated to the infrared thermography study performed by Paulik, et al., (Paulik, Buckholz et al. 1998) on BAT which observed a temperature increase of 0.8 °C after acute β3 stimulation, then there should be at least a 0.16 °C change in temperature of similarly treated WAT.

To measure such small temperature differences it is necessary to fully insulate the sample and to reduce or even eliminate outside temperature influences. This necessitates the control of sample injection, in order to minimize the thermal effects of the fluid itself. The purpose of this study was to design and test a device and procedure that allows remote temperature measurement of very small temperature changes during chemical stimulation of cells for detection of increased metabolic activity. Such information could be useful for evaluating potential lead compounds for treating metabolic disorders.
Once the setup was validated, cell tests were performed using 3T3-L1 cultured adipocytes. The goal of the study was to try to reproduce the remodeling effects that have been previously reported by treating the cells chronically with a potent β₃ agonist for seven days. Chronic treatment, as opposed to a single dose, should have the effect of increasing the overall metabolic capability of the cell and therefore the total heat output. The goal is to validate the method of heat measurement with a cell model and show that detection of heat is possible with cells that are not conventionally thought to release significant amounts of energy in the form of heat.
Chapter 2: Device Description and Validation

2.1 Materials and Methods

2.1.1 Construction

A closed, insulated chamber was constructed to shield the cells and camera from outside temperature effects and from external infrared radiation sources that could effect measurements (Figure 2). The opening in the side is for accessing the chamber to insert and remove the cell plate. This opening is normally closed with an insulation block designed to fit in the opening. Cell culture plate and sample injection temperature control was integrated into the design of the chamber. Temperature control was achieved by placing thermoelectric heaters (Peltier plates) adjacent to aluminum blocks to evenly transfer the heat to the cell plate, and injection syringes such that all components are at the same temperature. The cell culture dish rests on an aluminum block in the center of the chamber, while two separate aluminum blocks house injection syringes oriented to introduce samples directly into the cell plates (Figures 3 and 4). The injection syringes are positioned symmetrically on both sides of the cell culture plate so that each of the six cell wells can be injected independently and/or simultaneously. This design allows one side of the cell well to be treated with the agonist of interest and the other with a control vehicle so that the induced effect can be calculated from a differential measurement. The treatment and control wells can also easily be randomized in this arrangement. The camera (FLIR SC 500, FLIR Systems Inc., Boston, MA) is positioned 0.3048 m (one foot) directly above the cell plate at the minimum focusing distance while still being able to simultaneously image all six of the cell culture wells. The injection syringe block can be positioned near to the cell well during injection and then retracted such that the
syringes did not obstruct the camera’s field of view. Thermal images were acquired at a rate of 1 per second using the Thermacam Researcher software (FLIR Systems Inc., Boston, MA) and images were saved as SEQ files. The thermal image analysis was performed using the Thermacam Researcher software and by embedding a VBA macro (Appendix A) into Microsoft Excel (Microsoft Corp., Redmond, WA) to extract and analyze the data.

The chamber casing was made out of 0.5 inch sheets of R3 Styrofoam (Figure 2). Sheets were cut to desired dimensions and layered on top of each other within a steel frame. The Styrofoam was used because it is readily available, cost effective, easy to work with, and provided the necessary thermal insulation.

**Figure 2.** Autocad rendering of the design.
2.1.2 Electronic Control Module

A BASIC Stamp® (Model# BS2P40, Parallax Inc., Rocklin, CA) module was used as the main temperature controller. A small circuit (Figure 5) was designed to convert
the output signal of the BASIC Stamp into a 12-volt signal that activated a set of relays that powered thermoelectric modules (Peltier Plates). Thermistors were implanted into each of the aluminum blocks to provide a temperature feedback to the BASIC Stamp. The temperature feedback was achieved by using a small RC time circuit that charged a capacitor to 5 volts and measured the amount of time it took for the capacitor to discharge, which is dependent on the thermistor’s resistance. A temperature value is calculated from the discharge time and used to control the power to the peltier plate. The aluminum blocks are also independently monitored by a set of thermocouples attached to a datalogger (model: TC-08 (USB) PicoTech, Cambridgeshire, UK). Details are provided in Appendix B.

The temperature is maintained between below 37 °C and above 34 °C. To ensure that the cells are slightly below the physiological temperature range. This is done so that any increase in temperature would be measurable and so that cells would be close enough to physiological temperature to keep proteins and molecular machinery operating optimally. There may be differences in temperature between cells maintained at 30 °C versus 38 °C.

2.1.3 Validation Testing Procedures

To validate the design and construction of the measurement setup, several iterations of testing and optimization were performed. These control tests used empty six-well cell plates and water solutions. Empty cell plates were injected with 10 µl of nanopure water and recorded with the infrared camera. These tests were performed to characterize the system behavior without cells or treatment compounds. Ideally, these control experiments should yield very consistent results with all recorded well
temperatures being similar. Prior to injection, it is important that the injection blocks have the same temperature, which is verified with the thermocouple data logging system. The experiments were performed to determine the thermal inconsistencies/differences between different plates location, followed by optimization and adjustment of the hardware and control procedures.

2.2 Results

Several initial tests were completed to determine if any temperature fluctuations or heating patterns existed inherently in the design. The tests revealed a repeatable difference in temperature, with the treatment side being warmer than the control side even in the absence of cells and agonist (Figures 6 & 7). Further tests were performed with the temperature controller on and off after injection to determine whether there were significant differences between the two configurations. The tests with the power off show an initial spike after injection then a gradual cooling (Figure 6). The treatment side
contains wells 1, 3 and 5 (red and orange) and the control side consists of wells 2, 4 and 6 (blue). After injection into an empty cell plate with water, the power to the plates was cut off, which accounts for the downward trend. The average value for each well is presented in the top corner of figure 6. The average values are color coded to match their respective well and placed in a similar configuration to how they appear on the camera. It is apparent from this chart that the control side was consistently cooler than the treatment side despite there being no difference in treatment. The power on tests (Figure 7) see these same trends with an eventual return to higher temperatures as the sample is evaporated. In both sets of data the treatment wells were warmer than the control wells.

The physical design was examined for the possible causes of the temperature irregularities observed in figures 6 and 7. A small air draft was identified in the unit and was repaired. Five subsequent trials with the power on failed to show any improvement over the previous tests, with the control side still cooler than the treatment side (Figure 8). A secondary investigation of the physical design revealed that the aluminum block supporting cell plate was not sufficiently insulated from the steel bar supporting the camera. After padding an immediate effect was an increased temperature of the aluminum cell plate. This change necessitated an adjustment of the set point on the temperature control unit as the cell plate was now hotter than the injection plates. The insulation of the camera support bar had the desired effect of reducing temperature fluctuations (Figure 9), with no major outliers and greatly reduced variance (Table 1). The row marked “split” is an indicator of the average temperature of the treatment wells vs. the control wells. It is computed by dividing the average of the treatment wells from the average of the control wells and then subtracting 1. This gives a positive or negative
Figure 6. Average well temperatures for four separate trial runs with the temperature controller off.

value that is proportional to the difference between the wells. Positive numbers indicate tests where the treatment wells were hotter on average while negative values indicate a test where the control wells were hotter. Statistical analysis showed that there was no difference (p>0.05) between the control and treatment side.

To determine if these thermal effects are from temperature inconsistencies in the heating plates, an infrared imaging test was run with no cell plate present. Temperature images were recorded every second and data was extracted from the area over the peltier plates, and from the entire area (Figure 10).

After the data was extracted, it was apparent that there was very little measurable should receive equal temperature and therefore any differences between different cell plates should be due to differences in emissivity and reflectivity. There was also no
difference between the two sides of the aluminum plate where the treatment and control cell plates rest (Figures 11 & 12).

![No Snapwell Plates on Well Averages](image)

**Figure 7.** Average well temperatures for three separate trial runs. After injection into an empty cell plate with water, the power to the plates was kept on, which accounts for the upward trend.

2.3 Discussion

The results of our experiments suggests that the thermal inconsistency within the system have been minimized and that the statistical difference between well locations is very close to zero. This provides the best possible environment for sensing the receptor activated heat release in cell cultures, as any temperature change recorded would be due to metabolic heat production rather than due to uncertainties in the measurement setup.

Another step that could be used to mitigate any further or unresolved thermal inconsistencies would be to randomize the treatment and control wells instead of restricting them to one side of the cell well.
Figure 8. Average well temperatures for four separate trial runs (n=4). This indicates that the cell plates difference between the different areas (Figure 11).

Figure 9. Average well temperatures for three separate trial runs (n=3).
While the described modifications to the enclosure were able to remove thermal inconsistencies between the statistical means of the treatment and control sides, closer evaluation of the data reveals that the control side did have a larger range of values (Figure 13). This could indicate that further insulation of the enclosure may facilitate better results.

**Table 1.** Summary of all results.

<table>
<thead>
<tr>
<th>Well</th>
<th>Initial Test Well Averages (°C)</th>
<th>Post Patch 2 (°C)</th>
<th>Post Patch 3 (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well 1</td>
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<td>36.39399</td>
<td>34.05685686</td>
</tr>
<tr>
<td>Well 3</td>
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<tr>
<td>Well 5</td>
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<tr>
<td>Well 2</td>
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<td>33.92555889</td>
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</tr>
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<td>0.037280394</td>
</tr>
<tr>
<td><strong>Split</strong></td>
<td>0.014264017</td>
<td>0.007408975</td>
<td>-0.001833748</td>
</tr>
</tbody>
</table>

**Figure 10.** Infrared image of aluminum plate that is used to heat cells.

The total average temperature for all wells over the entire experiment was 34.18 °C and the standard deviation for the same data was 0.17 °C. It has been reported that adherent BAT treated with CL were observed to have a 0.8°C increase (Paulik, Buckholz et al. 1998).
Figure 11. Average temperature values for the aluminum heating plate for the areas indicated in Figure 10. The temperatures are so close that their plots completely overlap.

Figure 12. Average temperatures of the treatment vs. control side.

These cells were not aspirated so a large amount of energy was required to increase the temperature of the media that was measured. Based on a power test using the standard deviation of the data reported here, the enclosure should be able to establish
Figure 13. Control and treatment means vs. individual well means.

significance with only 3 samples for an increase of approximately 0.3 °C, assuming standard deviation remains small for the cell tests.

\[ n = \frac{ \left( t_{\alpha/2} + t_\beta \right)^2 \cdot s^2}{E} \]

Where \( n \) is the sample number, \( t_{\alpha/2} \) is the t chart value for a 95% confidence interval with 5 degrees of freedom, \( t_\beta \) is the t chart value for a beta of 0.2, \( s \) is the standard deviation of the sample, and \( E \) is the expected difference between treatment and control.

The data collected here suggests that the enclosure should be able to detect changes in cellular thermal output with a relatively small sample size. However, the sensitivity is still relatively small compared with microcalorimetry which can be in the range of mK (Wadso 2001). Based on the results of previous IR studies, this high degree of sensitivity is not required for testing receptor activated heat generation of large groups
of adherent cells, and the speed and ability of IR to measure many samples at once with little preparation is preferable.

This design yielded a viable method for measuring thermal effects in cells quickly and easily. Programming and data analysis can be streamlined with the use of Visual Basic (Microsoft, Redmond, WA) macros and pre-designed templates. Additionally, the chamber design could be scaled-up to use cell plates with more wells. The rapid processing of many samples with controls and with varying concentrations simultaneously within a single test would be preferred over existing techniques. Current methods limit researchers to testing samples one at a time (with microcalorimeters), and the present approach has the additional advantage of being able to determine spatial temperature distributions at any given time. This investigative technique could be more powerful with higher performance infrared cameras with increased resolution.

2.4 Conclusion

An experimental approach for receptor activated cell heat detection that has been designed, constructed, optimized, and successfully tested is described here. The thermal inconsistencies initially found in the design were identified and rectified. The apparatus is a tool to measure the temperature change of cells in a six well cell culture plate immediately after the stimulant sample is introduced. This technique provides several advantages over established microcalorimetric techniques and previously described infrared techniques. Future designs could implement more sophisticated temperature control units (such as a proportional/integrative/derivative controller) and a different syringe heating design to allow for the use of cell plates with more wells. This type of approach could be useful for quickly examining cellular metabolic effects of anti-obesity
targets such as the ones disused in chapter one, or some combination thereof. The creation and testing of different chemical modifications of known targets could lead to different potential lead compounds but these variations must be tested for efficacy (Atgie, Faintrenie et al. 1998; Kasuga, Nakagome et al. 2007).
Chapter 3: Cell Tests

3.1 Introduction

Validation of the enclosure described in chapter 2 allowed for the testing of cell cultures. 3T3-L1 adipocytes were treated with the most acute β3 agonist known, CL 316-243, for a period of seven days in order to measure the potential increase in metabolic capacity facilitated by the remodeling of the cells. To quantify and validate the cell remodeling, the cells were stained with two fluorescent dyes; mitotracker deep red (ex.644 em.665) and lipidtox green (ex. 495 em. 505). Fluorescence was measured using flow cytometry and a fluorescence plate reader. Brightfield images of the fixed cells were also acquired as a qualitative indicator of the cell remodeling. Mitotracker deep red intensities should increase after the treatment as the number of mitochondria is expected to increase. Conversely, the lipidtox green intensity should decrease, as there should be less fat in the cells.

3.2 Materials and Methods

The acute β3 agonist CL 316243 was used to treat 3T3-L1 adipocytes chronically for seven days to bring about the previously described structural remodeling to modulate the cells energy consumption profile and thereby increase the cells ability for thermogenesis. The cells were monitored in the above-described set up using the same protocol.

Murine 3T3-L1 preadipocytes were plated and grown 2 days past confluence in Dulbecco’s modified Eagle’s medium (DMEM) and 10% bovine serum that was changed every 48 hours. Differentiation of the cells into adipocytes was induced by changing the media to DMEM with 10% fetal bovine serum (FBS), 0.5 mmol/l 3-isobutyl-
methylxamine, 1 µmol/l dexamethasone, and 1.7 µmol/l insulin (MDI). After 48 hours, the DMEM-MDI was changed to DMEM and 10% FBS. This media was used through the rest of experimentation. DMEM was purchased from Invitrogen (Carlsbad, CA). FBS was purchased from Atlanta Biologicals (Lawrenceville, GA), and calf serum was purchased from Invitrogen. DMEM and 10% FBS adipocyte media was used for differentiated cells and was purchased from Zen-Bio (Research Triangle Park, NC).

Cells were treated with CL 316-243 (Sigma-Aldrich) on a seven-day treatment program; with the final treatment reaction recorded using an infrared camera. This was performed as constant or periodic measurements could introduce contamination to the cells, (the cover of the cells is removed during imaging). Additionally, the aspiration of media for prolonged periods during imaging could introduce undesirable artifacts. CL treatments were administered in 10 µl aliquots at a concentration of 100 nM, with the final treatment concentration being 25 µM due to the media aspiration for imaging. The 100 nM concentration was chosen to compare with a previous IR cell culture study (Paulik, Buckholz et al. 1998).

The treatment and control vehicle was nano pure water. There could be osmotic shock effects to the cells as a result of the treatments. These effects should be accounted for in the experimental design, as control tests are done with the vehicle as well. It may be beneficial in the future to use cell media as the vehicle to avoid osmotic shock issues.

Two cell plate configurations were tested, a normal 6 well plate and a 6 well snapwell plate (Catalog # 07200708, Fischer Scientific, Pittsburg, PA). Snapwell plates consist of a normal 6 well plate with small polyester membranes for cell growth that are supported by a conical polycarbonate scaffold, such that the membrane suspends above
the bottom of the well submerged in media. A lifting mechanism was fashioned out of polycarbonate such that snapwell treated cells could remain in their media until they were ready to be imaged. The lifting mechanism lifted the cells out of the media thus reducing the heat transferred to the media and to the cell plate itself. Evaporative cooling is minimized using a fourth peltier plate that resides in the upper insulation and evaporates a small pool of water into the chamber increasing the humidity to approximately 75% and the partial pressure of water in the environment. The snapwell plate was tested in order to minimize the effects of conductive heat loss from the cells when they heat. It is noteworthy that the cells grown in the snapwell plates receive the normal (100 nM) CL dose during final injection because the media is not aspirated. Image analysis of the snapwell plates is only done for the small insert; likewise, image analysis on the normal plates is typically done on the area where treatment is applied, if the treatment does not cover the entire well area.

The agonist and vehicle were loaded into the syringes, inserted into place and allowed to equilibrate for 10 minutes. The cells were then aspirated and placed inside the chamber and allowed to equilibrate for 10 minutes. The treatments and vehicle were administered manually and power to the temperature control unit was turned off. Power was turned off to avoid controlling the variable that was being measured (temperature). Cells were imaged for ~30 minutes then discarded. Images were collected and analyzed as described in Chapter 2. It is noteworthy that treatment typically covered 85 – 70% of the cell well in the normal 6 well plates. These regions of treatment were gated and measured. The snapwell plates were only measured on the small polyester membrane. It
was assumed that the cells received a homogeneous CL treatment, as the molecular weight of CL is small (465.79 g/mol).

To verify the effectiveness of the treatment, cells were stained with HCS lipidtox green and mitotracker deep red (protocol in Appendix D) (Cat# H34475 and M22426, Invitrogen Corporation, Grand Island, NY). The goal was to verify that the treatment with CL over seven days was indeed bringing about the previously described remodeling of the cells. By staining the Lipid droplets, which are expected to decrease in size, and the mitochondria, which are expected to proliferate, a general indicator of remodeling could be attained. The cells’ fluorescence intensity was measured using Flow cytometry (FACSAria, BD Biosciences, San Jose, CA) and a plate fluorescence reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany). The flow cytometer excitation and emission channels were set to 480 and 520, respectively for the lipidtox green and 650 and 680, respectively for the mitotracker red dyes.

An additional effort was made to quantify the treatment effects of the beta agonist on the morphology of the cells. Brightfield images (20x) were taken of two cell plates that received the same 0, 4, and 7-day treatments. These images were processed using an image processing technique to quantify the number and size of lipid droplets by thresholding bright areas. A single thresholding value was selected by averaging the optimal value from six random images (two images from each treatment). Once the average threshold value was selected it was applied to 9 random images from the two plates for each treatment for a total of 27 images. The equivalent radius count, average equivalent radius, equivalent radius standard deviation, and the maximum and minimum equivalent radius were extracted from each thresholded image.
3.3 Results

Brightfield imaging (Figure 14) shows that the cells appeared to go through the remodeling, which produced somewhat smaller, leaner cells that should be more capable of heat output (Granneman, Li et al. 2005). There appeared to be a fractioning of the large adipose depots into smaller ones, however large droplets did not completely separate. This morphology indicates a partial remodeling of unilocular to multilocular depots.

Figure 14. Brightfield image of 3T3-L1 adipocytes on the 1\textsuperscript{st} and 7\textsuperscript{th} day of treatment at 40x. Notice the cells shift from unilocular to multilocular fat depots.

Fluorescent images were successful in staining targeted structures (Figure 15a). Lipid droplets were clearly visible and the red mitochondrial stain was present and visible, but appeared diffuse, which indicates that other structures may have also been stained. Two concentrations of mitotracker red and lipidtox were initially tested. The recommended concentration for the mitotracker red was 25-500 nM (Invitrogen product sheet), The concentrations tested were 250 nM (Figure 15a) and 25 nM (Figure 15b). The recommended lipidtox concentration was 1:200 (Invitrogen product sheet). The concentrations tested were 1:400 (Figure 15b) and 1:200 (Figure 15a). The lipidtox was clearly visible at both concentrations, whereas the lower mitotracker concentration was
not (Figure 15b). The higher mitotracker concentration (250 nM) was visible (Figure 15a), but it appears to have lost mitochondrial specificity, as individual mitochondria are not clearly discernable. A lower concentration, or shorter incubation time would be required to achieve a good stain (Appendix D).

**Figure 15.** Fluorescent images of stained 3T3-L1 adipocytes with no CL treatment. The fluorescence intensities increase from zero to four days of treatment, while the 7 day treatment decreases for the 100 nM concentration and increases for the 200 nM concentration (Figure 16). It is noteworthy that the detector was saturated (at 65000 relative fluorescence intensity units) in several samples of the lipidtox green samples. The mitotracker deep red fluorescence intensities increased slightly through the treatment, however this increase was not significant (Figure 17).

Image analysis of the brightfield images using average threshold values (1436) yielded quantitative data on the number and size of lipid droplets (Table 2). The equivalent radius ($R_e$) was used to calculate the average radius, minimum and maximum values. The total value is the product of the radius count and radius average. The average count number of lipid droplets from the thresholded images showed higher values per image for 4 and 7 day values, with the 4 day having the highest average count of 1594,
just about the 7 day treatment count of 1576. The average equivalent radius shrunk throughout the treatment from $0.77 \, \mu m$ for the 0 day to $0.45 \, \mu m$ for the 7 day.

**Figure 16.** Average Fluostar fluorescence intensities for Lipidtox green stained cells treated with 100 and 200 nM concentrations of CL for 0, 4, and 7 days. For infrared imaging results the average values for treatment and control in time showed an upward trend in the 7-day treatment as expected (Figures 18 & 19), however the large sample variance prevented the drawing of strong, statistically significant conclusions from the results. For maximum temperatures measured, statistical significance ($p < 0.05$) between the test and control samples was only shown for the 4 day treatment, (for both with and without snapwells) with the test being higher than the control, as expected (Table 3). However, for the seven day treatment, the maximum temperatures indicated only a tendency ($p < 0.10$) for the test to be warmer than the control (Table 3).
Figure 17. Average fluostar fluorescence intensities for Mitotracker Deep Red stained cells treated with 100 and 200 nM concentrations of CL for 0, 4 and 7 days.

Table 2. Quantitative results from thresholded images of 3T3-L1 cultured adipocytes are shown.

<table>
<thead>
<tr>
<th>Droplet Count</th>
<th>Re Average (µm)</th>
<th>Re Std. Dev (µm)</th>
<th>Re Min (µm)</th>
<th>Re Max (µm)</th>
<th>Re Total (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1290.00</td>
<td>0.77</td>
<td>0.70</td>
<td>0.17</td>
<td>4.57</td>
</tr>
<tr>
<td>4</td>
<td>1593.55</td>
<td>0.58</td>
<td>0.46</td>
<td>0.14</td>
<td>3.03</td>
</tr>
<tr>
<td>7</td>
<td>1576.00</td>
<td>0.45</td>
<td>0.34</td>
<td>0.14</td>
<td>2.42</td>
</tr>
</tbody>
</table>

The average temperature values also show a statistical tendency (p <0.10) for the tests to be warmer than the control at 7 days for normal cell plates and at 4 days for snapwell plates (Table 3). Additionally, it was shown that there was no statistical difference between the control and the zero day treatment of both the snapwell and normal cell plates (Table 3).
Figure 18. Average temperatures of three treatment wells and three control wells injected with vehicle. Standard deviation is plotted every 50 data points.

Figure 19. Average temperatures of three treatment snapwells and three control wells injected with vehicle. Standard deviation is plotted every 50 data points.
Table 3. T-test testing between control and treatment for each individual plate for average and maximum temperatures. A * indicates statistical significance (p < 0.05), a ■ highlight a tendency (p<0.10). SEM is standard error of the mean.

<table>
<thead>
<tr>
<th>Snap</th>
<th>Time</th>
<th>Ttreat Max</th>
<th>Tcontrol Max</th>
<th>SEM</th>
<th>p</th>
<th>Ttreat Average</th>
<th>Tcontrol Average</th>
<th>SEM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0</td>
<td>34.035</td>
<td>33.755</td>
<td>0.10</td>
<td>0.11</td>
<td>32.748</td>
<td>32.536</td>
<td>0.14</td>
<td>0.36</td>
</tr>
<tr>
<td>N</td>
<td>4</td>
<td>34.735</td>
<td>34.532</td>
<td>0.04</td>
<td>0.03*</td>
<td>33.477</td>
<td>33.298</td>
<td>0.08</td>
<td>0.20</td>
</tr>
<tr>
<td>N</td>
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<td>34.409</td>
<td>33.664</td>
<td>0.21</td>
<td>0.07*</td>
<td>33.078</td>
<td>32.213</td>
<td>0.25</td>
<td>0.07*</td>
</tr>
<tr>
<td>Y</td>
<td>0</td>
<td>32.086</td>
<td>32.003</td>
<td>0.21</td>
<td>0.80</td>
<td>30.970</td>
<td>30.935</td>
<td>0.29</td>
<td>0.93</td>
</tr>
<tr>
<td>Y</td>
<td>4</td>
<td>32.803</td>
<td>31.665</td>
<td>0.21</td>
<td>0.02*</td>
<td>32.061</td>
<td>30.906</td>
<td>0.32</td>
<td>0.06*</td>
</tr>
<tr>
<td>Y</td>
<td>7</td>
<td>32.612</td>
<td>31.838</td>
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<td>31.968</td>
<td>30.982</td>
<td>0.44</td>
<td>0.19</td>
</tr>
</tbody>
</table>

3.4 Discussion

3.4.1 Cell Remodeling

The initial staining of the cells not treated with CL with Mitotracker Deep Red and HCS Lipidtox Green appeared to be successful (Figure 15) as there clearly red and green regions of the cells that appeared to correspond to the specific structures. Analysis of adipocytes that had been trypsinized was difficult because their high fat content made them difficult to separate using conventional centrifugation. Fatty cells tended to float and were difficult to separate from media via aspiration, as they tended to agglomerate to the pipette tip. This resulted in low numbers of cells for analysis using flow cytometry. Consequently, there was an insufficient volume to scan in the fluostar plate reader after flow cytometry. Allowing the cells to remain attached permitted the use of the Fluostar plate reader to measure fluorescence intensity. This data provided a quantitative measure as to the degree of physical remodeling caused by CL treatment.

Lipidtox green fluorescence data was difficult to interpret. The quantification is difficult because there are two different physical phenomenon that occur during the remodeling process that could have different effects on fluorescence intensity. The first
effect is the decrease in overall lipid content of the cells. This decrease should have the
effect of lowering fluorescence intensity. The second phenomenon that could affect
fluorescence intensity is the fractioning of lipid droplets into many smaller ones. This
changes the average surface-to-volume ratio, which could exert an upward pressure on
fluorescence intensities. These simultaneous and potentially conflicting effects could
account for the rising and then falling of lipidtox green fluorescence intensities observed
on the 100 nM treatment (Figure 16).

Another factor that could potentially affect the fluorescence intensity of the lipid
droplets is the focal plain used to measure the cells. Fluorescence intensity is strongly
dependant on the focal plain. The large differences in droplet size may lead them to
agglomerate in different focal plains (for example, smaller droplets might tend to
accumulate in one focal plain and larger droplets in another), generating a potential for
bias toward one morphology or another.

Mitotracker deep red fluorescence intensities were expected to gradually increase
over the duration of the treatment due to mitochondrial biogenesis. This increase was
observed in the data (Figure 17), but was not statistically significant due to a large
variance. It is unclear if mitochondrial stains were successful in selectively staining only
mitochondria, as the stain often appeared as a diffuse glow, rather than small distinct
glowing structures. This diffuse effect may also occur due to insufficient magnification
(higher magnification lenses were not available for adherent cells).

Image analysis of brightfield images of cells yielded quantitative data that
describes the remodeling of the cells (Table 2). The equivalent radius of the thresholded
images were extracted and averaged for a total of 27 images (Appendix F). This quantitative data describes the number and size of lipid droplets of all the cells in the

![Fluorescent image of 3T3-L1 adipocytes treated with 200 nM Cl for 7 days.](image)

**Figure 20.** Fluorescent image of 3T3-L1 adipocytes treated with 200 nM Cl for 7 days. The particle counts showed an increase in droplet numbers throughout the treatments from 1290 on day 0 to 1593 on day 4 and finally, 1576 on day 7, as expected and noted by previous studies (Granneman, Li et al. 2005). The complete dissolution of small lipid droplets during the seven-day treatment could account for the slight decrease from the 4\textsuperscript{th} day to the 7\textsuperscript{th}. The image analysis showed that the equivalent radius average decreased over the course of the treatments from 0.77 µm on the zero day treatment to 0.58 µm on the fourth day treatment and finally, 0.45 µm on the seventh day. This shift in the average equivalent radius indicates that the treatment created a change in the cells. Additionally, the equivalent radius maximum decreased over the treatment indicating that the larger droplets were not present in the treated cells. The maximum fell from 4.57 µm
to 3.03 \( \mu \text{m} \) on the fourth day and then 2.42 \( \mu \text{m} \) on the seventh day of treatment. The image analysis of brightfield images produced more conclusive quantitative results than the fluorescent imaging that the cells underwent the remodeling process.

3.4.2 IR Results

The infrared images (Figures 21 & 22) show differential rates of heating in the zero, four and seven day treatments. Heating above initial temperatures was observed in most instances, but treatment wells seemed to heat faster although not significantly in all cases. Contrary to the efforts described in the previous chapter, the control wells in some instances appeared cooler then treatment wells before the final treatment (Figures 18 & 19). This was not expected, as up until this point, all of the wells for each plate have received the same treatment/feeding schedule.

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**Figure 21.** Infrared images of cell plates at zero day (a, b, c), 4 day (d, e, f) and 7 day treatments (g, h, i) at time zero (a, d, g), ten minutes (b, e, h), and twenty minutes (c, f, i) after injection. Treatment wells are on the right and control wells are on the left.
Figure 22. Infrared images of snapwell cell plates at zero day (a, b, c), 4 day (d, e, f) and 7 day treatments (g, h, i) at time zero (a, d, g), ten minutes (b, e, h), and twenty minutes (c, f, i) after injection. Treatment wells are on the right and control wells are on the left.

Compiled data for both the snapwell and non-snapwell tests, ignoring differences between treatment schedules and well types, showed a statistical significance (p < 0.05) between the control and the treatment wells (see Appendix E). Additional testing could determine if there was significance between different cell plate type and treatment schedule. A t-test between treatment and control for each plate indicated that there was no difference between treatment and control for the zero day tests for both the snapwell and the normal plates (Table 3). This was in line with what was expected, as a unilocular adipocyte should not typically posses the molecular machinery in sufficient quantities to facilitate an appreciable (measurable) metabolic gain and thermogenic response upon
stimulation. An unexpected finding was that the four-day treatment showed a slightly higher average temperature than the seven-day treatment for all tests (Table 3). Temperature increases of 0.86 °C were observed in this study using a standard 6 well culture plate (Table 3), however due to a larger standard deviation, this increase is only a statistical tendency (p < 0.10). The snapwell tests showed even larger differences, with a max increase of 1.15 °C during the four-day test (Table 3). Additionally, the 4-day tests showed significant heating over control (p < 0.05 for maximum well temperatures), whereas the seven-day treatments only showed a tendency to heat more than controls (Table 3). This was not expected, as the seven-day treatments were expected to have more mitochondria and a higher expression of proteins associated with fatty acid transport and breakdown, and therefore a greater thermogenic response. One possible explanation of this is that beta 3 adrenoceptors are down regulated during stimulation (Granneman and Lahners 1995; Klaus, Muzzin et al. 1995; Bengtsson, Redegren et al. 1996) and therefore the cells possessed fewer receptors by the 7th day of treatment.

One previous IR study on cell cultures examined heating effects on BAT resulting from CL stimulation. Paulik et. al. found a 0.8°C increase in temperature over the control (Paulik, Buckholz et al. 1998). There are several noteworthy differences between the Paulik’s cell culture study and this one. The previous study used rotenone as a control, which inhibits mitochondrial proton transport (Ahmad-Junan, Amos et al. 1994). This should have provided for a cooler control than the vehicle tests reported here, as even basal metabolism would come to a halt as the ATP was depleted. Temperature control of injection allows for immediate measurement whereas in the previous study a ten minute period was used to allow for equilibration. The methods used here turned off power to the
temperature control device in order to prevent controlling what is being measured. This study aspirated the media in larger 6 well plates, whereas the previous study cultured cells in 96 well plates and allowed 50 µl/well to remain during imaging. The aspiration of the media should allow for more precise kinetic analysis as the heat is not conducted through the media in order to be measured. Also, aspiration of the media should allow for detection of a larger temperature increase. It is likely that BAT measured using the enclosure and method reported here would yield even larger temperature gains compared to the previous study. It is noteworthy that the CL concentration of 100 nm was used in both studies (Paulik, Buckholz et al. 1998).

3.5 Conclusions and Future Considerations

The data presented here indicates that there was a significantly higher temperature for some samples of cultured adipocytes following CL treatment. Improvements to the design and additional testing could provide more insight to the process. More robust cell models could be used to take advantage of the higher throughput of these studies, and additional thermal insulation and a more sophisticated control unit may provide more significant results.

Future efforts to stain the cells to quantify the cell remodeling could utilize different mitochondrial stains that change fluorescence intensity depending on mitochondrial activity, such as JC-1 or rhodamine derivatives (Reers, Smith et al. 1991). This dynamic fluorescence would provide a fast direct measurement of change within the cell. Measurements with mitochondrial reactive dyes would be an interesting addition to this work but not a substitute for direct temperature measurement, as these dyes have a
high degree of non-specific staining, and therefore introduce background noise. The dyes can also be cytotoxic or might be metabolized by the cells.

The enclosure and thermal control of both the cells and the treatments is what differentiates these studies from those previously reported. The thermal control of the injection temperatures allows for immediate temperature measurement after injection and could offer new insight into the kinetics of thermogenesis. A larger population of trials would be necessary to establish more significant trends in heating. To accommodate cell plates with more wells would only require a redesigned set of injection blocks. The geometry of new injection block would require a careful design, as plates with more wells would necessitate syringes that protrude farther into the interior of the enclosure.

While chapter 2 established that there was no difference in the averages between treatment and control wells, the larger variance of the control side implies that further measures could be used to completely insulate the cells. This could be accomplished by adding insulation to the enclosure, or by placing the enclosure itself inside of a thermally monitored environment. It is noteworthy that lab temperature fluctuated considerably, and that those fluctuations did affect chamber temperature, so it is likely that the design could benefit from further thermal insulation.

The down-regulation of the β3 receptors during stimulation could present a challenge to the development of a beta adrenoceptor anti-obesity drug. Additional challenges to the use of this target include the elevated plasma FFA levels resulting from lipid transport out of WAT that may be detrimental to health. Because of this, it seems that β3 adrenergic stimulation alone would not be a desirable treatment modality for
obesity. It is likely that some combination of targets would have more beneficial outcomes.

In general, the recent advances of infrared imaging technology have increased the capability of this type of temperature measurement systems at greatly reduced cost. Previous research and the study described here is likely only the beginning of IR technology uses in biotechnology. Agricultural and food bioprocess IR imaging applications as well as clinical applications have been investigated (Chaerle 2000; Jones 2002; Lenhard 2006; Gerbo 2008). Future improvements to the above described model and technique would likely yield better and more precise information.

The spatial resolution of IR thermography has several benefits. The image processing software reports the hottest or coldest temperature or the average in a defined area of the image, all of which can be performed in real time, post processing or both. This is a significant advantage over thermocouple temperature measurement in situations where the maximum or minimum temperatures of a system may be desired, but can not be easily located. Performing these tasks in post processing allows for data to be analyzed many different ways without the need for moving sensors or replicating experiments.

The spatial resolution of IR thermography could potentially be very useful in studies investigating effects of autocrine signaling (signals from adipocytes to other adipocytes) on adipocyte metabolism. WAT is known to secrete a series of adipokines, 17 of which are thought to have an autocrine function (Wang, Mariman et al. 2008). These studies could take advantage of the spatial resolution of IR thermography by observing effects on peripheral cells caused by other adipocytes secreting adipokines. Additionally, it may be possible to culture adipocytes with other cell types to measure
endocrine or paracrine functions. This would be an excellent application for the snapwell plates, as one type of cell could be cultured in the bottom of the well and the adipocytes could be cultured separately in the snapwell insert. This configuration could accommodate the special growth or treatment requirements of different types of cells while allowing for a common environment for signaling and measuring by simply moving the insert.

In addition to the above-described applications, a system is envisioned that would take advantage of the onboard processing and logic output capabilities of today’s cameras. Selective hyperthermia using photothermal ablation has been suggested as a possible cancer treatment with the aid of chromophores that selectively accumulate in cancer tissue and are easily heated by lasers or other electromagnetic stimulation. Infrared thermography has shown to be useful for measuring temperature changes of carbon nanotubes (Boldor, Gerbo et al. 2008). A proposed sensitizer could therefore prove to be a useful method of laser dosage control in the eventual clinical manifestation of these treatments. Current models of IR cameras have real time analysis software and can be integrated with specialized software packages such as Labview (National Instruments, Austin, TX) and built in controllers. It is easy to envision a system whereby skin temperature is used as a feedback in a control circuit for laser ablation of tissue in a clinical setting. More complex techniques of control feedback equations could be developed to incorporate sophisticated IR techniques such as lock in thermography for increased sensitivity and for use with lasers that have a higher penetration depth such as the Nd:YAG and other diode lasers currently being developed.


Appendix A: VBA Macro for Infrared Image Extraction in Excel

Sub macro()
    Dim sess As Object
    Dim row As Integer
    Dim col As Integer
    row = 3
    col = 2

    'get a reference to the termacam research object
    Set sess = _Worksheets("Sheet1").OLEObjects("Object 1").Object

    ' Move to the first image in the session
    sess.GotoFirstImage

    ' Start a loop that iterates through all images in the session
    Do While True
        ' Store IR images time and spotmeter temperatures in the cells
        Worksheets(2).Cells(row, col + 1).Value = _sess.GetNamedValue("AR01.avg")

        If sess.IsLastImage Then
            Exit Do
        End If
        sess.Stepforward
        row = row + 1
    Loop
End Sub
Appendix B: Basic Stamp Program for Temperature Control

' $STAMP BS2p
' $PORT COM1

result1 VAR Word
AvgResult1 VAR Word
result2 VAR Word
AvgResult2 VAR Word
result3 VAR Word
AvgResult3 VAR Word
i VAR Byte

Main:
GOSUB Test1
GOSUB Check1
GOSUB Test2
GOSUB Check2
GOSUB Test3
GOSUB Check3
GOTO Main

Check1:
IF AvgResult1 > 1650 THEN Relay1On 'red #2
IF AvgResult1 < 1650 THEN Relay1Off
RETURN

Check2: 'this is for the center plate and is thermocouple #1
IF AvgResult2 > 1270 THEN Relay2On
IF AvgResult2 < 1270 THEN Relay2Off
RETURN

Check3:
IF AvgResult3 > 1530 THEN Relay3On 'green #3
IF AvgResult3 < 1530 THEN Relay3Off
RETURN

Relay1On:
DEBUG "Relay1 ON",CR
HIGH 3
HIGH 6
RETURN
Relay1Off:
DEBUG "Relay1 OFF",CR
LOW 3
LOW 6
RETURN

Relay2On:
DEBUG "Relay2 ON",CR
HIGH 4
HIGH 7
RETURN

Relay2Off:
DEBUG "Relay2 OFF",CR
LOW 4
LOW 7
RETURN

Relay3On:
DEBUG "Relay3 ON",CR
HIGH 5
HIGH 8
RETURN

Relay3Off:
DEBUG "Relay3 OFF",CR
LOW 5
LOW 8
RETURN

Test1:
FOR i = 1 TO 10
HIGH 15  ' charge the cap
PAUSE 1  ' for 1 ms
RCTIME 15, 1, result1
AvgResult1 = AvgResult1 + result1
NEXT
AvgResult1 = AvgResult1/10
DEBUG DEC ? AvgResult1  ' display result
RETURN

Test2:
FOR i = 1 TO 10
HIGH 14  ' charge the cap
PAUSE 1 ' for 1 ms
RCTIME 14, 1, result2
AvgResult2 = AvgResult2 + result2
NEXT
AvgResult2 = AvgResult2/10
DEBUG DEC ? AvgResult2
RETURN

Test3:
FOR i = 1 TO 10
HIGH 13 ' charge the cap
PAUSE 1 ' for 1 ms
RCTIME 13, 1, result3
AvgResult3 = AvgResult3 + result3
NEXT
AvgResult3 = AvgResult3/10
DEBUG DEC ? AvgResult3
RETURN
Appendix C: Protocol for Treating and Imaging Cells

Materials:

- 6 well Cell plates
- Adipocyte Media (2.5 ml /well) - aliquot into 50 ml centrifuge tube (Fischer Scientific)
- 10ml pipettes (2 for just feeding - more if cells are treated and you wish to avoid contamination of treatment – 4 total for 3 plates)
- cell waste beaker
- 25 µM Cl 316 243 solution for treatment (Sigma Aldrich)
- 10 ul pipette tips

CL treatment and Feeding Procedure:

1) Make sure hood vent is turned ON then pull open the hood and allow it to run for 5 minutes

2) Wipe down area under hood thoroughly

3) Gather ALL required materials and place them under the hood

4) Aspirate media starting with control cells then aspirate treatment cells with 10ml pipettes to avoid contamination

5) With a new pipette, add 2.5 ml of new media being careful not to touch the cell well with the tip of the pipette. If this happens, discard your pipette and get a new one. This will help avoid contamination of the media.

6) For treatment, vortex or shake the 25 µM CL 316 243 stock and then add 10 µl to each well being treated being sure to clearly mark which plates are treated and which ones are not

7) Replace cells in incubator and clean all surfaces thoroughly and put media back in the refrigerator and clean the cell beaker with soap and bleach

Treatment and Imaging Procedure:

1) Turn on the electronic control unit and allow it to equilibrate overnight.

2) Examine the temperature measurement system to ensure that all plate temperatures have equilibrated. An adjustment to the control units code may be required.
3) Turn on the IR camera and allow it to equilibrate for 20 minutes. Check to ensure that it is properly focused and that the emissivity is set to 0.98.

4) Once the temperatures are equilibrated, aspirate the media of the cell wells and load it in the chamber. Close the chamber.

5) Load the injection syringes with 10 µl of the treatment or control media.

6) Allow the system to equilibrate for about 10 minutes.

7) Start recording using the digital control software, and press the syringed to inject the samples. Retract the sample injection blocks from the field of view of the camera and turn off the electronic power supply to the peltier plates. For snapwell plates, insert the polycarbonate lifting mechanism to lift the cells from the media.

8) Allow the system to record as many samples as desired.

9) Stop Recording.

10) Power on the electronic control circuit so the system can heat back up.

11) Remove the cell plate and discard.

12) Once the set up temperature has re equilibrated, repeat steps 4-12 until all plates have been recorded.
Appendix D: Protocol for Staining and Measuring Fluorescence Intensity

This is a protocol for dual labeling 3t3-l1 cells to selectively stain the mitochondria and the fat depots. This will be useful for quantifying the remodeling of the cells subject to beta-adrenergic agonists using flow cytometry and a fluorescence plate reader. The expected effect of the treatment is an increase in the number of mitochondria and a decrease in the size of lipid depots.

**Required materials:**
- Cells – in six or twelve well plates
- DMSO
- DMEM or adipocyte media
- PBS
- 0.25% Tripsin 1X
- Formaldehyde
- HCS lipidtox green
- Mitotracker deep red
- Falcon flow cytometry tubes
- Paraformaldehyde 1%
- 1ml pipette and tips
- 1.5ml eppendorf tubes

**Part 1: Mitotracker Staining**

Preparing stock solutions:

Before opening vial, allow the product to warm to room temperature. To prepare the stock solution, dissolve the lyophilized product in high-quality, anhydrous dimethylsulfoxide (DMSO) to a final concentration of 1 mM. You can store the frozen stock at <-20°C and protect from light.

1.1 Dilute the 1 mM stock solution to the final working concentration in growth medium (DMEM or adipocyte media). For live cell staining concentrations of 25-500 nM are recommended for fixed cells higher concentrations are recommended (100-500nM). The concentration should be as low as possible to avoid artifacts and overlap.

The mitotracker comes in 50 µg tubes and has a molecular weight of 543.58. So to make a 1 mM stock you should add the 50 µg to:

\[50\mu g \times 1000 \frac{l}{M} \times \frac{1M}{543.58g} = 91.8\mu l\]

91.8 µl of DMSO.

Staining two twelve well plates with 0.6 ml of solution in each well will require 14.4 ml of 25nM solution. This will require 0.36 µl of 1mM stock solution (to make 15 ml use
To avoid doing serial dilutions, you could use 1 µl in 40 ml of solution for a 25 nm solution.

1.2 Remove media from grown cells and replace with the media with the mitotracker label. This media should be pre-warmed to 37 C. Incubate the cells for 15-45 minutes in the incubator.

1.3 Replace media and dye with fresh pre warmed media and observe the cells under the microscope. Cells may require higher concentrations or longer incubation times.

Part 2: Harvesting and fixing the cells.

A. Observe and take brightfield images of cells seeded in 12mm-cell culture dishes before harvesting (to ensure uniform cell density among all wells).

B. Harvest cells
   1) Aspirate
   2) Wash with 0.6 mL PBS
   3) Add 0.6 mL 0.25% trypsin.
      i. Allow 5-7 minutes for cells to detach from the bottom of the dish.
   4) Add 0.6 mL of DMEM-RS (+3% FBS)
   5) Transfer cells in solution to 1.5mL eppendorf tube using 1mL pipettor (or 5 mL cytometer tube using transfer pipet)
   6) Spin down at 1,800 rpm for 5 minutes on big centrifuge (or 2,400 rpm on mini-spin)
   7) Aspirate carefully using 1 mL pipettor

C. Wash procedure
   1) Resuspend the cells in 600 µL DMEM-RS+FBS
   2) Spin down at 1,800 rpm for 5 minutes on big centrifuge (or 2,400 rpm on mini-spin)
   3) Aspirate carefully using 1 mL pipettor

D. Fixing cells
   1) Add 300 µL 1% PFA (paraformaldehyde) in buffer to each tube
   2) Incubate for 10-30 minutes at room temperature

E. Wash procedure
   1) Resuspend the cells in 600 µL DMEM-RS+FBS
   2) Spin down at 1,800 rpm for 5 minutes on big centrifuge (or 2,400 rpm on mini-spin)
   3) Aspirate carefully using 1 mL pipettor

F. Wash procedure
   1) Resuspend the cells in 600 µL DMEM-RS+FBS
   2) Spin down at 1,800 rpm for 5 minutes on big centrifuge (or 2,400 rpm on mini-spin)
   3) Aspirate carefully using 1 mL pipettor

Part 3: Lipidtox Staining

3.1 Dilute the LipidTOX™ neutral lipid stain 1:200 in buffer. Prepare a volume sufficient to completely cover cells.
**Note:** The 1:200 dilution is a recommended starting point. It may be necessary to optimize the dilution for your system. You should have this dilution before starting this procedure.

3.2 Add LipidTOX™ neutral lipid stain and incubate the cells at room temperature for at least 30 minutes before imaging.
Appendix E: SAS Code for Compiled Data

The Mixed Procedure

Model Information

Data Set WORK.TWO
Dependent Variable tavg
Covariance Structure Diagonal
Estimation Method REML
Residual Variance Method Profile
Fixed Effects SE Method Model-Based
Degrees of Freedom Method Residual

Class Level Information

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Fit Statistics

-2 Res Log Likelihood 46.5
AIC (smaller is better) 48.5
The SAS System 16:05 Thursday, January 24, 2008

The Mixed Procedure

Fit Statistics

AICC (smaller is better) 48.7
BIC (smaller is better) 49.7

Type 3 Tests of Fixed Effects

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I have a question: I thought that at time 0 you should see some treatment differences, but you don’t with either of the plate’s type (snap vs. nonsnap). If you are not suppose to see a difference at time 0 between trt. and ctr., let me know because we should treat it in a different way. Basically not include time 0 into the model.
The UNIVARIATE Procedure
Variable:  Resid  (Residual)

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Basic Statistical Measures

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The UNIVARIATE Procedure
Variable: Resid (Residual)

Quantiles (Definition 5)

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Multiply Stem Leaf by 10**-1

The UNIVARIATE Procedure
Variable: Resid (Residual)

Normal Probability Plot

---

-2 -1 0 +1 +2

---
Appendix F: Brightfield Images for Quantitative Analysis

Zero Day Images
Four day images
Vita

Nicholas Gerbo was born in Covington, Louisiana, in March of 1983. He attended St. Peter’s middle school and St. Paul’s high school. He received his bachelor of science in biological engineering in December 2005 from Louisiana State University.