Coupling Between Nutrient Availability and Thyroid Hormone Activation

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COUPLING BETWEEN NUTRIENT AVAILABILITY AND THYROID HORMONE ACTIVATION

By

Lattoya Josephine Lartey

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

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COUPLING BETWEEN NUTRIENT AVAILABILITY AND THYROID HORMONE ACTIVATION

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Thyroid hormone is essential for metabolism, growth, development and cognitive function. Thyroid hormone is secreted in two forms, the biologically active 3,5,3′-triiodothyronine (T3) and the inactive thyroxine (T4) that must be converted to T3 in order to gain biological activity. This reaction is catalyzed by type II deiodinase (D2), a thioredoxin-fold containing selenoenzyme; its activity plays an essential role in thyroid hormone activation.

The thyroid gland is regulated by the hypothalamic-pituitary-thyroid (HPT) axis. It responds to food availability via leptin induction of thyrotropin releasing hormone (TRH) in the hypothalamus and thyroid stimulating hormone (TSH) in the pituitary. These actions stimulate the production and secretion of T3 and T4 from the thyroid. This current work describes the exciting new finding that food availability also activates thyroid hormone action via the induction of D2 as measured, in part, through the conversion of T4 to T3. Herein, we have characterized the mechanism whereby D2 is activated via insulin/IGF-1 signaling through the PI3K-mTORC2-Akt pathway.

To understand how nutritional signals affect D2 expression and activity, we studied mouse skeletal muscle and a mesothelioma cell line (MSTO-211H) and show that D2 is transcriptionally upregulated during the transition from fasting to refeeding or upon
switching from 0.1 to 10% FBS, respectively. The underlying mechanism is the transcriptional de-repression of DIO2 through the activation of the mTORC2 signaling pathway, since lentiviral knockdown of rictor abrogates this effect. Forkhead box O1 (FOXO1), a downstream target of mTORC2, transcriptionally represses DIO2; this was determined using its specific inhibitor AS1842856 and through adenoviral infection of constitutively active FOXO1. These results were confirmed by ChIP studies in MSTO-211H cells indicating that within 4h of exposure to 10% FBS-containing media, FOXO1 binding to the DIO2 promoter markedly decreases and the DIO2 promoter is activated. Studies using the insulin-receptor FOXO1 KO mouse indicate that insulin is a key signaling molecule in this process. Similar results were found using IGF-1 as a stimulant. This study concludes that FOXO1 represses DIO2 during fasting and DIO2 de-repression is stimulated by nutritional activation of the PI3K-mTORC2-Akt-FOXO1 pathway.

Through these studies, we have been able to characterize a novel mechanism of D2 regulation that is both locally and temporally regulated. It mechanistically describes how the production of T3 fluctuates in response to nutritional stimuli independently of the HPT axis. This mechanism linking thyroid hormone activation via D2 induction to food availability describes how the body adjusts T3 production to an appropriate level in the conditions of fasting and refeeding.
DEDICATION

I dedicate this work to my mother, Jacqueline Omega Robinson, for your unwavering belief in my abilities. Every prayer you have said on my behalf has given me the strength to continue along this journey. Thank you for your motivation, for your encouragement and for being my biggest cheerleader. I have worked to accomplish this goal not only for myself, but for you and for every sacrifice that you have made to ensure that I have every opportunity that you did not. We can celebrate this achievement together because it has been a joint effort and without your love and support, this would not have been possible.
ACKNOWLEDGEMENTS

I am immensely grateful to GOD ALMIGHTY for without His blessings and guidance, this study would not have been possible. I am grateful for being given the opportunity to study GOD’s greatest creation.

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<td>protein kinase B</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>CA</td>
<td>constitutively active</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CHX</td>
<td>cyclohexamide</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DIT</td>
<td>diiodotyrosine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>D1</td>
<td>type I deiodinase</td>
</tr>
<tr>
<td>D2</td>
<td>type II deiodinase</td>
</tr>
<tr>
<td>D3</td>
<td>type III deiodinase</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FOXO</td>
<td>family of forkhead box ‘other’ transcription factors</td>
</tr>
<tr>
<td>HPT</td>
<td>hypothalamus-pituitary-thyroid axis</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>insulin like growth factor receptor 1</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
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<tr>
<td>LIRKO</td>
<td>liver-specific insulin receptor knock-out</td>
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<tr>
<td>LIRFKO</td>
<td>liver-specific insulin receptor/FOXO1 double knock-out</td>
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<tr>
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<td>monocarboxylate transporter 8</td>
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<tr>
<td>MCT10</td>
<td>monocarboxylate transporter 10</td>
</tr>
<tr>
<td>MIT</td>
<td>monoiodotyrosine</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MSTO-211H</td>
<td>mesothelioma cell line</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC</td>
<td>mTOR complex</td>
</tr>
<tr>
<td>NSC</td>
<td>non silencing control</td>
</tr>
<tr>
<td>OATP</td>
<td>organic anion transporting polypeptide family</td>
</tr>
<tr>
<td>PE</td>
<td>PBS EDTA buffer</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>RAPTOR</td>
<td>regulatory-associated protein of mammalian target of rapamycin</td>
</tr>
<tr>
<td>RICTOR</td>
<td>rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid-X receptor</td>
</tr>
<tr>
<td>S6K</td>
<td>ribosomal S6 kinase</td>
</tr>
<tr>
<td>TRs</td>
<td>thyroid hormone receptors</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin releasing hormone</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
</tr>
<tr>
<td>TRE</td>
<td>thyroid hormone response element</td>
</tr>
<tr>
<td>TRX</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>T3</td>
<td>3,5,3’-triiodothyronine, thyroid hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>4EBP</td>
<td>4-(eIF4E) binding proteins</td>
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Chapter 1. Introduction

1.1 Thyroid hormone

Thyroid hormone acts on nuclear thyroid hormone receptors (TRs) to control the transcription of genes that modulate processes required for normal growth, development, cognitive function and metabolic function (Brent 2012, Mullur, Liu et al. 2014). Thyroid hormone is produced in and secreted from the thyroid gland. Synthesis of thyroid hormone occurs when iodide from the circulation is transported into the thyroid; in a series of oxidative coupling reactions, iodine is incorporated into tyrosine residues of the thyroglobulin molecule to form iodotyrosines, monoiodotyrosine (MIT) or diiodothyrosine (DIT). These hormonally inactive intermediates are coupled through oxidation to form thyroid hormone. The fusion of one MIT molecule with one DIT molecule or of two DIT molecules produces thyroid hormone in two forms, 3,5,3'-triiodothyronine (T3) or thyroxine (T4) (Fig. 1.1), respectively (Larsen and Ingbar 1992). T4 is the major secretory product of the thyroid and serves as an inactive precursor of T3; outside of the

![Structural formulas of thyroid hormones and related compounds.](image)
thyroid parenchyma, T4 can be activated to T3 to gain biological activity (Bianco and Kim 2006).

This process of thyroid hormone production and secretion is controlled by the hypothalamus-pituitary-thyroid (HPT) axis. Originating in the paraventricular nucleus of the hypothalamus, thyrotropin releasing hormone (TRH) is secreted in the median eminence and acts on the anterior pituitary gland to stimulate the release of thyroid stimulating hormone (TSH). TSH acts on follicular cells in the thyroid gland to stimulate the synthesis and release of T4 and T3 (Mullur, Liu et al. 2014) (Fig. 1.2). Both thyroid hormones signal back to the hypothalamus and pituitary to negatively regulate the secretion of TRH and TSH (Fekete and Lechan 2014).

1.2 Thyroid hormone action

The response of a particular cell to T3 is mediated by various factors including the transmembrane transport of thyroid hormone via thyroid hormone transporters, the
expression of nuclear thyroid hormone receptors (TR’s), and the levels of deiodinase expression (Brent 2012, Warner and Mittag 2012). Specific thyroid hormone transporters such as monocarboxylate transporter 8 (MCT8), monocarboxylate transporter 10 (MCT10), and organic anion transporting polypeptide family (OATP’s), are required for uptake of T4 and T3 into the cell (van der Deure, Peeters et al. 2010). Their location and distribution on T3 target tissues regulate the cell response to thyroid hormone.

T3 diffuses into the nucleus of the target cell and controls gene transcription by binding to nuclear TRs thereby modulating biological processes in the body (Brent 2012). TRs belong to the steroid/thyroid hormone receptor superfamily of ligand-dependent transcription factors and are located on the promoter regions of T3 target genes at sites containing thyroid hormone response elements (TRE’s) (Brent 2012). There are two TR genes, TRα and TRβ, resulting in different TR isoforms with varying degrees of tissue distribution and expression levels (Brent 2012). They function as heterodimers with retinoid-X receptors (RXR). Without T3, corepressors bind to the TR complex preventing gene transcription. However, upon the binding of T3 to the TR, coactivators replace the corepressors and gene transcription is initiated (Fig. 1.3). Therefore, the levels of TR occupancy determine the extent of intracellular T3 activity. T3 responsive genes are commonly involved in development, differentiation and metabolism and differ based on the gene expression profile of a particular cell type or tissue.
Intracellular T3 activation and deactivation is also regulated by the action of iodothyronine deiodinases (~29-33 kDa), a family of dimeric thioredoxin (TRX) fold-containing selenoproteins consisting of types I, II, and III deiodinases (hereby referred to as D1, D2, and D3, respectively) (Callebaut, Curcio-Morelli et al. 2003). These enzymes share approximately 50% sequence homology and catalyze the reductive dehalogenation of iodothyronines. The deiodinases belong to a family of 25 selenoproteins containing the rare selenocysteine (Sec) amino acid. The deiodinases are integral membrane proteins, however, their cellular locations differ; the expression and activity levels of the deiodinases are temporal and tissue specific which allow for cellular customization of a T3 footprint (Bianco and Kim 2006, Gereben, Zavacki et al. 2008). D3 is located in the plasma membrane and nucleus and its active center is located in the cytosol; it is primarily expressed in the central nervous system, placenta, skin, uterus and the brain (Jo, Kallo et al. 2012). It serves to inactivate T3 by the removal of an iodine residue from the inner tyrosyl ring of the phenolic iodothyronine forming rT3 from T4 or T2 from T3. D2
is located in the endoplasmic reticulum with its active center located in the cytosol; it is primarily expressed in the central nervous system, hypothalamus, pituitary, thyroid, brown adipose tissue and skeletal muscle (Larsen and Zavacki 2012). D2 activates the prohormone, T4 to the active T3 molecule through the removal of an iodine residue from the outer phenolic ring of the iodothyronine. D1, located in the plasma membrane and its active center is in the cytosol; it is primarily found in the liver, kidney and thyroid. D1 has dual functions and is able to either activate T4 to T3 or convert rT3 to T2 by the removal of an outer ring iodine or inactivate T4 and T3 by inner ring deiodination to produce rT3 and T2, respectively (Fig. 1.4) (Zavacki, Ying et al. 2005, Bianco and Kim 2006). Based on kinetic studies, D2 is thought to be the main T4 activating enzyme, while D1 is thought to have a scavenger role mainly deiodinating conjugated iodothyronines in process of elimination (Arrojo e Drigo and Bianco 2011). Therefore,

![Diagram of deiodinase-mediated metabolism of iodothyronines.](image)

**FIGURE 1.4** Deiodinase mediated metabolism of the iodothyronines. Schematic depiction of the action of the deiodinases. The size of the deiodinase indicates the predominant pathway.
due to the deiodinase-mediated local activation of thyroid hormone, serum T3 levels are not always reflective of the state of T3 target tissues.

1.3 Type II deiodinase

Various mechanisms regulate D2 in a temporal and tissue specific manner including intracellular mediators such as cAMP (Bartha, Kim et al. 2000, Canettieri, Celi et al. 2000), transcription factors such as Nkx-2.5 and GATA-4 in the human heart (Dentice, Morisco et al. 2003), hormones such as T3 (Kim, Harney et al. 1998), and post translational factors such as ubiquitination (Gereben, Goncalves et al. 2000) (Fig. 1.5, Fig. 1.6). Physiologically, D2 plays a role in the hypothalamic-pituitary feedback, it is required for thermogenesis in BAT, and it provides a source of plasma T3 (Larsen and Zavacki 2012). Because D2 is able to increase intracellular thyroid hormone production thereby expanding the nuclear T3 pool, understanding pathways that control D2 are relevant for tissues involved in energy expenditure and metabolism such as brown adipose tissue and skeletal muscle (Bianco and McAninch 2013).
### D2 Regulatory Factors and Conditions

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<th>Transcriptional effect</th>
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<tbody>
<tr>
<td>CAMP</td>
<td>increase</td>
<td>†</td>
<td>(3-6)</td>
</tr>
<tr>
<td>ntc.&lt;sup&gt;2.5&lt;/sup&gt; GATA-4</td>
<td>increase in human, but not rat</td>
<td>†</td>
<td>(25)</td>
</tr>
<tr>
<td>SRY-1</td>
<td>increase</td>
<td>†</td>
<td>(0)</td>
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<tr>
<td>AP-1</td>
<td>decrease</td>
<td>†</td>
<td>(6, 53)</td>
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<tr>
<td>TPA</td>
<td>increase/decrease cell type dependent</td>
<td>†</td>
<td>(35, 37)</td>
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<td>p65 (RelA)</td>
<td>increase</td>
<td>†</td>
<td>(36)</td>
</tr>
<tr>
<td>LPS</td>
<td>increase hypothalamus</td>
<td>†</td>
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<tr>
<td>hypothyroidism</td>
<td>increase</td>
<td>†</td>
<td>(12, 18, 45, 46)</td>
</tr>
<tr>
<td>T3</td>
<td>decrease</td>
<td>†</td>
<td>(12, 16, 34)</td>
</tr>
<tr>
<td>rT3</td>
<td>potent post-translational downregulator</td>
<td>†</td>
<td>(34)</td>
</tr>
<tr>
<td>TRH</td>
<td>increase</td>
<td>†</td>
<td>(34)</td>
</tr>
<tr>
<td>TSH</td>
<td>increase</td>
<td>†</td>
<td>(35, 55)</td>
</tr>
<tr>
<td>17β-estradiol + progesterone</td>
<td>increase rat uterus</td>
<td>†</td>
<td>(51)</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>increase/decrease cell type dependent</td>
<td>†</td>
<td>(34, 37, 52, 56, 59)</td>
</tr>
<tr>
<td>CRH</td>
<td>increase in the presence of dexamethasone</td>
<td>†</td>
<td>(52)</td>
</tr>
<tr>
<td>Iodine deficiency</td>
<td>increase CNS</td>
<td>†</td>
<td>(60)</td>
</tr>
<tr>
<td>Stress</td>
<td>increase CNS</td>
<td>†</td>
<td>(64)</td>
</tr>
<tr>
<td>Trauma</td>
<td>increase CNS and peripheral nervous system</td>
<td>†</td>
<td>(62, 63)</td>
</tr>
<tr>
<td>Fasting</td>
<td>increase hypothalamus</td>
<td>†</td>
<td>(66, 86)</td>
</tr>
<tr>
<td>Light</td>
<td>decrease pl. pineal; increase hypothalamus</td>
<td>†</td>
<td>(67-69)</td>
</tr>
<tr>
<td>Melatonin</td>
<td>decrease</td>
<td>†</td>
<td>(69)</td>
</tr>
</tbody>
</table>

*cAMP, cyclic adenosine monophosphate; P4A, protein kinase A; TFF-1, thyroid transcription factor; AP-1, activator protein-1; TPA, 12-0-tetradecanoyl-phorbol 13-acetate; LPS, lipopolysaccharide; T3, triiodothyronine; rT3, reverse triiodothyronine; TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone; CRH, corticotropin-releasing hormone; T4, thyroxine.

**FIGURE 1.5** D2 regulatory factors. A comprehensive list of regulatory factors that up- or downregulate D2 in various tissues. (adapted from Gureben and Salvatore, 2005).

### D2 Regulatory Pathways

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Stimuli</th>
<th>Mechanism</th>
<th>Pathway/Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAT</td>
<td>Bile acids</td>
<td>Transcriptional</td>
<td>TGR5-CAMP (13)</td>
</tr>
<tr>
<td></td>
<td>Chemical chaperones</td>
<td>Transcriptional</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold exposure</td>
<td>Transcriptional</td>
<td>β-adrenergic receptor-cAMP (4, 85)</td>
</tr>
<tr>
<td>Bone (tibia growth plate)</td>
<td>Indian hedgehog (Ihh)</td>
<td>Post-transcriptional</td>
<td>Ubiquitination- USP9 (27)</td>
</tr>
<tr>
<td>Brain</td>
<td>LPS</td>
<td>Transcriptional</td>
<td>NF-kappaB (75)</td>
</tr>
<tr>
<td></td>
<td>Thyroxine (T4)</td>
<td>Post-transcriptional</td>
<td>Ubiquitination (6-14), TEB4 (25), WSB1 (22)</td>
</tr>
<tr>
<td>Lung (airway cells)</td>
<td>LPS</td>
<td>Transcriptional</td>
<td>Injury (35, 36)</td>
</tr>
<tr>
<td></td>
<td>ER stress</td>
<td>Transcriptional</td>
<td>PERK-eIF2a (15)</td>
</tr>
<tr>
<td></td>
<td>Chemical chaperones</td>
<td>Post-transcriptional</td>
<td>Reversal of ER stress (15)</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>Bile acids</td>
<td>Transcriptional</td>
<td>TGR5-CAMP (13)</td>
</tr>
<tr>
<td></td>
<td>Kaempferol (KPF)</td>
<td>Transcriptional</td>
<td>cAMP (41)</td>
</tr>
<tr>
<td></td>
<td>Forskolin (FSK)</td>
<td>Transcriptional</td>
<td>cAMP (98)</td>
</tr>
<tr>
<td></td>
<td>Cold exposure</td>
<td>Transcriptional</td>
<td>Adrenergic receptors (103)</td>
</tr>
</tbody>
</table>

*Description of the regulatory pathways and factors that affect D2 expression levels.*

**FIGURE 1.6** D2 regulatory pathways. A comprehensive list of regulatory pathways that regulate D2 in various tissues. (adapted from Arrojo, Fonseca et al. 2013).
Previous studies in floating rat brown adipocytes have shown that insulin increases D2 activity in a concentration dependent manner (Mills, Barge et al. 1987). Additionally, studies also indicate that rats injected with insulin have increased D2 activity in the BAT (Silva and Larsen 1986). Since insulin is an indicator of food/nutrient availability in the body, this suggests that D2 may be affected by nutrient availability in the BAT. However, studies have yet to be conducted to determine the mechanism of D2 regulation in response to insulin and/or nutrient availability.

1.4 Thyroid hormone and food availability

In the adult, alterations in thyroid hormone levels primarily affect energy expenditure and metabolic processes which manifest as changes in the rate of metabolism (Bianco and McAninich 2013). In humans, the thyroid contributes approximately 20% of the daily T3 production and the remaining 80% is contributed by the two deiodinases, D1 and D2, with the latter playing the major role (Bianco, Salvatore et al. 2002). A major condition in which thyroid hormone plays a role is in fasting and/or caloric restriction (LoPresti, Gray et al. 1991). In this state, there is a reduction in energy expenditure, a decline in leptin and decreases in plasma insulin and T3 levels (LoPresti, Gray et al. 1991, Rosenbaum, Murphy et al. 2002, Chan, Heist et al. 2003). These effects correlate with the downregulation of the HPT axis that contribute to the decreased serum T3. Leptin, an adipocyte-derived hormone, has been shown to be a key factor stimulating the HPT axis (Boelen, Wiersinga et al. 2008). The drop in serum leptin alters the neuroendocrine response to fasting by suppressing the preproTRH gene in the neurons of
the paraventricular nucleus of the hypothalamus resulting in a decrease in TRH and consequently thyroid hormone (Legradi, Emerson et al. 1997, Boelen, van Beeren et al. 2012). However, upon refeeding, thyroid hormone levels are restored as seen in patients recovering from anorexia nervosa (Schebendach, Golden et al. 1997).

Both thyroid hormone and deiodinase expression levels are affected by fasting in animal models and in human subjects. In rats, in vivo kinetic studies indicate that reduced serum T3 levels during fasting are a result of decreased thyroid hormone secretion (Kinlaw, Schwartz et al. 1985). The expression of D3 accelerates thyroid hormone inactivation in multiple tissues, as well as increased sulfation and glucuronidation of T3 in the liver (Vella, Ramadoss et al. 2011). Additionally, hepatic D1 is decreased during fasting, a result of its sensitivity to T3 (Zavacki, Ying et al. 2005). Recent studies in mice have shown that in fasting, D2 is reduced in the pituitary and BAT but is unaffected in the cerebrum, cerebral cortex and hypothalamus (Galton, Hernandez et al. 2014). Finally, studies of human muscle biopsies taken from fasting subjects indicate that D2 mRNA decreases with fasting and is increased after insulin infusion (Heemstra, Soeters et al. 2009). However, studies have yet to define a specific mechanism for the action of D2 during fasting. Therefore, due to D2’s ability to contribute to intracellular T3 activation and consequent contribution to serum T3 levels, we aim to understand the mechanism regulating D2 in the skeletal muscle during fasting and refeeding.
1.5 Insulin signaling

To study the mechanistic regulation of D2 in the skeletal muscle during fasting and refeeding, we turned our attention to insulin, an indicator of nutrient/food availability and a major effector of skeletal muscle that decreases during fasting. Insulin is a multifunctional protein that stimulates cell growth and differentiation, stimulates lipogenesis, glycogen and protein synthesis and increases glucose uptake in the fat and muscle. The insulin receptor belongs to a subfamily of receptor tyrosine kinases that include the insulin-like growth factor (IGF)-1 receptor and the insulin receptor-related receptor (IRR) (Saltiel and Kahn 2001). These receptors exist as homo- or heterodimers that become autophosphorylated upon the binding of insulin, which stimulates a signaling

![Diagram of Insulin/Growth Factors](image)

**FIGURE 1.7 The mTOR signaling pathway.** The mTOR signaling pathway is a complex signaling pathway that regulates various processes including translational initiation, cell metabolism, growth, cell survival and proliferation (Laplante and Sabatini 2009).
cascade (Nakae, Kido et al. 2001). Insulin exerts its affects through various nutrient sensing pathways, one of these being the mTOR signaling pathway. The mTOR signaling pathway is a complex network that regulates various processes including translational initiation, cell metabolism, growth, cell survival and proliferation through the PI3K/mTOR pathway (Laplante and Sabatini 2009, Laplante and Sabatini 2009, Agulnik 2012) (Fig. 1.7). mTOR is a 289-kDa Ser/Thr kinase that is the catalytic core of two distinct multi component complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Wander, Hennessy et al. 2011). Numerous studies have linked mTORC1 to protein synthesis and growth (Thoreen, Chantranupong et al. 2012) and to the downstream activation of a myriad of metabolic regulatory genes including HIF-1α and SREBP1/2 (Duvel, Yecies et al. 2010). mTORC2 phosphorylates the AGC kinases and mediates cell survival and proliferation through phosphorylation of its downstream effector, Akt (Su and Jacinto 2011). It also phosphorylates PKCα to affect cytoskeleton arrangement (Ma and Blenis 2009).

Our understanding of the role of insulin during fasting and the regulation of D2 in the skeletal muscle during fasting led to the hypothesis that D2 may be a downstream target of insulin and the mTOR signaling pathway. If proven true, this would implicate mTOR signaling as a major mediator of metabolic pathways linking nutrient availability and the modulation of thyroid hormone action. Therefore, the aim of this current work is to examine the regulation of thyroid hormone activation via D2 stimulation during fasting and refeeding through an examination of the mTOR signaling pathway. The goals of this work are to:
1. Determine how D2 is affected by fasting and refeeding *in vivo* and *in vitro*.

2. Determine if the mTOR signaling pathway affects D2 expression and activity.

3. Determine the transcriptional mechanism responsible for the insulin stimulated increase of D2 activity.

In exploring these aims, we have identified that D2 is transcriptionally upregulated by insulin/IGF-1 and signals through the PI3K-mTORC2-Akt pathway to phosphorylate FOXO1 and relieve its negative repression on the *DIO2* promoter. This novel mechanism links nutritional availability to thyroid hormone activation - a plausible connection as thyroid hormone regulates various aspects of metabolism, many aspects of which are directly affected by nutrient status. Through these novel studies, we have elucidated a new role for mTOR signaling as well as a novel regulatory mechanism for D2. The findings from this work have been published in *The Journal of Biological Chemistry* (Lartey, Werneck-de-Castro et al. 2015).
Chapter 2. Methods

2.1 Mice

All studies performed were approved by the Institutional Animal Care and Use Committee by the University of Miami in compliance with the National Institutes of Health Standards. Male, 3-month old C57BL/6J mice (Jackson Laboratory) were housed at room temperature (22°C) on a 12h dark/light cycle. Mice were randomly divided into 3 groups (control, fasting, refeeding) \( n = 7 \) and acclimatized for 5 days. Animals were fed \textit{ad libitum} with standard chow diet (3.5kcal/g, 28.8% protein, 58.5% carbohydrate, 12.7% fat; 5010 LabDiet laboratory autoclavable rodent diet; PMI Nutrition, Richmond, IN) and water.

2.1.1 Fasting/refeeding protocol in mice

As indicated, food was withdrawn at 21:00h for the fasting and refeeding groups. After 36h, control and fasted mice were sacrificed by asphyxiation in a CO\(_2\) chamber. Mice from the refeeding group were weighed, refed for an additional 8h and sacrificed. The soleus muscle and cerebral cortex were rapidly dissected and frozen in liquid nitrogen and stored at -80°C until analysis.
2.1.2 Blood biochemistry

Blood was collected by cardiac puncture and plasma stored at -80°C until analysis. Insulin was measured using Mouse Insulin ELISA (Mercodia, AB) per manufacturer’s instruction.

2.1.3 IR/LIRKO/LIRFKO mice

As indicated, freely feeding 11 week old male insulin receptor (IR) floxed, liver-specific IR knockout (LIRKO) and liver specific IR/FOXO1 double knockout (LIRFKO) mice (I, Zhang et al. 2015) were used as well. They were sacrificed by decapitation following brief sedation with isofluorane. The liver was rapidly dissected and frozen in liquid nitrogen and stored at -80°C until analysis.

2.2 Cell culture

MSTO-211H cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in humidified atmosphere with 5% CO₂ at 37° C, in RPMI 1640 from CellGro (Manassas, VA) supplemented with FBS 10% and 100nM sodium selenite (Curcio, Baqui et al. 2001, Arrojo e Drigo, Fonseca et al. 2011).

2.2.1 Fasting/refeeding protocol in MSTO-211H cells

For starvation assays, cells were serum starved for 48h in 0.1% FBS (Atlanta Biologicals, Flowery Branch, GA) containing medium. For exposure to 10% FBS assays, cells were
serum starved for 48h in 0.1% FBS containing medium and then re-exposed to 10% containing FBS medium. For insulin stimulation assays, cells were serum starved for 48h in 0.1% FBS containing medium and then re-exposed to 300 or 600nM insulin (Sigma-Aldrich, St. Louis, MO) supplemented in 0.1% FBS containing medium. For IGF-1 stimulation assays, cells were serum starved for 48h in 0.1% FBS containing medium and then re-exposed to 50ng/mL IGF-1 (Sigma-Aldrich, St. Louis, MO) supplemented in 0.1% FBS containing medium.

### 2.2.2 Signaling pathway inhibition in MSTO-211H cells

When indicated, cells were serum starved for 48h in 0.1% FBS prior to treatment with various inhibitors:

#### Table 1. Pharmacological inhibitors utilized

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Company</th>
<th>Biochemical/Physiological Action</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY294002</td>
<td>Sigma-Aldrich</td>
<td>Specific cell permeable phosphatidylinositol 3-kinase inhibitor.</td>
<td>50µM</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Sigma-Aldrich</td>
<td>Membrane permeable hydrophobic fungal metabolite that selectively inhibits phosphoinositide 3-kinase.</td>
<td>100nM-1µM</td>
</tr>
<tr>
<td>GSK690693</td>
<td>Sigma-Aldrich</td>
<td>An ATP competitive, potent pan-AKT inhibitor against Akt 1,2 and 3.</td>
<td>10nM</td>
</tr>
<tr>
<td>PP242</td>
<td>Sigma-Aldrich</td>
<td>A potent and selective mTOR inhibitor targeting the ATP domain of mTOR.</td>
<td>2µM</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Sigma-Aldrich</td>
<td>A macrocyclic triene antibiotic with potent immunosuppressive and anticancer activity; complexes with FKB12 to bind and inhibit the mTORC1.</td>
<td>25nM</td>
</tr>
<tr>
<td>AS1842856</td>
<td>EMD Millipore</td>
<td>A cell-permeable oxodihydroquinoline that</td>
<td>20µM</td>
</tr>
</tbody>
</table>
preferentially inhibits the transcriptional activity of FOXO1 via direct binding to the active FOXO1.

MSTO-211H cells were treated with inhibitors at the concentration listed above.

2.3 D2 activity assay

For the measurement of D2 activity in MSTO-211H cells, harvested cells were re-suspended in DPBS containing 1 mM EDTA (PE), 0.25M sucrose and 10 mM dithiothreitol (DTT). Cells were sonicated and lysates analyzed for protein concentration via Bradford assay (Bio-Rad Laboratories). 25µg of total protein was incubated for 1h at 37°C in the presence of 20mM DTT, 0.1nM T4 and 100K cpm $^{125}$I-T4 (Perkin Elmer Life and Analytical Sciences, Inc.- NEX111H500UC). Assays were stopped with the addition of horse serum and 50% trichloroacetic acid and free $^{125}$I was counted on the 2470 automatic γ-counter Wizard2 (Perkin Elmer) in duplicates as previously described (Werneck-de-Castro, Fonseca et al. 2015). For the measurement of D2 activity in the soleus muscle, the assay was performed with the following modifications: protease inhibitor cocktail (Roche) was added before sonication and 200 µg of total protein was incubated for 3h at 37°C in the presence of 20mM DTT, 1mM PTU, 0.5 nM T4 and 200K cpm $^{125}$I-T4.

2.4 Gene expression analysis

RNA was extracted from MSTO-211H cells or animal tissue using RNAqueous- Micro Kit (Life Technologies Inc.) or RNeasy Mini Kit (Qiagen), respectively. RNA was
quantified with a NanoDrop and reverse transcribed using High Capacity cDNA (Applied Biosystems) or First Strand cDNA Kit for RT-PCR (AMV) Kit (Roche). Genes of interest were measured by qPCR (BioRad iCycler iQ Real-Time PCR Detection System) using the iQ SYBR Green Supermix (BioRad) or qPCR (Applied Biosystems Step One Plus Real-Time PCR System) using the SYBR Green FastMix ROX (Quanta Biosciences). Relative quantitation was measured using the standard curve method and the iCycler or Step One Plus software. Primer sequences are provided below:

Table 2. Primers used for qPCR analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Abbreviation</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Type II Deiodinase</td>
<td>Dio2</td>
<td>F: 5’- TCCTAGATGCCTACAAACAGGTTA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GTCAGGTTGGCTGAACAAAG-3’</td>
</tr>
<tr>
<td>Mouse CycloA</td>
<td>CycloA</td>
<td>F: 5’-GCCGATGACGAGCCCTTG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-TGCCGAGCTGCCATTATG-3’</td>
</tr>
<tr>
<td>Mouse RNA Polymerase II</td>
<td>RNApolII</td>
<td>F: 5’-ATGTGCGAGAACATGACCG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-GCCACATGAAACAGGTT-3’</td>
</tr>
<tr>
<td>Mouse TATA Binding Protein</td>
<td>Tbp</td>
<td>F: 5’-ACCCCTTCAACATGACTCTTAG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-ATGATGACTGCAAGCAATCGC-3’</td>
</tr>
<tr>
<td>Human Type II Deiodinase</td>
<td>DIO2</td>
<td>F: 5’-GGCTGACCGCATGGACAATAA-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-CTACCCCGTAACGCTATGTGA-3’</td>
</tr>
<tr>
<td>Human CycloA</td>
<td>CYCLOA</td>
<td>F: 5’-GGCAAATGTGGCCCAACAC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5’-TGCCATCTGGACCCCAAAGC-3’</td>
</tr>
</tbody>
</table>

All primers were made using Invitrogen Custom Primers.

2.5 Western blot analysis

Cells/tissues were lysed in 0.25M sucrose PE containing 10mM DTT. The lysates were diluted with 4X sample loading buffer (Invitrogen) and 5-25µg of total protein were run on 4-12% NuPAGE Bis-Tris Gels (Life Technologies, Carlsbad, CA). Samples were transferred to Immobilon-FL PVDF transfer membrane (Millipore, Billerica, MA) and
probed with primary antibodies as indicated at a 1:1000 dilution overnight at 4°C. Next, membranes were incubated with fluorescently labeled secondary antibodies (LiCOR Biosciences, Lincoln, NE) at a 1:2500 dilution for 1h at room temperature. All blots were imaged using LiCOR Odyssey instrument per manufacturer’s instructions (LiCOR Biosciences, Lincoln, NE). Primary antibodies used are provided below:

Table 3. Primary antibodies used for western blotting

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Company</th>
<th>Dilution</th>
<th>Isotype/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-S6rp (S235/236)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>p-S6rp (240/244)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>S6rp</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Mouse IgG</td>
</tr>
<tr>
<td>p-Akt (S473)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>p-Akt (T308)</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>Pan Akt</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>p-4EBP1</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>4EBP1</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>Rictor</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>Raptor</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>p-FOXO1</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GeneTex</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

Antibodies were used and diluted according to manufacturer’s instructions.

2.6 T4 to T3 conversion in intact cells

The production of T3 from outer ring-labeled T4 in intact cells was analyzed by measuring $^{125}$I in the medium as described elsewhere (da-Silva, Harney et al. 2007) except that the assay was stopped 12h after addition of $^{125}$I-T4 and the free T4 concentration was 30 pM.
2.7 Lentivirus-mediated shRNA knockdown of rictor and raptor

Rictor and raptor knockdown were established by transduction with GIPZ lentiviral shRNAmir vectors in MSTO-211H cells using GIPZ lentiviral shRNAmir for non-silencing control (NSC), human rictor (clone ID. V2LHS_120392 and clone ID. V3LHS_367492) or human raptor (clone ID. V3LHS_636800) from Thermo Scientific (Lafayette, CO). For generation of stable knockdown cell lines, MSTO-211H cells were plated at 1.5 x 10^5 cells/well and transduced with lentiviral particles at a multiplicity of infection (MOI) of ~23 and ~15 for rictor, respectively, and an MOI of ~13 for raptor and diluted in 1mL of serum free RPMI containing 8ug/mL polybrene (Sigma Aldrich). After 6h, 1mL of complete RPMI was added to each well (6-well plates). 72h later, the transfection cocktail was replaced with complete medium containing puromycin (1ug/mL) to select for shRNA expressing cells. Stable cell lines were generated after puromycin selection for 7 days.

2.8 Adenoviral-mediated transduction of WT and CA FOXO1

Adenoviral particles expressing wildtype (WT) and constitutively active (CA) FOXO1 as described (Zhang, Patil et al. 2006) were used to generate transient transfections for WT and CA FOXO1 in MSTO-211H. Cells were plated at 2.5 x 10^5 cells/well in 6-well dishes and transduced with adenoviral particles of concentration 2.0 x 10^{10}PFU/mL and 1.8 x 10^{10}PFU/mL, respectively, that was diluted in 1mL of serum free RPMI. Complete
media containing 10% FBS was added after 6h and the media was changed again after 24h. At 48h, cells were re-fed or stimulated with insulin for experimental procedure.

2.9 Chromatin immunoprecipitation (ChIP) of FOXO1

ChIP was performed in MSTO-211H cells using EZ-ChIP Chromatin Immunoprecipitation Kit (EMD Millipore) according to the manufacturer’s guidelines. Primers for RT-qPCR (Applied Biosystems Step One Plus Real-Time PCR System) were designed for predicted FOXO1 binding sites in the \textit{DIO2} promoter region: forward, 5’-CTGTCTGGAGGAAGTGGATTT-3’, and reverse, 5’-CCATGACTCCAACCTTTGT-3’ and performed using the SYBR Green FastMix ROX (Quanta Biosciences). \(\alpha\)-FKHR (H-128) (Santa Cruz Biotechnology) was used to detect binding of FOXO1 to the \textit{DIO2} promoter and ChIP enrichment is reported as input percent.

2.10 Statistical analysis

All data were analyzed using PRISM software (GraphPad Software) and expressed as mean \(\pm\) SEM. The Student’s \(t\) test was used to compare two groups. One-way analysis of variance (ANOVA) was used to compare more than two groups. Significance was held at \(p<0.05\) (two tailed).
Chapter 3. The effects of fasting and refeeding on D2 activity and expression in the soleus muscle of mice and the MSTO-211H cell line

3.1 Introductory remarks

Numerous metabolic changes occur when transitioning from the fed to fasting state that elicit a myriad of intracellular biochemical interactions. In the fed state, the primary goals of the body are to maintain glucose homeostasis, to promote the storage of fuels and to synthesize proteins. These goals are achieved through the secretion of insulin from the β-cells of the pancreas, the stimulation of glycogen synthesis, the uptake of glucose by the muscle and adipose tissue and the acceleration of glycolysis. During fasting there is a decrease in insulin secretion which mobilizes hepatic glycogen and glucose secretion. These alterations in metabolic processes serve to optimize energy utilization and conserve energy (Berg, Tymoczko et al. 2002).

In humans, fasting results in changes along the HPT axis in addition to alterations in thyroid hormone action and signaling in the periphery. It is marked by a decrease in serum T3, an increase in serum rT3 and minor or no change in serum T4 (Chan, Heist et al. 2003, Boelen, Wiersinga et al. 2008). The production of rT3 from T4 (Fig. 1.4) serves to decrease cellular metabolism and alleviate the energetic burden in a fasted state (Martinez, Sonanez-Organis et al. 2013). TRH and TSH decrease due to diminished leptin secretion which serves to transcriptionally decrease the expression of preproTRH in the paraventricular nucleus of the hypothalamus (Legradi, Emerson et al. 1997, Boelen, van Beeren et al. 2012). In rodents, fasting decreases serum T4 and T3 levels
and affects the expression of hepatic thyroid hormone responsive genes indicative of altered T3 content and action on a tissue specific basis (Carr, Seelig et al. 1983, Galton, Hernandez et al. 2014).

Since the iodothyronine deiodinases mediate T3 production in a tissue specific manner, we sought to examine the mechanistic role of D2 during fasting. Previous studies have shown that in fasted rodents, Dio2 expression increases in the hypothalamus (Diano, Naftolin et al. 1998) yet decreases in the pituitary (Boelen, Kwakkel et al. 2006) indicating that there is likely a tissue specific response to fasting. To our knowledge, there is yet to be an examination of the effects of fasting and refeeding on Dio2 expression and activity in the skeletal muscle; this is important given this tissue contribution to plasma T3. Thus, in our fist experiments, we sought to examine the effects of fasting and refeeding on Dio2 expression and activity levels in the soleus muscle of mice.

3.2 Results

3.2.1 Short-term fasting lowers soleus Dio2 expression and activity levels in the soleus muscle of wild type mice

To assess the role of D2 in fasting, wild type mice were subjected to a 36h fast and provided with water ad libitum. Measurement of body weight before and after the fasting period resulted in a ~5-fold decrease in change in body weight (Fig. 3.1A). Serum insulin levels significantly decreased in comparison to control animals (Fig. 3.1B),
which correlated to a decrease in phosphorylation of Akt and S6rp, targets along the mTOR signaling pathway, a major insulin signaling pathway (Fig. 3.1C). Dio2 expression was significantly reduced in the muscle of fasted animals (Fig. 3.1D) while the measurement of D2 activity in the same tissue resulted in decreased D2 activity as well (Fig. 3.1E).

**FIGURE 3.1** The effects of short term fasting on the soleus muscle of mice. (A) Change in body weight of control, fasted and refeed mice measured before and after experimental period; n=7; ***p<0.001. (B) Serum insulin levels measured in control, fasted and refeed mice; n=10, 12, 7, respectively; ***p<0.001. (C) Western blot analysis of control, fasting and refeeding groups using α- total S6rp, α-phospho S6rp (S235/236/240/244), α-pan Akt and α-phospho Akt (S473) antibodies. (D) CycloA4 was used as a housekeeping gene for the measurement of relative mRNA levels of Dio2/CycloA4 in the soleus muscle; n=3 (control), n=6 (fasting); ***p<0.001. (E) D2 activity measured in soleus muscle of control and fasted mice; n=3, 7 respectively; ***p<0.001.
3.2.2 Refeeding restores Dio2 expression and activity levels in the soleus muscle of fasted animals

With 8h of refeeding, animals that were fasted for 36h had normalization of body weight (Fig. 3.1A), a significant increase in serum insulin levels by ~100-fold (Fig. 3.1B) as well as a restoration of signaling along the mTOR signaling pathway (Fig. 3.1C).

Using RNA Polymerase II as a housekeeping gene (Fig. 3.2A), there was a significant increase in Dio2 expression (Fig. 3.2B) and activity upon refeeding (Fig. 3.2C). The

**FIGURE 3.2** The effects of refeeding on the soleus muscle of mice. (A) RNA Polymerase 2 was used as a housekeeping gene; n=7. (B) The measurement of relative mRNA levels of Dio2/RNAPol2 in soleus muscle; n=7; *p<0.05. (C) D2 activity measured in soleus muscle of fasted and refed mice; n=7; *p<0.05. (D) CycloA was used as a housekeeping gene for the measurement of relative mRNA levels of Dio2/CycloA in the cortex; n=7.
changes in Dio2 expression are tissue-specific given that under all 3 conditions of control, fasting, and refeeding, Dio2 expression remained unchanged in the cerebral cortex (Fig. 3.2D).

3.2.3 Fasting and refeeding is modeled in the MSTO-211H cell line

Typically, skeletal muscle cell lines exhibit immeasurably low D2 activity levels (Grozovsky, Ribich et al. 2009), therefore, in order to define a mechanism for this phenomenon, a mesothelioma cell line (MSTO-211H), noted for their high endogenous DIO2 expression levels (Curcio, Baqui et al. 2001), was used to examine the role of D2 in fasting and refeeding. To simulate fasting, MSTO-211H cells were serum starved in 0.1% FBS for 48h. To simulate refeeding, cells that were serum starved were re-exposed to media containing 10% FBS. Under conditions of 0.1% FBS (fasting), there was a progressive decline in DIO2 expression (Fig. 3.3A) and activity levels (Fig. 3.3B). Following re-exposure to 10% FBS (refeeding), D2 activity (Fig. 3.3C) was significantly increased by ~3.5-fold along with DIO2 expression levels by ~2-fold (Fig. 3.3D), both of which returned to baseline by 24h. Varying the concentration of FBS that cells were re-exposed to resulting in a concentration dependent increase in D2 activity (Fig. 3.4A) which correlated with signaling of typical serum dependent targets along the mTOR signaling pathway (Fig. 3.4B).
FIGURE 3.3 Fasting and refeeding is modeled in the MSTO-211H cell line. (A) DIO2 mRNA expression levels and (B) D2 activity measured during 48h of starvation in 0.1% FBS; n=3; ***p<0.001 vs 0h, **p<0.01 vs 0h. (C) D2 activity following the addition of 10% FBS; n=3; *p<0.05 vs 0h, ***p<0.001 vs 0h. (D) DIO2 expression levels measured by fold change of DIO2/CYCLOA following the addition of 10% FBS; n=3; *p<0.05 vs 0h, **p<0.01 vs 0h.

FIGURE 3.4 The response of MSTO-211H cells to FBS. (A) D2 activity following 48h of serum starvation in 0.1% FBS prior to the addition of 0.1 or 10% FBS supplemented media; Cells were harvested at the indicated time points; n=3; *p<0.05, **p<0.01. (B) Western blot analysis at the indicated time points using α-total S6rp, α-phospho S6rp (S235/236), α-pan Akt and α-phospho Akt (S473) antibodies.
3.2.4 D2 activity half-life is unaffected by re-exposing MSTO-211H cells to 10% FBS

D2 is regulated by numerous transcriptional and post-transcriptional factors (Fig. 1.5) one of which is ubiquitination leading to proteasomal degradation (Sagar, Gereben et al. 2007). Being that this mechanism can control the half-life of D2 by affecting its degradation, we examined whether it may be involved in the induction in D2 activity that is seen upon re-exposure of cells to 10% FBS. MSTO-211H cells were treated with cyclohexamide (CHX), used to prohibit translation, at 0, 4, 8, and 12h following their re-exposure to 10% FBS. They were harvested 0, 30, 60 and 90 minutes after CHX addition and D2 activity was measured. Results show that there was no significant effect on D2 activity half-life (Fig. 3.5 A-D). Therefore, we have shown that the stimulation of D2 occurs via a transcriptional mechanism as post-translational mechanisms affecting D2 activity are unaffected.
3.2.5 Insulin and IGF-1 induce D2 activity and increase T3 production in MSTO-211H cells

FBS is used in eukaryotic cell culture to supply nutrients and growth factors necessary for normal cell growth and development, therefore, FBS contains numerous factors that could possibly contribute to the induction of D2 activity upon re-exposure of MSTO-211H cells to 10% FBS. Thus, we decided to examine the role of 2 members of the insulin-like growth factor family (IGF), insulin and insulin-like growth factor-1 (IGF-1) (Brahmkhatri, Prasanna et al. 2015). These proteins regulate numerous cellular functions such as growth and development and are primarily mediated by the insulin
receptor and/or IGF-1 receptors (Jacobson, De et al. 2009, Brahmkhatri, Prasanna et al. 2015).

These targets were chosen based on previous studies in primary cultures of brown adipocytes showing that insulin stimulates D2 activity in a concentration dependent manner by increasing the $V_{\text{max}}$ (Mills, Barge et al. 1987). Additionally, intravenous injection of insulin into diabetic rats increased D2 activity by ~8-fold peaking 3-4h after the injection (Silva and Larsen 1986). These studies indicate that insulin (and/or its familial members) may be playing a role in the induction of D2 activity as seen in our model.

To examine the effect of insulin on this phenomenon, we supplemented MSTO-211H cells with 0, 100, 300 or 600nM insulin in 0.1% FBS containing media for 8h following starvation in 0.1% FBS for 48h. This resulted in a concentration-dependent increase in D2 activity (Fig. 3.6A) with a noticeable 50% increase in D2 activity using 300nM insulin. Next, we examined the effect of IGF-1; MSTO-211H cells that had been serum starved in 0.1% FBS for 48h were exposed to 50ng/mL IGF-1 supplemented in 0.1% FBS. This resulted in a significant time-dependent increase in D2 activity, peaking during its 8-12h time points (Fig. 3.6B). To examine whether the increase in D2 activity effects T3 production, we decided to measure T3 production in this system using an in vitro assay to measure T4-T3 conversion in the media of intact cells through the assessment of the cleavage of $^{125}$I (da-Silva, Harney et al. 2007). A 4-5-fold increase in T3 production in cells was observed 12h following its stimulation with either 300nM insulin or 50ng/mL IGF-1 (Fig. 3.6C). These results show that the insulin/IGF-1 induced increase in D2 activity positively affects T3 production.
3.3 Summary

Short term fasting decreases body weight, serum insulin levels and D2 activity and expression in the soleus muscle of mice. Conversely, refeeding rescues this phenotype and upregulates body weight, serum insulin levels and D2 activity and expression. MSTO-211H cells can be used as a cell model to study the mechanism by which fasting (serum starvation in 0.1% FBS) and refeeding (re-exposure to 10% FBS)
decreases and increase D2 activity, respectively, via insulin/IGF-1 signaling. This is important because we model the physiologically relevant observation of D2 regulation in skeletal muscle during fasting and refeeding in a cell line. Using this model, we have found that D2 activity is transcriptionally regulated through insulin and/or IGF-1 stimulation and increases cellular T3 production. These results will allow us to conduct a mechanistic study to decipher how D2 is positively regulated via insulin/IGF-1 signaling.
Chapter 4. Insulin/IGF-1/FBS stimulates D2 activity via PI3K-dependent pathways

4.1 Introductory remarks

Insulin is a pleiotropic hormone that controls many aspects of cellular metabolism including stimulation of growth and differentiation, promotion of substrate storage, inhibition of protein breakdown and the regulation of blood glucose content (Saltiel and Kahn 2001). This is accomplished through the activation of a variety of pathways, many of which are also responsive to IGF-1, a member of the insulin-like growth factor family (Siddle 2011). Although they have distinct cellular roles, these ligands share binding specificity to the insulin receptor (IR) and the insulin-like growth factor receptor 1 (IGF-1R) (Nakae, Kido et al. 2001, Shukla, Grisouard et al. 2009, Siddle 2011). These homo- or heterodimeric receptors are located on the cell surface and activate kinase activity eliciting various metabolic responses (Nakae, Kido et al. 2001). Thus, our observation that D2 activity is stimulated by both insulin and IGF-1 is highly conceivable.

In support of this finding, previous studies have shown that D2 activity is stimulated by insulin in rat floating brown adipocytes (Mills, Barge et al. 1987), which was also seen in the BAT of intact rats when injected with insulin (Silva and Larsen 1986). Mechanistically, insulin stimulates the sympathetic nervous system to release norepinephrine which transcriptionally upregulates D2 expression and activity (Martinez-deMena and Obregon 2005). However, studies to determine the mechanism mediating this interaction in the skeletal muscle have yet to be conducted.
To determine the molecular pathway regulating the insulin/IGF-1 induced increase in D2 activity, we shifted our attention to the mTOR signaling pathway for two main reasons. First, its activation is mediated by insulin/IGF-1. Second, we observed altered signaling along this pathway in the fasted/re-fed state of mice in our previous experiments (Fig. 3.1C) and in MSTO-211H cells (Fig.3.4B). mTOR is a 289 kDa Ser/Thr kinase that controls cell growth, metabolism, and proliferation in response to nutrients, growth factors, and energy status (Zinzalla, Stracka et al. 2011). MTOR is part of two distinct multiprotein complexes, mTORC1 and mTORC2.

MTORC1 is a sensor of nutritional status, energy and stress (Bracho-Valdes, Moreno-Alvarez et al. 2011) and is activated by growth factors (e.g. insulin/IGF1) via signaling through PI3K, PDK1, Akt, the TSC1-TSC2 complex and Rheb (Zinzalla, Stracka et al. 2011). MTORC1 contains six protein components including mTOR- the catalytic core with Ser/Thr kinase activity, regulatory-associated protein of mammalian target or rapamycin (RAPTOR)- a scaffolding protein, mammalian lethal with sec-13 protein 8 (mLST8), DEP domain containing mTOR-interacting protein (DEPTOR), Tti1/Tel2 complex, and proline-rich Akt substrate 40 kDa (PRAS40) (Laplante and Sabatini 2012). The mTORC1 signaling cascade results in the stimulation of cap-dependent translational initiation through its two major downstream targets, ribosomal S6 kinases (S6K1 and S6K2) and eukaryotic initiation factor 4E (eIF4E)- binding proteins (4E-BP1 and 4E-BP2) (Ma and Blenis 2009). The functional roles of mTORC1 have been well characterized and through its targets, mTORC1 controls an array of metabolic processes including gene transcription, ribosome biogenesis, protein synthesis, lipid
synthesis, nutrient transport, autophagy and other processes related to growth (Duvel, Yecies et al. 2010, Zinzalla, Stracka et al. 2011).

MTORC2 is activated by growth factors (e.g. insulin/IGF-1) via signaling through PI3K and ribosomal association (Zinzalla, Stracka et al. 2011). It contains seven protein components including mTOR- the catalytic core with Ser/Thr kinase activity, rapamycin-insensitive companion of mTOR (RICTOR)- a scaffolding protein, mammalian lethal with sec-13 protein 8 (mLST8), DEP domain containing mTOR-interacting protein (DEPTOR), Tti1/Tel2 complex, protein observed with rictor 1 and 2 (protor1/2) and mammalian stress-activated map kinase-interacting protein 1 (mSin1) (Laplante and Sabatini 2012). The best characterized downstream targets of mTORC2 are the AGC kinases including protein kinase B (PKB/Akt), protein kinase C (PKC) and serum glucocorticoid-induced protein kinases (SGK) (Su and Jacinto 2011). These targets serve to regulate actin cytoskeleton reorganization, cell migration and survival, protein synthesis, protein maturation, autophagy, and metabolic regulation (Oh and Jacinto 2011, Lamming, Demirkan et al. 2014).

To our knowledge, there are no existing studies that explore the role of mTOR in the regulation D2. Therefore, through the inhibition of targets along this pathway, we aim to determine the molecular signaling pathway that induces D2 activity via insulin/IGF-1 signaling.
4.2 Results

4.2.1 The inhibition of PI3K prevents the FBS and insulin-mediated stimulation of D2 activity in MSTO-211H cells

The role of PI3K in the insulin induced induction of D2 was examined using LY294002, a well-known reversible inhibitor of PI3K. MSTO-211H cells were serum starved for the standard 48h, prior to exposure to 10% FBS containing media with 50uM LY294002 or vehicle (DMSO). PI3K inhibition resulted in the prevention of the insulin induced stimulation of D2 activity (Fig. 4.1A). An examination of the phosphorylation of Akt and S6rp indicated that inhibition was successful as downstream signaling was prevented with treatment (Fig. 4.1B). Following the same protocol but stimulating cells with 300nM insulin following starvation produced similar results; PI3K inhibition prevented the insulin induced stimulation of D2 (Fig. 4.1C). Again, phosphorylation of Akt and S6rp indicated successful inhibition (Fig. 4.1D). As confirmation, we used 0.1-1µM Wortmannin in cells that had been serum starved before stimulation using 300nM insulin. The inhibition of PI3K using Wortmannin also prevented the insulin induced increase in D2 activity (Fig. 4.2A-C). These results prompted us to explore targets further along the mTOR signaling pathway.
FIGURE 4.1 The inhibition of PI3K signaling using LY294002 prevents the FBS and insulin-induced increase in D2 activity in MSTO-211H cells. (A) D2 activity following 48h of starvation in 0.1% FBS prior to the addition of 10% FBS along with 50 µM LY294002; n=3; **p<0.01, ***p<0.001. (B) Corresponding western blot analysis at the indicated time points using α- total S6rp, α-phospho S6rp (S235/236), α-pan Akt and α-phospho Akt (S473) antibodies. (C) D2 activity following 48h of starvation in 0.1% FBS prior to 300 nM insulin stimulation along with 50 µM LY294002; n=3; ***p<0.001, **p<0.01. (D) Corresponding western blot analysis at the indicated time points using α- total S6rp, α-phospho-S6rp (S235/236/240/244), α-pan Akt and α-phospho Akt (S473) antibodies.
4.2.2 The inhibition of Akt prevents the FBS and insulin-mediated stimulation of D2 activity in MSTO-211H cells

Next, we examined the role of Akt in this mechanism using GSK690693, a selective ATP-competitive inhibitor (Levy, Kahana et al. 2009, Carol, Morton et al.)
MSTO-211H cells were serum starved in 0.1% FBS for 48h and pretreated for 1h with 10nM GSK690693 prior to stimulation with 300nM insulin. The inhibition of Akt signaling prevented the insulin induced stimulation of D2 activity (Fig. 4.3A) and successful treatment was documented in an assessment of Akt and S6rp (Fig. 4.3B). A common feature of small molecule Akt inhibitors is the hyper-phosphorylation of the Akt pathway upon treatment which is believed to be the effects of a feedback mechanism (Levy, Kahana et al. 2009, Carol, Morton et al. 2010); this is documented in the corresponding western blot (Fig. 4.3B). Taken together, this indicates that the FBS/insulin induced stimulation of D2 activity is dependent on a target downstream of PI3K and Akt and led us to further explore this signaling pathway.
4.2.3 The inhibition of mTOR prevents the FBS and insulin-mediated stimulation of D2 activity in MSTO-211H cells

To examine the role of mTOR in this mechanism, we used PP242, a competitive ATP inhibitor of both mTORC1 and mTORC2 (Feldman, Apsel et al. 2009). Following the standard 48h serum starvation in 0.1% FBS, we added 2µM PP242 to MSTO-211H cells either exposed to 10% FBS (Fig. 4.4A) or stimulated with 300nM insulin (Fig. 4.4C); this again resulted in the prevention of the insulin induced stimulation of D2 activity. Western blots of Akt, S6rp and 4EBP1 confirm successful inhibition of mTOR.

![Western blots of Akt, S6rp and 4EBP1 confirming successful inhibition of mTOR](image)

**FIGURE 4.4 The inhibition of mTOR signaling using PP242 prevents FBS and insulin-induced increase in D2 activity in MSTO-211H cells.** (A) D2 activity following 48h of starvation in 0.1% FBS prior to the addition of 10% FBS along with 2µM PP242; n=3; **p<0.01, ***p<0.001. (B) Corresponding western blot analysis at indicated time points using α- total S6rp, α-phospho S6rp (S235/236/244), and α- pan Akt and α-phospho Akt (S473) antibodies. (C) D2 activity following 48h of starvation in 0.1% FBS prior to 300nM insulin stimulation along with 2µM PP242; n=3; **p<0.01, ***p<0.001, *p<0.05. (D) Corresponding western blot analysis at indicated time points using α- total S6rp, α-phospho S6rp (S235/236/240/244), α-total 4EBP1, α-phospho 4EBP1, α- pan Akt and α-phospho Akt (S473) antibodies.
targets (Fig. 4.4B,D). Being that PP242 is a dual mTOR inhibitor, next, we had to examine the roles of mTORC1 and mTORC2 individually.

4.2.4 The inhibition of mTORC1 signaling delays but does not prevent the induction of D2 activity in MSTO-211H cells

Using rapamycin, we explored the role of mTORC1 in this mechanism. Rapamycin is a natural product isolated from Streptomyces hygroscopicus that indirectly binds to and inhibits mTORC1; it was the first and is a well-known mTORC1 inhibitor (Guertin and Sabatini 2009, Seto 2012). MSTO-211H cells were serum starved for 48h in 0.1% FBS prior to exposure to 10% FBS with either 25nM rapamycin or DMSO (control). Treatment with rapamycin delayed but did not prevent the stimulation of D2 with peak activity seen at 24h (Fig. 4.5A). A corresponding western blot confirmed the selective inhibition of mTORC1 as seen in the inhibition of S6rp, a direct target of mTORC1, and not Akt, a direct target of mTORC2 (Fig. 4.5B). Next, we assessed DIO2 expression in cells under the same condition and saw a progressive increase in DIO2 expression over 24h, with expression also peaking at 24h (Fig. 4.5C). Finally, using 600nM insulin to stimulate cells along with 25nM rapamycin treatment also failed to inhibit the insulin induced stimulation of D2 (Fig. 4.5D).

As an alternative method of mTORC1 inhibition, we used GIPZ-lentiviral vectors to knock down raptor, an essential component of the mTORC1 complex. We created a stably expressing raptor knockdown cell line using MSTO-211H cells (GIPZ-Raptor-
shRNA-mirA (Fig. 4.6A,C) and subjected them to the typical starvation and re-exposure to 10% FBS/insulin stimulation protocols (Fig. 4.6B,D). Both conditions failed to prevent the induction of D2 activity via 10% FBS exposure or insulin stimulation. Since mTORC1 inhibition was unable to prevent the insulin induced increase in D2 activity, these results indicate that this mechanism is mTORC1 independent.

**FIGURE 4.5** The inhibition of mTORC1 signaling with rapamycin delays but does not prevent the induction of D2 activity in MSTD-211H cells. (A) D2 activity following 48h of starvation in 0.1% FBS prior to the addition of 10% FBS along with 25nM rapamycin; n=3; *p<0.05, **p<0.001. (B) Western blot analysis at the indicated time points using α-total S6rp, α-phospho S6rp (S235/236/240/244), α-pan Akt and α-phospho Akt (S473) antibodies. (C) DIO2 expression levels measured as fold change $DIO2/CYCLOA$ at the indicated time points; n=3; **p<0.01, ***p<0.001. (D) D2 activity following 48h of starvation in 0.1% FBS prior to 600nM insulin stimulation along with 25nM rapamycin; n=3; **p<0.01.
FIGURE 4.6 The inhibition of mTORC1 signaling does not prevent the induction of D2 activity in MSTO-211H cells. (A) Western blot analysis confirming knockdown of rictor using α-total raptor and GAPDH. (B) D2 activity measured in MSTO-211H cells stably expressing GIPZ-non silencing control (NSC) or GIPZ-raptor-shRNAmir A lentiviral vector; cells were serum starved in 0.1% FBS for 48h prior to the addition of 10% FBS; n=3; ***p<0.001 vs GIPZ-NSC, **p<0.01 vs GIPZ-NSC, *p<0.05 vs GIPZ-NSC. (C) Western blot analysis confirming knockdown of rictor using α-total raptor and GAPDH. (D) D2 activity measured at various time points in MSTO-211H cells stably expressing GIPZ- non silencing control (NSC) or GIPZ-raptor-shRNAmir A lentiviral vector; cells were serum starved in 0.1% FBS for 48h prior to 600mM insulin stimulation; n=3; *p<0.05 vs GIPZ-NSC, **p<0.01 vs GIPZ-NSC, ***p<0.001 vs GIPZ-NSC.
4.2.5 The inhibition of mTORC2 signaling prevents the FBS and insulin-mediated increase in D2 activity in MSTO-211H cells

To explore the role of mTORC2 in this mechanism, we used the GIPZ lentiviral system to knock down rictor, an essential component of the mTORC2 complex. Doing this inhibits mTORC2 as a pharmacological inhibitor is not available. Western blots confirmed successful rictor knockdown in the stably transfected MSTO-211H cell lines generated (GIPZ-Rictor-shRNAmirA, GIPZ-Rictor-shRNAmirB) (Fig. 4.7A,C). Following 48h of starvation in 0.1% FBS, re-exposure to 10% FBS (Fig. 4.7B) or stimulation using 600nM insulin (Fig. 4.7D) resulted in the prevention of the insulin induced stimulation of D2 activity in comparison to control group (NSC). This experiment shows that insulin signals through mTORC2 to regulate D2 and indicates that the mechanism is acting via an mTORC2 dependent pathway.
4.3 Summary

In summary, these results describe the signaling mechanism of the insulin/IGF-1 mediated stimulation of D2. These experiments used a combination of molecular biology techniques (lentiviral transfection) and pharmacological inhibitors to elucidate this mechanism. Various targets along the mTOR signaling pathway were examined to
determine how different signaling molecules regulate the insulin induced stimulation of D2 activity. We explored PI3K signaling using LY294002 and Wortmannin, Akt signaling using GSK690693, and mTOR signaling using the dual mTOR inhibitor PP242. To explore the role of mTORC1, rapamycin was used along with studies in stably transfected MSTO-211H cells with raptor knockdown. To explore the role of mTORC2, studies in stably transfected MSTO-211H cells with rictor knockdown were conducted. The results show that signaling along the PI3K-mTORC2 pathway stimulates D2; this is an mTORC2 dependent mechanism. The stimulation of mTORC2 (nutrient availability/refeeding) upregulates D2 activity while cellular starvation (fasting) inhibits its activity. This finding links insulin signaling to thyroid hormone activation via D2 upreguation and highlights the involvement of mTORC2.
Chapter 5. FOXO1 transcriptionally represses DIO2

5.1 Introductory remarks

Thus far, these studies have shown that the insulin/IGF-1/FBS induced stimulation of D2 in soleus muscle and MSTO-211H cells acts via an mTORC2 dependent pathway. Next, we needed to determine the downstream targets of mTORC2 that could transcriptionally upregulate D2 activity. In the search for a transcriptional activator of D2, we came across some interesting results from a collaborator, Terry G. Unterman, Ph.D., in possession of transgenic mice implicating FOXO1 in this mechanism.

The family of forkhead box ‘other’ (FOXO) transcription factors form a subgroup of the Forkhead transcription factor family that includes FOXO1/FKHR/FOXO1a, FOXO3/FKHRL1/FOXO3a, FOXO4/AFX and FOXO6; they share high sequence homology, structure and function (Tzivion, Dobson et al. 2011). These proteins are localized in the nucleus and are involved in a vast array of cellular processes including cellular proliferation, apoptosis, reactive oxygen species response, cancer, regulation of cell cycle and metabolism (Karger, Weidinger et al. 2009, Tzivion, Dobson et al. 2011). They serve to positively or negatively regulate gene transcription and are regulated via post-translational modifications such as phosphorylation (Calnan and Brunet 2008). As major targets of insulin action, they are highly expressed in insulin responsive tissues such as the pancreas, liver, skeletal muscle and adipose tissue (Kousteni 2012).
Therefore, these transcription factors are essential in metabolic regulation in response to nutrient availability (Barthel, Schmoll et al. 2005).

FOXO1 was the first member of this family to be identified (Zhao, Herrera et al. 2001) and has been characterized for its distinct role in controlling hepatic insulin sensitivity and lipid metabolism (Nakae, Kitamura et al. 2003, Matsumoto, Han et al. 2006, Zhang, Patil et al. 2006). In response to insulin, its transcriptional activity is inhibited via phosphorylation of FOXO1 regulatory sites through Akt and is subject to nuclear expulsion (Matsuzaki, Daitoku et al. 2003, Kousteni 2012). Once in the cytoplasm, it is ubiquitinated and tagged for proteasomal degradation (Matsuzaki, Daitoku et al. 2003). Therefore, via Akt phosphorylation, FOXO1 is a downstream target of mTORC2.

To corroborate our suspicion that FOXO1 may be involved in this regulatory mechanism, previous studies have shown that FOXO3 induces Dio2 expression by binding to the Dio2 promoter region in muscle cells (Dentice, Marsili et al. 2011). Since FOXO transcription factors share similar DNA binding sites and sequences (Dentice, Marsili et al. 2011, Tzivion, Dobson et al. 2011), we thought that FOXO1 might also regulate Dio2 expression and activity. Taken together, these observations suggested that there may be a regulatory role for FOXO1 in the insulin/IGF-1/FBS -induction of D2 activity and we sought to explore its involvement in this mechanism.
5.2 Results

5.2.1 *The effect of a liver specific knock out of the insulin receptor on Dio2 expression*

A collaborator, Terry G. Unterman, Ph.D., in possession of transgenic mice harboring either the insulin receptor floxed (IR), the liver-specific insulin receptor knockout (LIRKO) or the liver-specific insulin receptor and FOXO1 double knockout (LIRFKO) (I, Zhang et al. 2015), examined the livers of 11-week old male mice. In assessing *Dio2* expression, the LIRKO mice had significantly decreased mRNA levels in comparison to the IR floxed control mice (Fig. 5.1A). However, this was reversed in the LIRFKO mice, which indicated that insulin and FOXO1 couple to regulate D2 (Fig. 5.1A). Since FOXO1 affects D2 transcription and depends on insulin signaling, these

![Graph](image-url)
studies prompted our exploration of the role of FOXO1 in the insulin induced mediation of D2 in our own model.

5.2.2 The effect of FOXO1 inhibition on D2 activity

First, we sought to examine the correlation between D2 activity and FOXO1 phosphorylation. To begin, MSTO-211H cells were starved for 48h in 0.1% FBS containing media. We examined D2 activity in cells that remained at baseline in starvation media and in cells that were re-exposed to 10% FBS. As was also seen previously in figure 3.3C, re-exposure to 10% FBS significantly increased D2 activity (Fig. 5.2A), which was coupled with an increase in FOXO1 phosphorylation, as confirmed via western blot (Fig. 5.2B). These results show that an increase in D2 activity is associated with the phosphorylation of FOXO1 indicating that D2 may be negatively regulated of FOXO1.

Next, we used AS1842856, a selective, potent, small-molecule inhibitor of FOXO1 (Nagashima, Shigematsu et al. 2010) to examine D2 activity when FOXO1 is pharmacologically inhibited. During starvation in 0.1% FBS, the binding of FOXO1 is expected to be the highest; the addition of 20µm AS1842856 causes a significant increase in D2 activity over a period of 36h (Fig. 5.2C). Similar results are also seen when the same concentration of AS1842856 was added to cells following 48h of starvation (Fig. 5.2D). These results show that the inhibition of FOXO1 stimulates D2 activity indicating that D2 may be under the transcriptional control of FOXO1.
5.2.3 Constitutive activation of FOXO1 abrogates the FBS and insulin-induced stimulation in D2 activity in MSTO-211H cells.

Next, Terry G. Unterman, Ph.D. provided wildtype (WT) and constitutively active (CA) FOXO1 expressing adenoviral vectors (Zhang, Patil et al. 2006) and they were used to create transient transfections in MSTO-211H cells. The CA FOXO1 vector contains mutated phosphorylation sites rendering it unresponsive to phosphorylation via the
insulin signaling pathway (Zhang, Patil et al. 2006). These transiently transfected MSTO-211H cells were serum starved and re-exposed to 10% FBS or stimulated with 600nM insulin. The results show that the expression of CA FOXO1 prevents the induction of DIO2 expression whether the cells are re-exposed to 10% FBS (Fig. 5.3A) or stimulated with 600nM insulin (Fig. 5.3B). Under the same conditions, constitutive activation of FOXO1 also prevented the induction of D2 activity (Fig. 5.3C-D). These results indicate that the constitutive activation of FOXO1 prevents D2 stimulation.

**FIGURE 5.3 Constitutive activation of FOXO1 abrogates the FBS and insulin-induced stimulation in D2 activity in MSTO-211H cells.** (A) The measurement of DIO2/CYCLOA relative mRNA levels in cells transduced with WT or CA FOXO1 adenoviral vectors following the addition of 10% FBS; n=3; ***p<0.001. (B) The measurement of DIO2/CYCLOA relative mRNA levels in cells transduced with WT or CA FOXO1 adenoviral vectors following 600 nM insulin stimulation; n=3; ***p<0.001. (C) Above: D2 activity measured in cells transduced with WT or CA FOXO1 adenoviral vectors following the addition of 10% FBS; n=3; **p<0.01. Below: Corresponding western blots of phosphorylated and total FOXO1. (D) Above: D2 activity measured in cells transduced with WT or CA FOXO1 adenoviral vectors following 600nM insulin stimulation; n=3; **p<0.01. Below: Corresponding western blots of phosphorylated and total FOXO1.
5.2.4 FOXO1 binds to the DIO2 promoter during starvation and is released upon re-exposure to 10% FBS.

To show that this is indeed a transcriptional affect and that FOXO1 binds to the DIO2 promoter, ChIP analysis was performed. Using the PROMO/ALGGEN database (Messeguer, Escudero et al. 2002, Farre, Roset et al. 2003), primers were generated to a predicted FOXO1 binding site located -570bp away from the DIO2 transcription start site (Fig. 5.4A). MSTO-211H cells were serum starved for 48h in 0.1% FBS and were either maintained in 0.1% FBS or re-exposed to 10% FBS for 4h. Results show that there was specific binding of FOXO1 in cells maintained in baseline conditions in comparison to cells re-exposed to FBS (Fig. 5.4B). RNA polymerase II and IgG were used as positive and negative controls, respectively. This indicates that changing the media, thereby 

![Figure 5.4](image)

**FIGURE 5.4 FOXO1 binds to the DIO2 promoter during starvation and is relieved upon re-exposure to 10% FBS.** (A) Predicted binding site of FOXO1 on DIO2 promoter, transcription start site (TSS) depicted. (B) Chromatin immunoprecipitation of MSTO-211H cells with anti-FOXO1 antibody followed by RT-qPCR analysis with result presented as percent input by control antibody; n=2; ^p<0.05.

stimulating insulin/IGF-1 signaling, decreases the binding of FOXO1 on the DIO2 promoter. This proves that FOXO1 is the transcriptional mediator that regulates the insulin/IGF-1 mediated induction of D2.
5.3 Summary

These experiments show that an increase in D2 activity is correlated with the phosphorylation of FOXO1 and that the inhibition of FOXO1 using AS184256 leads to the stimulation of D2 activity. Additionally, CA of FOXO1 prevents the stimulation of D2 mRNA and activity following re-exposure to 10% FBS or insulin stimulation.

Finally, using ChIP studies, it was determined that mTORC2 activation, by re-exposing cells to 10% FBS for 4h, decreases the binding of FOXO1 to the DIO2 promoter region. Taken together, these mechanistic studies indicate that FOXO1 is the transcriptional mediator downstream of mTORC2 that mediates the insulin/IGF-1/FBS stimulation of D2.

Herein, we have elucidated the transcriptional mediator that regulates the insulin/IGF-1 stimulated induction of D2; FOXO1 negatively regulates D2 via transcriptional repression. When FOXO1 is phosphorylated via insulin/IGF-1 signaling to mTORC2 and Akt, FOXO1 is removed from the DIO2 promoter region and is excluded from the nucleus; D2 transcription and translation is increased.
Chapter 6. Discussion

6.1 General conclusions

The goal of this study was to examine the link between nutrient availability (insulin signaling), fasting and D2. Using a combination of animal and cells models, a novel mechanism linking insulin signaling to thyroid hormone activation via D2 induction has been elucidated. Stemming from the observation of the effects of fasting and refeeding on D2 in the skeletal muscle of mice, this work has described the molecular signaling pathway mediating this affect using MSTO-211H cells, which highlights the physiological relevance of this mechanism. We have elucidated the mechanism wherein DIO2 is transcriptionally regulated by insulin/IGF-1 via signaling through the PI3K-mTORC2-Akt pathway to phosphorylate FOXO1 in the nucleus. This relieves its transcriptional repression on the DIO2 promoter and leads to increased D2 activity and ultimately, T3 production. However, during fasting, or cellular starvation, FOXO1 remains bound to the DIO2 promoter region preventing its expression and activity (Fig. 6.1). This novel mechanism describes a regulatory process that mediates T3 production on a tissue specific basis; it describes a new mechanism of T3 regulation during fasting that is independent of the HPT axis. This starvation compensatory mechanism explains how T3 levels are kept proportionate to the availability of energy substrates. It also explains the observation that in muscle biopsies in fasted patients, insulin partially restores muscle D2 activity (Heemstra, Soeters et al. 2009).
Thyroid hormone plays an important role in skeletal muscle as it controls an array of T3 responsive genes that regulate numerous metabolic processes. In vivo studies in healthy men treated with T3 determined that genes regulating transcriptional control, mRNA maturation, protein turnover, signal transduction, cellular trafficking and energy metabolism were T3 responsive (Clement, Viguerie et al. 2002). Functionally, T3 also controls the expression of numerous contractile genes such as troponin C, tropomyosin and serca1/2 (Simonides and van Hardeveld 2008, Visser, Heemstra et al. 2009, Werneck-de-Castro, Fonseca et al. 2015), developmental genes such as myogenin and myoblast determination protein 1, and metabolic genes such as glucose transporter type 4 (GLUT4), mitochondrial uncoupling protein 3 (UCP3) and proliferator-activated receptor...

The expression and function of D2 has also been studied in the skeletal muscle. Previous studies have determined that D2 is expressed in skeletal muscle although its activity is rather low in both mice and humans (Grozovsky, Ribich et al. 2009). The D2 expressed in skeletal muscle functions in a regulatory manner and is required for myogenesis and muscle regeneration (Dentice, Marsili et al. 2011, Marsili, Tang et al. 2011). In relation to fasting, previous studies have indicated that D2 mRNA is decreased in human skeletal muscle (Heemstra, Soeters et al. 2009). However, prior to this current work, an examination of the regulation of D2 during fasting and refeeding in the skeletal muscle had yet to be conducted. Therefore, the importance of this work is that it defines the mechanism by which D2 mRNA and activity is decreased during fasting and conversely increased during refeeding in the skeletal muscle.

In the skeletal muscle, pituitary, BAT, and brain, D2-generated T3 provides an additional source of T3 to the periphery (Bianco, Salvatore et al. 2002). In our study, T3 production was stimulated by insulin and IGF-1 in MSTO-211H cells (Fig. 3.4C), which has been shown in our lab to contribute to the peripheral T3 concentration (data not shown). Herein, we have shown that this induction of D2 increases T3 production in our cell model (Fig. 3.6C). However, due to the limitations of our cell model, we were unable to correlate this increase with an intracellular modification in T3 responsive genes.

The data generated from the LIRKO and LIRFKO mice indicate that insulin is a key molecule initiating this stimulatory cascade (Fig. 5.1A). However, in this work, we
have established that both insulin and IGF-1 activate this mechanism (Fig. 3.4A-B.), likely attributable to dual receptor recognition. Typically, insulin and IGF-1 function in different physiological processes although they activate the same signaling pathways (Siddle 2011). However, our protocols of insulin stimulation or re-exposure to fresh media containing 10% FBS efficiently mimic insulin/IGF-1 binding to stimulate the mTOR signaling pathway. Therefore, this work highlights the parallel actions of these molecules in the stimulation of the mTORC2 signaling pathway to regulate D2 induction.

MTOR signaling has been implicated in a variety of transcriptionally regulated metabolic processes affecting glycolysis, oxidation of the pentose phosphate pathway, de novo lipid biosynthesis, bioenergetics and anabolic cellular processes through its downstream targets S6K, HIF1α and SREBP1/2 (Duvel, Yecies et al. 2010). However, these observations implicate mTORC1 signaling - the complex that is typically involved in metabolic regulation (Laplante and Sabatini 2009, Howell and Manning 2011, Laplante and Sabatini 2012). Recently, more studies have emerged that explore and define a metabolic role for mTORC2. For example, it was recently identified in embryonic stem cells that mTORC2 is involved in early adipogenesis; mTORC2 phosphorylates BTSA (a BSD domain-containing protein), an Akt interacting protein that induces the transcription of FoxC2 thereby stimulating brown adipogenesis (Lamming and Sabatini 2013, Yao, Suraokar et al. 2013). In addition, in the liver of fasting transgenic rictor knock out mice, phosphoproteomic profiling indicated significant alterations in the expression of genes related to intermediary metabolism, ribosomal biogenesis and proteasomal biogenesis (Lamming, Demirkan et al. 2014). Therefore, the
finding that this current work is regulated via mTORC2 enhances the functionality of this complex thereby expanding its role as a metabolic regulator.

6.2 Clinical implications

6.2.1 Hypothyroidism

According to the American Thyroid Association, an estimated 20 million Americans suffer from some type of thyroid disorder with an overwhelming 60% of this population unaware of their condition. Thyroid illnesses disproportionately affect women and statistics show that one in eight women will develop a thyroid condition in their lifetime. The most common thyroid disorder is hypothyroidism, which is characterized by subnormal production and secretion of thyroid hormone and may clinically manifest with fatigue, lethargy, sleepiness, decreased cognition and depression.

The standard treatment given to hypothyroid patients is levothyroxine, an identical synthetic pharmaceutically made L-T4 (Jonklaas, Bianco et al. 2014). In these patients, deiodination becomes the predominant source of T3. Therefore, the present findings are extremely important for our understanding of the generation and maintenance of serum T3 levels, particularly in L-T4 treated hypothyroid individuals.

The thyroid plays a role in regulating serum T3 levels. However, the thyroid of a hypothyroid patient is unable to contribute a sufficient amount of T3; thus this may result in increased dysfunction and worsen their symptoms. Such patients are at a greater risk to exhibit low levels of T3.
6.2.2 Insulin resistance

More than eighty percent of T3 in the serum is contributed by T3 generated in the periphery. The importance of the present work is that insulin signaling in the skeletal muscle is critical for T3 activation and the impairment of this pathway decreases the production of T3. Irregular insulin signaling is seen in insulin resistance, a metabolic condition in which tissues fail to respond to insulin. The repercussions of this condition include decreased insulin-mediated glucose uptake and impaired suppression of hepatic glucose output (Peppa, Koliaki et al. 2010). Since the activation of PI3K-mTORC2-Akt-FOXO1 is an insulin dependent pathway, it raises the question of whether or not thyroid hormone activation is normal in patients who are insulin resistant, such as type 2 diabetics or obese patients. Insulin resistance is a common hallmark of both of these conditions and if the tissues are resistant to insulin, this would impair D2 signaling and T3 activation.

6.3 Future directions

The present findings lay the ground work for clinical studies to examine if and how various diets affect T3 production. Since this work describes how insulin signaling can activate T3, it would be interesting to study if/how different types of diets affect thyroid hormone. The goal of this type of study would be to find out if there is a
particular food composition that will increase T3 production. This would be useful for hypothyroid patients as it may provide a natural alternative to increase thyroid hormone signaling.

6.4 Concluding remarks

In closing, this work improves our understanding of the role of D2 in response to fasting and refeeding in the skeletal muscle. The complete characterization of this pathway describes the intricate signaling pathway that mediates D2-generated T3 in response to food availability and collectively contributes to the larger discussion of the
metabolic role of T3 (Fig. 6.2). It increases our knowledge of how the body uses insulin/IGF-1 to regulate the production of D2-generated T3.
References


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