ROLE OF FOXO1 IN SKELETAL MUSCLE METABOLISM AND GROWTH

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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PgCert.

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DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Robert James Southgate

14/03/2007
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<tr>
<td>Akt</td>
<td>Acute Transforming Retrovirus Thymoma</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>β-HAD</td>
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<td>cAMP</td>
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<td>COX4</td>
<td>Cytochrome Oxidase 4</td>
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<td>CREB</td>
<td>Carbohydrate Regulatory Element</td>
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<td>CS</td>
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<td>siRNA</td>
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<td>Vehicle</td>
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<tr>
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<td>Wild-Type</td>
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<td>4-Hydroxytamoxifen</td>
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ABSTRACT

There are multiple binding domains on the promoter region of the peroxisome proliferator activator receptor gamma (γ) coactivator-1 α (PGC-1α) gene, including a trio of insulin responsive elements that are activated by the Forkhead box class-O (FoxO1) winged helix transcription factor, which is known to be regulated by acute transforming retrovirus thymoma (Akt). The first two experimental chapters (Chapters 2 and 3) of this thesis indicate that in skeletal muscle biopsy specimens from healthy humans and cultured human skeletal myotubes, insulin phosphorylates Akt (Ser473) and FoxO1 (Thr24, Ser256), leading to reduced nuclear abundance of FoxO1 total protein. This is associated with an insulin-mediated repression of the mRNA expression of PGC-1α and downstream genes associated with oxidative phosphorylation. In contrast, in muscle taken from insulin resistant humans or in palmitate treated insulin resistant myotubes, neither Akt nor FoxO1 was phosphorylated by insulin resulting in a failure for nuclear exclusion of FoxO1 total protein, and an inability for insulin to repress the mRNA expression of PGC-1α and downstream genes. To determine whether the regulation of FoxO1 was Akt dependent, the work that constitutes Chapter 4 describes experiments where Akt2 −/− and wild type mice were treated with or without insulin. Insulin phosphorylated Akt and FoxO1 (Thr24, Ser256) resulted in a reduced nuclear expression of FoxO1 total protein in wild type but not Akt2 −/− skeletal muscle.

The mammalian target of rapamycin (mTOR) is regulated by growth factors to promote protein synthesis. In mammalian skeletal muscle, the Forkhead-O1 transcription factor (FoxO1) promotes protein catabolism by activating ubiquitin-protein ligases. Using both C2C12 mouse myoblasts stably expressing FoxO1-ER fusion proteins that are rapidly activated by 4- OH
tamoxifen and transgenic mice that specifically overexpress constitutively active FoxO1 in skeletal muscle (FoxO++/+), Chapter 5 provides evidence that FoxO1 inhibits mTOR signaling and protein synthesis. Activation of constitutively active FoxO1 induced the expression of eukaryotic initiation factor 4E binding protein 1 (eIF4E-BP1) mRNA and protein, but reduced eIF4E-BP1 phosphorylation (Thr37/46). The reduction in eIF4E-BP1 phosphorylation was associated with a reduction in the abundance of Raptor and mTOR, Raptor-associated mTOR, reduced phosphorylation of the downstream protein p70S6 kinase and attenuated protein synthesis. The FoxO++/+ mice, characterized by severe skeletal muscle atrophy, displayed increased nuclear FoxO1 abundance and eIF4E-BP1 protein expression which was associated with reduced eIF4E-BP1 phosphorylation, Raptor, and mTOR protein abundance. These results provide the first evidence that FoxO1 \textit{per se} inhibits protein synthesis via increased expression of the translational protein eIF4E-BP1 and reduced signaling through mTOR/Raptor complexes.

In conclusion, insulin decreases the expression of genes involved in oxidative metabolism in healthy but not insulin resistant muscle, due to a decrease in FoxO1 phosphorylation and nuclear exclusion secondary to reduced Akt activity. FoxO1 may be an important therapeutic target in human disease where catabolism of muscle is observed, as FoxO1 appears to inhibit protein synthesis and translation/initiation pathways.
CHAPTER 1

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Transcription factor regulation of gene expression

Gene activation is a multi-step process involving a very large number of proteins functioning in discrete complexes (Spiegelman & Heirich 2004). Eukaryotic cells carry a monolithic proportion of genetic information. This information encodes in the range of 6000-100,000 proteins which are necessary to promote and perpetuate life and this situation is true from yeast to man. In order for the organism to propagate and thrive, its genome must also contain vast amounts of cis-regulatory DNA that can respond to spatial and temporal patterns of gene expression initiated by upstream metabolic requirement, developmental programs and a plethora of external stimuli (Lemon & Tjiam 2001). To maintain regulation of these genetic adaptive responses, eukaryotes have organized co-linear DNA into discrete chromosomes each packaged into chromatin. Transcription factors can be thought of as biological molecular machines that navigate through chromatin to promote target gene expression. Transcription factors bind to DNA in a sequence specific manner and when bound they essentially mark a gene for activation or repression through the recruitment of co-activator or co-repressor proteins (Speigelman & Heirich 2004).

FoxO transcription factors: identification of the Forkhead DNA binding domain

Initial evidence for a new DNA-binding motif came when Hepatocyte Nuclear Factor-3 (HNF-3) α, β, and γ were found to share a novel, highly conserved DNA-binding region (Lai et al., 1990; Lai et al., 1991) with the nuclear protein product of the Drosophila homeotic fork head gene,
involved in the formation of terminal structures of the early fly embryo. The *Drosophila*
Forkhead (Fkh) gene was reported to be essential for proper formation of terminal structures of
the embryo (Weigel *et al*., 1989; Weigel & Jackel 1990). Mutations of the Fkh gene caused
homeotic transformation of gut structures into head-derived elements. The nuclear localisation of
Fkh in these early studies suggested that Fkh regulated the transcription of other subordinate
genes (Weigel *et al*., 1989).

Shortly after the initial description of *Drosophila* Forkhead, a small family of hepatocyte
enriched DNA-binding transcription factors (Hepatocyte Nuclear Factor-3 [HNF-3]) were
described in rodents (Lai *et al*., 1990). The DNA binding domain of the HNF factors encompass
a region of about 110 amino acid residues. At the time of reporting, the DNA binding domain of
HNF factors did not resemble any other known DNA binding motif (Lai *et al*., 1990). However,
later studies employing amino acid sequence comparisons, noted a high degree of similarity
between the DNA binding domains of Forkhead and HNF proteins (Weigel & Jackle 1990). As a
result, this particular DNA binding domain sequence was termed the Forkhead/HNF-3 domain.
The Forkhead/HNF-3 DNA binding domain was later reported to be present in many proteins
from yeast to human suggesting conservation of this domain across species types (Lai *et al*.,
1993). This conserved region also known as the ‘winged helix’ domain is a variant of the helix-
turn-helix motif (Brennan 1993). The structure of the fork head/HNF-3 domain was resolved via
X-ray crystallography analysis on the complex of the fork head domain of HNF-3γ with its
cognate DNA at resolution of 2.5 angstroms (Å), (Clark *et al*., 1993). The forkhead/HNF-3 or
‘winged helix’ domain is responsible for DNA binding specificity and binds DNA as a monomer,
with two loops or ‘wings’ on the C-terminal side of the helix-turn-helix (Anderson *et al*., 1998).
The ‘winged helix’ domain flanks a core derived of α-helical and β-helical elements. This
structure represents a unit that cannot be split without losing DNA-binding properties as revealed by foot-printing and deletion studies (Lai et al., 1990, 1991; Kaufmann et al., 1994).

**Forkhead Box transcription factors: unified nomenclature for winged helix/Forkhead transcription factors**

Following the initial identification of the Forkhead domain and its sequence homology to HNF-3, researchers identified in excess of 100 members of the Forkhead gene family, again, spanning the spectrum of species from yeast to human (for review see Kaufamnn and Knockel 1996). The rapidly developing interest in the Forkhead DNA binding motif resulted in the significant accumulation of reported sequences by many different laboratories leading to the use of multiple names and classifications for proteins sharing the Forkhead DNA binding domain. Cleary, this rapidly developing interest in the Forkhead domain resulted in confusion when following the literature and impeded the ability to name newly characterized winged helix/Forkhead transcription factors. This problem was recognized at the first International Meeting on Forkhead/Winged Helix proteins held in La Jolla, California, in November 1998. As a result it was proposed that a standardized nomenclature be developed for these proteins. Fox (Forkhead box) was adopted as the unified symbol for all chordate winged helix/Forkhead transcription factors (Kaestner et al., 2000).

Fox proteins were assigned to individual subclasses based on phylogenetic analysis (Kaestner et al., 2000). As described by Kaester et al., (2000) a letter designated subclasses, and within each subclass proteins were given an Arabic numeral. Thus, the name of any particular Fox protein is ‘Fox, subclass N, member X, or for example FoxO1. It was deemed appropriate those
abbreviations for the chordate Fox proteins contain all uppercase letters for human (e.g. FOXO1), but only the first letter capitalized for those expressed in the mouse (e.g. Foxo1) and the first and subclass letters capitalized for all other chordates (e.g. FoxO1) Kaestner et al., (2000). For the purposes of clarity, the protein will herein be referred to as FoxO within this thesis.

As noted by Barthel et al., (2005), several features distinguish FoxO proteins from other Forkhead transcription factors. FoxO proteins contain a unique insert of five amino acids within the region of the DNA binding domain that is directly involved in sequence specific interaction with DNA binding sites. Whereas consensus sequences for binding most Forkhead proteins include a highly conserved core sequence (AAACA), the sequences flanking this core vary. The consensus binding sequence for FoxO proteins [(T/C)(G/A)AAACAA] diverges from that of other Forkhead proteins, providing a mechanism by which FoxO proteins can preferentially bind with a distinct set of target sites in the genome (Barthel et al., 2005).

**FoxO transcription factors: conservation across species**

As already noted, the Forkhead DNA binding domain is present in many proteins spanning various species. This conservation can also be applied to the FoxO subclass of Forkhead proteins. For example, FoxO1 (the mammalian ortholog of *Caenorhabditis elegans* DAF-16) was originally identified as a result of chromosomal translocation, t (2; 13) (q35; q14) and t (1; 13) (p36; q14) between PAX3 and/or PAX7 and FoxO (at that time called Forkhead homolog of Rhabdomyosarcoma [FKHR]), resulting in alveolar rhabdomyosarcoma tumor development (Galili et al., 1993; Borkhart et al., 1997; Anderson et al., 1998). Furuyama et al., (2000) & Biggs et al., (2001) confirmed FoxO protein expression in the mouse and assessed tissue
expression patterns. Orthology between human FoxO isoforms and DAF-16 was later confirmed (Lee et al., 2001) in experiments in C. elegans showing that FoxO could partially replace DAF-16 in the insulin signaling cascade, which is subsequently discussed.

**Regulation of FoxO transcriptional activity**

FoxO proteins have emerged as important downstream targets of insulin and growth factor action (Barthel et al., 2005), with numerous studies indicating that FoxO orthologs lie downstream of intracellular insulin signaling in *Drosophila* (Junger et al., 2003; Kramer et al., 2003; Puig et al., 2003; Hwangbo et al., 2004), the nematode *C. elegans* (Ogg et al., 1997; Lee et al., 2003; Murphy et al., 2003) and the mouse (Futuyama et al., 2000; Biggs et al., 2001). FoxO transcription factors interact with insulin response sequences (IRSs) on target gene promoters (Unterman et al., 1994; O’Brien et al., 1990; Daitoku et al., 2004; Puigserver et al., 2003). It is noteworthy that FoxO proteins are most highly expressed in insulin sensitive tissues such as skeletal muscle, liver, and adipose tissue (Imae et al., 2003). Insulin/growth factor stimulation negatively regulates FoxO transcription factor mediated gene expression via activation of phosphatidylinositol 3-Kinase (PtdIns3-K; PI-3K) and subsequent phosphorylation and activation of Acute Transforming Retrovirus Thymoma (Akt). Thus, FoxO proteins are substrates of the serine/threonine kinase Akt, and as such have conserved Akt sensitive residues that are phosphorylated in response to insulin mediated Akt activation (Biggs et al., 1999; Brunet et al., 1999; Guo et al., 1999; Kops et al., 1999; Nakae et al., 1999; Rena et al., 1999; Takaishi et al., 1999; Tang et al., 1999; Zhang et al., 2002), resulting in prompt and sustained FoxO protein nucleic exclusion (cytosolic sequestration) and transcriptional depression of FoxO target genes (Rena et al., 2002). The nucleic exclusion of FoxO proteins is mediated via a cytosolic protein...
called 14-3-3 (Rena et al., 2001; Zhao et al. 2004). Early studies suggested that phosphorylation of FoxO proteins resulted in 14-3-3 binding (Brunet et al., 1999) and moreover, the Serine 256 residue/phosphorylation site on FoxO1 was shown to be critical for this binding (Rena et al., 2001). These early studies suggest that 14-3-3 proteins were negative regulators of FoxO1 protein transcriptional activity. However, more recent work suggests this not to be the case, as it appears that some 14-3-3 proteins interact with FoxO proteins to promote lifespan extension. In *C. elegans*, two newly discovered 14-3-3 proteins (PAR-5 and FTT-2) described in three independent reports suggest that 14-3-3 proteins are required for Sir2.1 induced transcriptional activation of DAF-16 (the *C. elegans* orthologs of SIRT1 and FoxO1 respectively) and the interactions of these proteins positively increase life span (Berdichevesky et al., 2006; Wang et al., 2006; Li et al., 2007). Of interest, the work of Berdichevesky et al., (2006) showed that in response to heat stress, Sir 2.1 binds DAF-16 in a 14-3-3 dependant manner to promote lifespan extension, but in contrast, the stress imposed by low insulin-like signaling did not promote this association. Work to determine if these effects are conserved in humans is warranted.

Whilst there is broad consensus that Akt-dependant phosphorylation is crucial to the regulation of FoxO function and transcriptional targeting, recent reports suggest that FoxO proteins are also the target of the Sir orthologs (SIRT), or Sirtuins (Motta et al., 2004; Brunet et al., 2004; Yang et al., 2005; Kobayashi et al., 2005). These reports provide convincing evidence that FoxO is acetylated in response to cellular stress by the acetyltransferase activity of the nuclear hormone receptor co-activators Cbp and p300. Expression of SIRT1 causes FoxO deacetylation, and this deacetylation promotes cellular survival under stress conditions and protects against apoptosis by dampening or fine-tuning FoxO transcriptional gene targeting. Interestingly, Matusuzaki et al., (2005) suggest that acetylation of FoxO leads to a decrease in its DNA binding affinity, and moreover, acetylated
FoxO proteins become more sensitive to Akt dependant phosphorylation. Taken together these data suggest that acetylation and phosphorylation cooperatively regulate the function of FoxO, whilst deacetylation regulates the contextual response/function of FoxO proteins. More work is required to elucidate the exact contextual cues and effects of FoxO acetylation/deacetylation.

**FoxO proteins in cellular differentiation**

As already eluded to, FoxO factors play a key role in transmitting insulin signaling downstream of acute transforming retrovirus thymoma (Akt). Given that FoxO proteins are ubiquitously expressed across tissues and species, the metabolic functions of FoxO proteins are somewhat predictable based on the work in *C. elegans*. Evidence *in-vivo* and from *in-vitro* cell culture experiments suggest an integrative role for FoxO proteins with regard to the processing of extra-cellular cues and downstream transcriptional cascades that control differentiation. Nakae *et al.*, (2003), reported that that FoxO1 is induced in the early stages of adipocyte differentiation but that its activation is delayed until the end of the clonal expansion phase (the end of the proliferative phase). Constitutively active FoxO1 (FoxO mutants refractory for Akt phosphorylation) prevents the differentiation of preadipocytes, while dominant-negative FoxO1 restores adipocyte differentiation of fibroblasts from insulin receptor-deficient mice. Further, FoxO1 haploinsufficiency protects from diet-induced diabetes in mice (Altomonte *et al.*, 2003). These authors proposed that FoxO1 plays an important role in the integration of hormone-activated signaling pathways with the complex transcriptional cascade that promotes adipocyte differentiation (Nakae *et al* 2003).
Bois & Grosveld (2003) demonstrated that FoxO is localised in the cytoplasm of proliferating primary mouse myoblasts, yet translocates to the nucleus by a phosphorylation-independent pathway following serum starvation, a condition that induces myoblast differentiation. FoxO phosphorylation during terminal differentiation appears to down regulate its fusion activity, as a dominant-active, phosphorylation refractory FoxO mutant dramatically augments the rate and extent of myotube fusion. However, this FoxO mutant exerts its effects only after other events initiated the differentiation process. Conversely, enforced expression of a dominant-negative FoxO mutant blocks myotube formation whereas wild-type FoxO has no effect (Bois & Grosveld 2003). These authors concluded that, in addition to the role of FoxO proteins in regulating cell cycle progress and apoptosis, FoxO controls the rate of myotube fusion during myogenic differentiation.

Hribal et al., (2003) reported that a constitutively active FoxO1 mutant inhibits differentiation of C2C12 cells and prevents myotube differentiation induced by constitutively active Akt. In contrast, a transcriptional inactive mutant FoxO1 partially rescues inhibition of C2C12 differentiation mediated by wortmannin (a chemical inhibitor of PI3-kinase immediately upstream of Akt), but not by rapamycin (a chemical inhibitor of mTOR), and is able to induce aggregation-independent myogenic conversion of teratocarcinoma cells. Inhibition of FoxO expression by siRNA resulted in more efficient differentiation, associated with increased myosin expression. These observations indicate that FoxO proteins are key effectors of Akt-dependent myogenesis.

In terminally differentiated muscle cells, FoxO proteins work at the opposite end of the spectrum to induce genes associated with atrophy. Skeletal muscle atrophy is a debilitating response to
fasting, disuse, cancer, and other systemic diseases. In atrophying muscles, the ubiquitin ligase, atrogin-1 (MAFbx), is dramatically induced, and this response is necessary for rapid atrophy. Sandri et al., (2004) and Stitt et al., (2004) reported that in cultured myotubes undergoing atrophy, the activity of the PI3K/AKT pathway decreases, leading to activation of FoxO transcription factors and atrogin-1 induction. IGF-1 treatment or Akt over-expression inhibits FoxO and atrogin-1 expression. Moreover, constitutively active FoxO3 (Sandri et al., 2004) and FoxO1 (Stitt et al., 2004) act on the atrogin-1 promoter to cause atrogin-1 transcription and marked atrophy in both myotubes and muscle fibers. When FoxO activation was blocked by a dominant-negative construct in myotubes, or by siRNA in mouse muscles in-vivo, atrogin-1 induction during starvation and atrophy of myotubes induced by glucocorticoids was prevented (Sandri et al., 2004; Stitt et al., 2004). Thus, FoxO proteins play a critical role in the development of muscle atrophy, and inhibition of these proteins appears to be an attractive approach for alleviating muscle wasting pathologies.

**FoxO proteins role in cell cycle arrest**

In cells undergoing proliferation, the main effect of the expression of active forms of FoxO family members is to promote cell cycle arrest at the G1/S boundary (Medema et al., 2000). Target genes that mediate FoxO-induced cell cycle arrest are the Cdk inhibitor p27KIP1 (Medema et al., 2000) and the Rb family member p130 (Kops et al., 2002b). FoxO factors’ ability to induce G1 arrest is diminished in p27/p130-deficient fibroblasts (Kops et al., 2002b), suggesting that p27 and p130 are both critical to mediate FoxO-dependent G1 arrest. In the presence of Transforming Growth Factor-Beta (TGF-β), FoxO factors also bind to the promoter of p21, a cell cycle inhibitor, and induce cell cycle arrest at the G1/S transition (Seoane et al.,
Interestingly, FoxO factors can also promote cell cycle arrest by repressing the expression of cyclin D1 and D2, two cell cycle positive regulators (Ramaswamy et al., 2002; Schmidt et al., 2002). Thus, FoxO factors play a major role in G1 arrest by both up regulating cell cycle inhibitors (p21 and p27) and by repressing cell cycle activators (cyclin D1/D2). Akt promotes cellular proliferation by sequestering FoxO transcription factors in the cytoplasm and preventing them from inducing a G1 arrest. FoxO factors also play a role at other cell cycle checkpoints. Cells in which FoxO3 is activated in the S phase display a delay in their progression through the G2 phase of the cell cycle (Tran et al., 2002). Micro array analysis led to the identification of several FoxO3 target genes that may mediate FoxO's effect at the G2/M boundary, such as cyclin G2 and growth arrest and DNA damage-inducible protein 45 (GADD45) (Furukawa-Hibi et al., 2002; Tran et al., 2002). Thus, FoxO factors mediate cell cycle arrest at the G1/S and G2/M transitions, two checkpoints that are critical in the cellular response to stress. FoxO-induced cell cycle arrest may allow time for repair of damaged DNA and for detoxification of cells.

Consistent with FoxO factors’ role in promoting cell cycle arrest at the G1/S and G2/M boundaries, the expression of active forms of FoxO proteins up regulates several genes involved in DNA repair (Ramaswamy et al., 2002; Tran et al., 2002). GADD45 may mediate part of FoxO3-induced DNA repair since FoxO3- induced DNA repair is diminished in GADD45-deficient fibroblasts (Tran et al., 2002). In addition, FoxO proteins have been reported to allow detoxification of reactive oxygen species (ROS) by up regulating the free radical scavenging enzymes, including Manganese Superoxide Dismutase (MnSOD) and catalase (Kops et al., 2002a; Nemoto and Finkel, 2002; Ramaswamy et al., 2002; Tran et al., 2002). Thus, FoxO transcription factors control two aspects of the cellular resistance to stress: repair of the damage caused by ROS and removal of ROS byproducts.
**FoxO proteins: role in metabolism**

Aspects of FoxO’s role in metabolism and metabolic gene expression in muscle constitute a significant part of this thesis and as such are reviewed in later chapters. Two interesting publications detailing these effects in the liver provided the rationale for some of the work in this thesis.

Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) plays a major role in mediating hepatic gluconeogenesis in response to starvation, during which PGC-1α is induced by the cyclic AMP response element binding protein. Although it is observed that insulin counteracts PGC-1α transcription, the mechanism by which insulin suppresses the transcription of PGC-1α was unclear. Daitoku *et al.*, (2003) showed that Forkhead transcription factor FoxO1 contributes to mediating the effects of insulin on PGC-1α promoter activity. Reporter assays demonstrated that insulin suppresses the basal PGC-1α promoter activity and that co-expression of Akt mimicked the effect of insulin in HepG2 cells. Insulin response sequences (IRSs) are addressed in the PGC-1 promoter as the direct target for FoxO1 in-vivo. Co-expression of FoxO1 stimulates the PGC-1α promoter activity via interaction with the IRSs, while co-expression of FoxO (3A), in which the three putative Akt sites in FoxO1 are mutated, mainly abolishes the suppressive effect of Akt (Daitoku *et al.*, 2003). Whereas deletion of the insulin responsive motifs prevents the promoter stimulation by FoxO, that activity is still partially inhibited by insulin. These results indicate that signaling via Akt to FoxO can partly account for the effect of insulin to regulate the PGC-1α promoter activity via the IRSs. Puigserver *et al.*, (2003) concluded that hepatic gluconeogenesis is absolutely required for survival during prolonged
fasting or starvation, but is inappropriately activated in type 2 diabetes mellitus. Glucocorticoids and glucagon have strong gluconeogenic actions on the liver. In contrast, insulin suppresses hepatic gluconeogenesis (Puigserver et al., 2003; Daitoku et al., 2003). Two components known to have important physiological roles in this process are FoxO1 and PGC-1α, but whether these factors collaborate in the regulation of hepatic gluconeogenesis was, until recently, unclear.

Using wild type and mutant alleles of FoxO1, Puigserver et al., (2003) demonstrated that PGC-1α binds and co-activates FoxO1 in a manner inhibited by Akt-mediated phosphorylation. Furthermore, FoxO1 function was required for the robust activation of gluconeogenic gene expression in hepatic cells and in mouse liver by PGC-1α (Puigserver et al., 2003; Daitoku et al., 2003). Insulin suppresses gluconeogenesis stimulated by PGC-1α but co-expression of a mutant allele of FoxO1 insensitive to insulin completely reverses this suppression in hepatocytes or transgenic mice. Puigserver et al., (2003), concluded that FoxO1 and PGC-1α interact in the execution of a program of powerful, insulin-regulated gluconeogenesis.

Interestingly, Frescas (et al., 2005) published data relating to nucleic trapping of FoxO in hepatocytes under conditions associated with those seen in Type 2 diabetes. This trapping exacerbated FoxO driven gluconeogenic gene expression. This recent work extended that of Daitoku et al., (2003) and Puigserver et al., (2003) by showing that either Sirtuin activation or hydrogen peroxide treatment overrides the phosphorylation-dependent nuclear exclusion of FoxO1 caused by growth factors and leads to nuclear translocation of FoxO1 in hepatocytes. Kinetic measurements of nuclear fluorescence recovery after photo-bleaching showed that FoxO1 was readily diffusible within the nucleus under normal conditions but became restricted within a nuclear sub-domain after treatment with the prototypical Sirtuin agonist resveratrol or oxidative
stress. Expression of FoxO1 target genes was increased accordingly, leading to activation of gluconeogenesis and increased glucose release from hepatocytes (Frescas et al., 2005).

Imae et al., (2003) investigated if the mRNA levels of FoxO isoforms in rat liver respond to the stimuli of several nutritional and hormonal factors. Imposed fasting for 48 h significantly elevated mRNA levels of FoxO1 (1.5-fold), FoxO3 (1.4-fold), and FoxO4 (1.6-fold). Re-feeding for 3 h recovered the induced mRNA levels of FoxO1 and FoxO3 to control levels, but did not affect the mRNA levels of FoxO4. FoxO1 and FoxO4 mRNA levels were proved to be highly reflective of their protein levels measured by western immunoblotting. Of the three FoxO genes, FoxO4 alone showed altered levels of mRNA (a 1.5-fold increase) in response to a protein-free diet. Streptozotocin-induced diabetes for 28 days decreased hepatic mRNA levels of FoxO1 and FoxO3 and increased the level of FoxO4 mRNA, but short-term (7 days) diabetes induction had fewer effects on the expression of these genes. Insulin replacement partially restored the FoxO1 and FoxO4 mRNA levels, but had no effect on the FoxO3 mRNA level. Dexamethasone, a synthetic glucocorticoid, administered daily for one week increased the mRNA levels of FoxO1 (1.8-fold) and FoxO3 (2.4-fold) (Imae et al., 2003). These results showed that the FoxO genes respond differently to nutritional and hormonal factors, suggesting a mechanism for the regulation of FoxO-dependent gene expression by these factors. Moreover, changes of FoxO1 and FoxO4 in the nucleus in response to fasting also suggest that the regulation of nucleus/cytoplasm translocation actually functions in-vivo.

Bastie et al., (2005), investigated the role of FoxO1 in the regulation of fatty acid (FA) metabolism in muscle cells. C2C12 cells expressing an inducible construct with either wild type FoxO1 or a mutant form refractory for the activity of Akt were generated. FoxO1 activation after
myotube formation altered the expression of several genes of Fatty acid metabolism. Acyl-CoA oxidase and peroxisome proliferator-activated receptor delta mRNA levels increased 2.2-fold and 1.4-fold, respectively, whereas mRNA for acetyl-CoA carboxylase decreased by 50%. Membrane uptake of oleate increased 3-fold, and oleate oxidation increased 2-fold. Cellular triglyceride content was also increased. The enhanced FA utilisation induced by FoxO1 was mediated by a seven fold increase in plasma membrane level of the fatty acid translocase FAT/CD36 and eliminated by cell treatment with the CD36 inhibitor sulfo-N-succinimidyl-oleate. Bastie et al., (2005) concluded that in C2C12 cells, FoxO1 effects on FA uptake exceeded those on oxidation, resulting in net triglyceride accumulation and that these effects were mediated, at least in part, by membrane enrichment in CD36. These data suggest that FoxO1 contributes to preparing the muscle cell for the increased reliance on FA metabolism that is characteristic of fasting. Dysregulation of FoxO1 in muscle could therefore contribute to insulin resistance due to dysregulated fatty acid uptake leading to intramuscular lipid accumulation, a known primary cause of insulin resistance (Pan et al., 1997).

To better understand the role of FoxO proteins in the metabolic parameters in the liver, Zhang et al, (2006) created transgenic mice expressing constitutively active FoxO1 in the liver using the alpha1-antitrypsin promoter. Fasting glucose levels were increased, and glucose tolerance was impaired in transgenic versus wild type mice. Interestingly, fasting triglyceride and cholesterol levels were reduced despite hyperinsulinaemia, and post-prandial (post meal/feeding) changes in triglyceride levels were markedly suppressed in transgenic versus wild-type mice. Activation of pro-lipogenic signaling pathways (atypical protein kinase C and protein kinase B) and the ability to suppress beta-hydroxybutyrate levels were not impaired in the transgenic mice. In contrast, de novo lipogenesis measured with $^3$H$_2$O was suppressed by approximately 70% in the liver of the
transgenic mice after re-feeding. Gene-array studies revealed that the expression of genes involved in gluconeogenesis, glycerol transport, and amino acid catabolism were increased, whereas genes involved in glucose utilization via glycolysis, the pentose phosphate shunt, lipogenesis, and sterol synthesis pathways were suppressed in transgenic mice compared to wild-type controls (Zhang et al., 2006). Studies utilising adenoviral vectors in isolated hepatocytes confirm that FoxO1 stimulates expression of gluconeogenic genes and suppresses expression of genes involved in glycolysis, the shunt pathway, and lipogenesis, including glucokinase and SREBP-1c (Zhang et al., 2006). Together, these results indicate that FoxO proteins promote hepatic glucose production through multiple mechanisms and contribute to the regulation of other metabolic pathways important in the adaptation to fasting and feeding in the liver, including glycolysis, the pentose phosphate shunt, and lipogenic and sterol synthetic pathways. These metabolic effects of FoxO proteins have been comparatively less well characterised in skeletal muscle.

**FoxO’s regulation of cell growth**

Studies conducted in lower order organisms (*Drosophila & C. elegans*) suggest that FoxO proteins may also play role in cell growth/size regulation via their interaction with a common set of effectors in the mammalian target of rapamycin mTOR signaling pathway. The mTOR pathway is emerging as a major contributor in the regulation of cellular growth. mTOR proteins are protein kinases that were first identified in *Saccharomyces cerevisiae* through mutants that confer resistance to growth inhibition induced by the immunosuppressive macrolide rapamycin (Kunz et al., 1993). Interestingly, in response to growth factor (e.g. insulin) activation, Regulatory Associated Protein of mTOR (Raptor), a 150-kD polypeptide identified by Kim et al. (2002),
forms a complex with mTOR and phosphorylates eukaryotic Initiation Factor 4- Binding Protein 1 (eIF4E-BP1) (Hara et al., 2002). De-phosphorylation of eIF4E-BP1 is a limiting factor in protein synthesis, as a result of increased association with eIF4E. Raptor is indispensable for TOR signaling in-vivo (Kim et al., 2002), and is absolutely required for the mTOR-catalysed phosphorylation of eIF4E-BP1 in-vitro (Hara et al., 2002; Nojima et al., 2003).

Jia et al. (2004) conducted studies in C. elegans to investigate if DAF 16 imposed any transcriptional regulation on DAF 15 (C. elegans orthologs of mammalian FoxO and Raptor respectively). Using mutants where DAF 2 (C. elegans insulin receptor orthologs) are active or inactive, rendering DAF 16 (FoxO) inactive or active respectively, these authors found that in the active state, DAF 16 (FoxO) inhibited DAF 15 (Raptor) expression suggesting that FoxO proteins may have the potential to act as transcriptional repressors of Raptor expression.

Junger et al. (2003) investigated dFOXO (Drosophila FoxO) transcriptional targets. These experiments were performed within a context of cellular stresses, such as nutrient deprivation or increased levels of reactive oxygen species. These authors provide evidence that during cellular stress, dFOXO is active and promotes growth inhibition via the promotion of increased expression of d4E-BP1 (the Drosophila ortholog of mammalian eIF4E-BP1). The finding that d4E-BP1 is a bone fide transcriptional target of dFOXO was confirmed by Teleman et al. (2005a) utilising oxidative stress induction and starvation protocols. This study investigated dFOXO mediated induction of d4E-BP in a similar contextual model to that of Junger et al. (2003) but related the 4E-BP induction back to metabolism. In this regard, Teleman et al. (2005a) reported that an increased storage of lipid was seen during starvation concomitantly with induction of 4E-
BP, and moreover this induction of lipid storage and 4E-BP was dFOXO dependant. These authors subsequently showed that a molecule called MELTED binds dFOXO in response to growth factor stimulation and subsequently recruits FOXO to the inner apical cell membrane thereby inhibiting dFOXO transcriptional activity. In MELTED mutant flies (flies with genetic ablation of the MELTED gene) dFOXO retained its transcriptional activity with regard to the promotion of d4E-BP1 (Teleman et al., 2005b).

Taken together, these data suggest that FoxO proteins regulate components or effectors of cell growth and inducers of translation initiation in response to environmental cues. These observations raise the possibility that FoxO1 may not only contribute towards catabolic processes via activation of ubiquitin ligase’s (as discussed earlier in this chapter), but also via repression of anabolic pathways.

**Summary**

In summary, FoxO proteins are conserved across species and tissue types and their transcriptional activity is dictated via growth factor mediated phosphorylation and subsequent nucleic exclusion. There is evidence to suggest that acetylation and deacetylation fine tune the phosphorylation dependant nucleic exclusion and in the case of deacetylation. This fine-tuning represents a means of mediating target gene expression according to specific stimuli. FoxO proteins have a specific role in the differentiation of various cell types, and once the differentiation process has concluded, FoxO proteins subsequently regulate cellular size. In proliferating cells the main effect of forced activation or constitutively active forms of FoxO is to promote cell cycle arrest at the G1/S boundary, whilst also up regulating genes associated with
DNA repair and detoxification. FoxO regulates metabolic profiling in the liver via the targeting of a repertoire of genes associated with gluconeogenesis, and this targeting appears to be increased insulin resistant models, thereby exacerbating the diabetic phenotype. It would appear that the overall role of FoxO in metabolic profiling in the liver is to promote hepatic glucose output, whilst mediating other metabolic pathways pertinent to adaptation involved in fasting to feeding.

In muscle cells, data suggest that FoxO proteins prepare the muscle cell for an increased reliance on fatty acid metabolism, a situation that is characteristic of fasting, and as such could contribute towards intramuscular lipid accumulation and insulin resistance by maintaining fatty acid uptake.
During times of intracellular stress (e.g. starvation, osmotic, ultra violet), FoxO transcription factors appear to induce a program of gene expression specific to the contextual stimuli. In this regard, low level stress promotes the induction of genes associated with oxidative stress protection and genes associated with metabolic adaptation. As the insulting stimuli persists (moves towards a chronic state) or intensifies, FoxO induces genes associated with cell cycle arrest (with concomitant changes in metabolic gene expression) thus inhibiting propagation and proliferation of cells containing damaged DNA. If the insult becomes chronic, FoxO promotes a program of apoptosis or growth restriction depending upon tissue specificity.
Aims of thesis

The primary aim of this thesis was to investigate the role of FoxO1 in skeletal muscle, with particular emphasis on FoxO1’s role in the promotion of metabolic and growth regulatory gene expression. The FoxO family of transcription factors is relatively newly described. The research effort identified the winged-helix DNA binding domain associated with FoxO transcription factors some 13 years ago and since then the literature on these diverse transcription factors has grown enormously.

Work conducted in hepatocytes (published by other authors) suggested that FoxO1 targeted genes that exacerbate the insulin resistant state via the induction of a program of gluconeogenesis. Other work suggested that the basal expression of PGC-1α, a FoxO1 target, was reduced in insulin resistant muscle tissue. We hypothesized that FoxO1 transcriptional activity would be increased in the muscle of insulin resistant subjects. The work that constitutes Chapters 2, 3, and 4 of this thesis support the hypothesis and suggest that the regulation of FoxO1 transcriptional activity by insulin, in muscle tissue, is perturbed in the insulin resistant state. The observations reported in this thesis represent an important extension of the previously published work.

After establishing that FoxO1 regulation was perturbed in insulin resistance in-vivo and in experimentally induced insulin resistance in-vitro, it seemed reasonable to investigate how FoxO1 regulated intrinsic genes and proteins associated with the processing and expression of components of the growth regulatory pathways present in muscle cells (Chapter 5). The rationale for these later studies was related to the observation that insulin resistance appears to induce metabolic and size changes in muscle, and yet total dissemination of the tissue is not realised. It
was deemed important to investigate how FoxO1 might slow or reduce growth regulatory pathways in order that tissue maintains structural probity albeit with a reduced function. These later studies elucidated a role for FoxO1 in regulation of components that prime for the induction of translation and initiation of protein synthesis from messenger RNA (mRNA) in muscle tissue.
CHAPTER 2

CONTENTS

Investigation of FoxO1 mediated PGC-1α mRNA expression in Human skeletal muscle biopsy samples following a euglycaemic hyperinsulinaemic clamp procedure

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Evidence is emerging linking muscle insulin resistance with mitochondrial dysfunction (Peterson et al., 2003), and it has recently been reported that genes associated with mitochondrial function are down regulated in patients with Type 2 diabetes (Patti et al., 2003). Co-activators are important intracellular molecules, and although they do not directly bind to DNA, they can modulate gene expression through specific protein-protein interactions (Ichida et al., 2003). One such co-activator receiving much attention due to its hypothesised role in Type 2 diabetes (Attie and Kendziorski 2003) is the peroxisome proliferator-activated receptor $\gamma$ coactivator-1$\alpha$ (PGC-1$\alpha$), which was originally identified as a co-activator of PPAR-$\gamma$ in-vitro (Puigserver et al., 1998). Recent studies have demonstrated a down regulation of PGC-1$\alpha$ mRNA and associated genes in adipose tissue (Semple et al., 2004; Hammarstedt et al., 2003) and skeletal muscle (Patti et al., 2003; Mootha et al., 2003) of insulin resistant humans, although this is not always seen (Hammarstedt et al., 2003). It is suggested that PGC-1$\alpha$ is the hierarchical regulator of the oxidative phosphorylation (OxPhos) genes; Nuclear Respiratory Factor-1 (NRF-1) and mitochondrial transcription factor A (tFAM) which constitute a family of genes responsible for mitochondrial biogenesis and slow twitch (oxidative) muscle fibre expression (Puigserver & Spiegelman 2003). PGC-1$\alpha$ exerts this regulatory role via association with NRF-1, to control expression of tFAM (Lin et al., 2002), the signal through which the nucleus regulates mitochondrial DNA transcription and replication (Gordon et al., 2003). Thus, it is not surprising that suppression of PGC-1$\alpha$ in skeletal muscle could contribute towards insulin resistance via reduced mitochondrial size and number.
The regulation of PGC-1α gene transcription is complex. It is known that calcium (Ca\(^{2+}\)) activates calcineurin (CnA) and calmodulin kinases (CaMK). CnA activates Nuclear Factor of Activated T cells (NFAT) (Shulz & Yutzey 2004) which works in a combinatorial fashion with myocyte enhancer factor 2 (MEF2), (Wu et al., 2001) to regulate oxidative fiber development. MEF2 acts as transcription factor for PGC-1α in a CaMK dependant manner (Czubryt et al., 2003) where CaMK phosphorylates and inhibits DNA binding of the class II histone deacetylases (HDAC). The class II HDAC’s are suggested to negatively regulate PGC-1α via inhibition of MEF2 binding on the PGC-1α promoter (Czubryt et al., 2003). Taken together these previous studies suggest that MEF2 links the CaMK and calcineurin pathways to PGC-1α transcription.

Increased adrenaline can also activate cyclic AMP (cAMP) to phosphorylate a cAMP responsive element-binding protein (CREB), which activates CRE on PGC-1α (Daitoku et al., 2003). Hence, it is likely that in patients with type 2 diabetes, physical inactivity leads to reduced NFAT, MEF2 and CREB binding to the PGC-1α promoter, thereby resulting in the decreased PGC-1α mRNA and downstream genes observed basally in the skeletal muscle of these patients (Patti et al., 2003; Mootha et al., 2003).

There is also a trio of insulin responsive elements on the promoter region of the PGC-1α gene that are activated by FoxO1 (Puigserver et al., 2003; Daitoku et al., 2003). FoxO1 is expressed in insulin sensitive tissues such as skeletal muscle, liver, and adipose tissue (Crunkhorn et al., 2004). In these tissues, FoxO factors play a key role in transmitting insulin signaling downstream of Akt, where insulin negatively regulates FoxO trans-activation in such a way that upon insulin stimulation, Akt phosphorylates FoxO proteins resulting in their cytosolic sequestration (nucleic
exclusion) (Puigserver et al., 2003; Imae et al., 2003; Rena et al., 1999) and subsequent proteosomal degradation (Matsuzaki et al. 2003). In hepatocytes, phosphorylation of FoxO1, by Akt in response to insulin, abolishes FoxO1 binding to PGC-1α and these events reduce the expression of the gluconeogenic genes PEPCK and G-6-Pase (Puigserver et al., 2003). Daitoku et al. (15) recently reported that, in Hep G2 cells, insulin suppresses basal PGC-1α promoter activity and co-expression of constitutively active Akt mimics the effect of insulin. Moreover, co-expression of FoxO1 stimulated the PGC-1α promoter activity via interaction with PGC-1α’s insulin response sequences (IRSs), whilst co-expression of FoxO1 mutant, where the three putative Akt phosphorylation sites in FoxO1 are non-functioning, abolished the suppressive effects of Akt on FoxO1 trans-activation of the PGC-1α promoter (Puigserver et al., 2003; Daitoku et al., 2003).

It is well known that, in healthy skeletal muscle, insulin phosphorylates Akt, a process that can be impaired in the skeletal muscle of patients with Type 2 diabetes (Zierath et al., 1994). Somewhat surprisingly, no previous studies have examined the Akt mediated regulation of FoxO1 and PGC-1α by insulin in skeletal muscle, but this is warranted given the recent importance of PGC-1α in the aetiology of insulin resistance and the fact that skeletal muscle accounts for >80% of insulin stimulated glucose disposal. The work constituting this chapter examined the effect of insulin on Akt and FoxO1 phosphorylation and PGC-1α mRNA expression in skeletal muscle from healthy and insulin resistant humans.
Figure 2.1 Control of PGC1-α transcriptional regulation in skeletal muscle.

Three promoter regions are calcium (Ca\textsuperscript{2+}) and protein kinase A (PKA) responsive. Upon contraction Ca\textsuperscript{2+} activates calcineurin (CnA) and calmodulin kinases (CaMK). CnA activates nuclear factor of activated T cells (NFAT) and myocyte enhancer factor 2 (MEF2), which act as transcription factors for PGC1-α. CaMK phosphorylates histone deacetylases (HDAC), which inhibit the MEF2-responsive element on PGC1-α. Increased adrenaline can activate cyclic AMP (cAMP) to phosphorylate cAMP-responsive element binding protein (CREB), which activates CRE on PGC1-α. There is also trio of insulin-responsive elements on the promoter region. These are activated by the forkhead box class-O (FoxO1) winged helix transcription factor, previously known as forkhead homologue of rhabdomyosarcoma (FKHR). When insulin binds to its receptor and activates PI3K, resulting in Akt phosphorylation, FoxO1 is phosphorylated and excluded from the nucleus. This process is impaired in insulin resistance.

As adapted from Southgate et al., (2005).
METHODS

Subjects

A total of 16 volunteers (7 patients with type 2 diabetes [TYPE 2] and nine healthy but sedentary age-matched control subjects [CON]) participated in this investigation that was approved by the Human Ethics Committee of RMIT University. Normal glucose tolerance was confirmed in the control group [CON] by an oral glucose tolerance test (OGTT; 75 g glucose). TYPE 2 subjects had a mean time of 4 ± 1 yr since diagnosis of disease (range 6 months to 10 yr). All participants had maintained a constant body mass in the 6 months prior to the experiment and each was instructed to abstain from any form of vigorous physical activity for 36 h prior to the hyperinsulinaemia euglycaemic clamp experiment and to maintain their habitual diet. The subjects reported to the laboratory for this experiment following a 12-14 h overnight fast.

Experimental protocol

Patients arrived and rested supine for approximately 30 min. An anticubital vein (infusion of glucose and insulin), and a dorsal hand vein (blood sampling) were subsequently cannulated. The hand was continuously heated in order to sample arterialised blood. After collection of baseline blood samples, a percutaneous biopsy from the vastus lateralis muscle was obtained. After resting for 5 min a primed (9 mU·kg⁻¹) continuous (40 mU·m⁻²·min⁻¹) infusion of insulin (Actrapid, Novo Nordisk, Australia) was commenced and a variable rate infusion of 20% glucose was used to maintain euglycaemia (5 mmol/L⁻¹) for 120 min. The blood glucose concentration in TYPE 2 was allowed to decrease during the insulin infusion to 5 mmol.L⁻¹ and then maintained at this concentration for the remainder of the clamp. A second muscle biopsy was obtained at the
conclusion of the clamp. To prevent a decrease in plasma potassium concentration during the
clamp, 30 mmol KCl (Slow-K; Novartis, Australia) was administered orally.

**RNA extraction**

Muscle tissue was homogenised and extracted for total RNA using the acid guanidium
thiocyanate-phenol-chloroform extraction method (Chomczynski *et al.*, 1987) and modified
according to methods described elsewhere (Febbraio & Koukoulas 2000). RNA samples were
reverse transcribed using a thermal cycler (Perkin Elmer GeneAmp PCR 2400 Thermal Cycler,
Rowville, Victoria, Australia) with TaqMan Reverse Transcription Reagents (Applied
Biosystems, Foster City CA, USA) in 40 µl reaction mixtures containing 1 x TaqMan RT Buffer,
5.5 mM MgCl$_2$, 500 µM 2’-deoxynucleoside 5’-triphosphate, 2.5 µM random hexamers, 0.4U µl$^{-1}$
RNase inhibitor, 1.25 U/µl$^{-1}$ multiscribe reverse transcriptase. Control (reverse transcriptase
negative) samples were also made and included in all future PCR analyses to check for genomic
DNA contamination.

**Real-Time-Polymerase-Chain-Reaction analysis**

Real-time PCR was employed to quantify the genes of interest. All reactions were performed
according to the multiplex comparative critical threshold ($C_T$) method. This method allows for
the detection of the reference gene (ribosomal 18S) and the gene of interest in the same well or
tube. Preliminary experiments were performed to determine the efficiency of amplification of
18S and the gene of interest. PCR reactions were performed (in duplicate) in 96 well plates on a BioRad i-CYCLER iQ RealTime-PCR Detection System in 25 µl reaction volumes consisting of: BioRad iQ™ Supermix PCR mix (2 x; BioRad), Applied Biosystems Pre-Developed Assay Reagent for 18S (Applied Biosystems, Foster City CA, USA), the forward and reverse primers and probes of the genes of interest (see Table 1 for sequences, Appendix 1) and sterile H₂O. Human probe and primers were designed (Primer Express version 1.0 Applied Biosystems, Foster City CA) from the human gene sequence accessed from Gen-Bank/EMBL. The specific probe/primer sequences are reported in Table 1 (Appendix 1).

**Western blot analyses**

Muscle tissue was homogenised (Polytron; Brinkman Instruments, Westbury, NY, USA) in ice-cold buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA, 0.5% Triton X-100, 10% glycerol (v: v), 2 mg.mL⁻¹ leupeptin, 100 mg.mL⁻¹ PMSF, and 2 mg.mL⁻¹ aprotinin. Homogenates were spun in order to pellet cellular debris at 16,000 g for 60 min at 4°C and the supernatant was removed and rapidly frozen in liquid nitrogen. Protein concentration of the muscle lysates was subsequently determined (Pierce, Rockford, IL, USA). Muscle lysates were solubilised in Laemmeli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to a nitrocellulose membrane, blocked with 3% BSA and immunoblotted overnight with total-Akt, phospho-Akt (Ser⁴⁷³), total-FoxO1, phospho-FoxO1 (Ser²⁵⁶) or phospho-FoxO1 (Thr²⁴) antibodies (1:1000; Cell Signaling, Beverley, MA). After incubation with horseradish peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences, Castle Hill, NSW, Australia), the immunoreactive proteins were detected
with enhanced chemiluminescence (Perkin Elmer, Rowville, Victoria, Australia) and quantified by densitometry. All phospho-proteins were expressed as a ratio of phospho-to-total protein.

**Maximal enzyme activities**

Citrate synthase (CS) and β-hydroxyacyl CoA dehydrogenase (β-HAD) activities were analyzed by homogenising 5-10 mg of skeletal muscle in 1:50 dilution (w/v) of a 175 mM potassium buffer solution and measuring the disappearance of NADH spectrophotometrically at a constant temperature of 25°C according to the methods of Lowry and Passonneau (1972).

**Statistical analysis**

Data are presented as mean ± Standard Error of Mean (SEM). Statistical analysis consisted of a paired t-test for figure 2.2, one-way ANOVA for figure 2.3 and two-way ANOVA (condition x treatment) for all other figures. Significance was accepted if $P \leq 0.05$. Where analyses revealed a significant interaction, a Newman Keuls post-hoc test was used to locate specific differences. All statistical analyses were performed with the application of Statistica for Windows (Statsoft, Statistica® Tulsa OK).
RESULTS

Subject characteristics

The subject characteristics are presented in Table 2 (Appendix 1). As expected fasting blood glucose, insulin, FFA and HbA\textsubscript{1c} were all higher (P<0.05) in Type 2 diabetic (TYPE 2) participants when compared with healthy control (CON) participants. Neither blood glucose nor plasma insulin levels during the clamp differed when comparing TYPE 2 with CON.

Glucose infusion rate

As expected, glucose infusion rate (GIR) was lower (P<0.05) in TYPE 2 compared with CON averaging 2.0 \pm 0.1 and 7.9 \pm 0.9 mg.kg\textsuperscript{-1}.min\textsuperscript{-1} respectively (Figure. 2.2).

Basal mRNA expression

The basal mRNA expression of PGC-1\textalpha and downstream metabolic and mitochondrial genes are presented in Figure 2.3. The mRNA expression in the muscle biopsy specimens from the TYPE 2 cohort was expressed relative to CON (Con = 1). The mRNA expression of PGC-1\textalpha, COX4, tFAM, and NRF-1 were all lower (P<0.01) in TYPE 2 compared with CON. Although not significant, the expression of FoxO1 (P=0.10) and PSARL (P=0.16) tended to be lower in TYPE 2 compared with CON.

Mitochondrial enzyme activity

CS maximal activity was lower (P<0.05) in TYPE 2 compared with CON averaging 3.6 \pm 0.3 and 5.5 \pm 0.4 \mu mol.g\textsuperscript{-1}.min\textsuperscript{-1} respectively. In contrast, \beta-HAD activity was not different when
comparing TYPE 2 with CON averaging 5.0 ± 0.7 and 6.0 ± 0.7 µmol.g⁻¹.min⁻¹ respectively (see Figure 1 of Appendix 1).

To determine if insulin resistance was associated with an index of mitochondrial capacity, we performed correlations between GIR and maximal activity of CS (see Figure 2 of Appendix 1). This revealed a significant correlation (r=0.62, P=0.01) (data not shown). To assess whether basal mitochondrial mRNA expression was related to mitochondrial capacity, we also performed correlations between mRNA expression and maximal activity of CS. Taking note that a higher delta CT is indicative of lower mRNA expression, tFAM mRNA was inversely correlated with CS (r=-0.52; P=0.04). Although not statistically significant, there was a tendency for NRF-1 to be correlated with CS (r=0.44; P=0.09) (see Appendix 1 for data).

**Basal & insulin stimulated intracellular signaling**

There was no difference in basal phosphorylated Akt when comparing CON with TYPE 2 (Figure 2.4). However, whilst insulin increased (P<0.05) Akt phosphorylation in CON, this was not the case in TYPE 2 (Figure 4). Basal phosphorylation of FoxO1 on serine residue 256 (Ser²⁵⁶) was higher (P<0.05) in CON compared with TYPE 2 (Figure 2.5). Insulin did not increase phosphorylation at this residue in either group *in-vivo* (Figure 2.5). Basal phosphorylation of FoxO1 on Threonine residue 24 (Thr²⁴) was not different when comparing groups in the basal state *in-vivo* (Figure 2.6). However, whilst insulin stimulation increased (P<0.05) FoxO1 (Thr²⁴) phosphorylation in CON, it did not do so in TYPE 2 (Figure 2.6).
**Insulin stimulated mRNA Expression**

The insulin stimulated mRNA expression of PGC-1α and downstream metabolic and mitochondrial genes *in-vivo* are presented in Figure 2.7. The *in-vivo* mRNA abundance of the insulin stimulated expression (post-clamp) was expressed relative to basal (pre-clamp) for both CON and TYPE 2. We observed, *in-vivo*, that insulin decreased (P<0.05) the mRNA expression of PGC1-α, COX4, and NRF-1 in CON. In contrast, insulin did not repress the expression of PGC1-α, COX4, and NRF-1 in TYPE 2.
Figure 2.2. Glucose Infusion Rate- human hyperinsulinaemic-euglycaemic clamp experiments. Mean glucose infusion rate during a 120 min euglycemic, hyperinsulinemic clamp in patients with type 2 diabetes (TYPE 2) and healthy control subjects. *Difference \((P<0.05)\) compared with CONTROL. Data are mean ± SE.
Figure 2.3. Basal gene (mRNA) expression prior to hyperinsulinaemic-euglycaemic clamp procedure.
Basal mRNA expression of protein peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), nuclear encoded cytochrome oxidase (COX4), forkhead box class-O (FoxO1), mitochondrial transcription factor A (tFAM), presenilins-associated rhomboid-like protein (PSARL), and nuclear respiratory factor-1 (NRF-1) in patients with type 2 Diabetes expressed relative to Control subjects. *Difference (P<0.05) compared with CONTROL. Data are mean ± SE.
Figure 2.4. Western blot analysis of Akt phosphorylation pre & post hyperinsulinaemic-euglycaemic clamp procedure.
Pre (BASAL)- and post (INSULIN)-clamp, expression of phosphorylated Akt (Ser$^{473}$) in patients with type 2 diabetes (TYPE 2) and healthy control subjects. *Difference ($P<0.05$) compared with CON (insulin). †Difference ($P<0.05$) compared with basal. Data are mean ± SE. Phospho-Akt. (Ser473) expressed as a ratio of total Akt.
Figure 2.5. Western blot analysis of FoxO1 (Ser^{256}) phosphorylation pre & post hyperinsulinaemic-euglycaemic clamp procedure.

Pre (BASAL)- and post (INSULIN)-clamp, expression of phosphorylated FoxO1 (Ser256) in patients with type 2 diabetes (TYPE 2) and healthy control subjects. *Difference (P<0.05) compared with CON . Data are mean ± SE. Phospho-FoxO1 (Ser256) expressed as a ratio of total FoxO1.
Figure 2.6. Western blot analysis of FoxO1 (Thr^{24}) phosphorylation pre & post hyperinsulinaemic-euglycaemic clamp procedure. Pre (BASAL)- and post (INSULIN)-clamp, expression of phosphorylated FoxO1 (Thr24) in patients with type 2 diabetes (TYPE 2) and healthy control subjects. *Difference (P<0.05) compared with CON. †Difference (P<0.05) compared with basal. Data are mean ± SE. Phospho-FoxO1 (Thr24) expressed as a ratio of total FoxO1.
Figure 2.7. **Insulin gene (mRNA) expression post clamp- insulin stimulated.** Insulin stimulated (post-clamp relative to pre-clamp, preclamp = 1) mRNA expression of protein peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), nuclear encoded cytochrome oxidase (COX4), forkhead box class-O (FoxO1), mitochondrial transcription factor A (tFAM), presenilins-associated rhomboid-like protein (PSARL), and nuclear respiratory factor-1 (NRF-1) in patients with type 2 diabetes (TYPE 2) and healthy control subjects *Difference (P<0.05) compared with all other conditions. Data are mean ± SE.*
DISCUSSION

The aim of this chapter was to determine if the regulation of PGC-1α and associated OxPhos gene (genes hypothesized to be essential for mitochondrial biogenesis) expression, by insulin, is similar in skeletal muscle to those data reported in the literature, in the liver, i.e. the objective was to investigate if insulin’s regulation of PGC-1α mRNA in a FoxO1 dependant manner is conserved in skeletal muscle.

The data obtained suggests that there is conservation between these two tissue types and indicates that the same signaling intermediates Akt, and FoxO1 have role in these processes. A confirmatory aspect of these initial data (the data that constitutes chapter 2) is realised via the observation that even in the small cohort studied, PGC-1α mRNA was reduced in the basal state in the context of type 2 diabetes (i.e. when comparing healthy non-diabetic individuals to diabetic individuals). This is in agreement with the data of Patti et al. (2003), and Mootha et al. (2003). Interestingly, the data of Patti et al., (2003), and Mootha et al. (2003) utilised gene set enrichment techniques a costly and time consuming means of identifying potential gene targets associated with the diabetic phenotype. Here, the data were obtained via standard RT-PCR techniques and as such adds support to the notion that PGC-1α mRNA quantification could in the future, be used as means of assessing an individuals diabetic status. Clearly, more experimental research in diabetic individuals needs to be undertaken before PGC-1α can be deemed a bone fide marker for Type 2 diabetes and insulin resistance, but it appears that PGC-1α has the potential to be used as marker of the diabetic phenotype in muscle tissue.
The data reported by Patti et al. (2003) also indicated that NRF-1 (a downstream gene target of PGC-1α) was also reduced basally in Type 2 diabetic muscle and first-degree relative of type 2 diabetic patients. Again, the data presented in chapter 2 confirm the findings of Patti et al. (2003), and support the hypothesis that a repertoire of mitochondrial biogenesis related genes are dysregulated in Type 2 diabetes and that PGC-1α acts as a hierarchical regulator of OxPhos gene expression.

The novel data presented here indicate that PGC-1α mRNA levels were not repressed post insulin stimulation in the patients with type 2 diabetes in contrast with the healthy individuals. The exact physiological consequence or role for this relative non-repression in the diabetic group remains to be elucidated.

The aim of the experiments described in this Chapter was not to assess if PGC-1α has a role in protection from oxidative stress. However, in an attempt to explain the physiological context of my findings, it is interesting to note that experiments recently conducted in endothelial cells suggest that PGC-1α over expression may serve to play a critical, protective role in vascular complications via its involvement in the transcriptional regulation of the mitochondrial antioxidant defense system (Valle et al., 2005). Also of interest, Manganese Superoxide Dismutase (MnSOD) an anti-oxidant defense gene has been proposed to be a transcriptional targeted of FoxO1 (Kops et al., 2002a). Although MnSOD was not measured in this study, the fact that it is a transcriptional target of FoxO1 adds support to the notion that FoxO1 and PGC-1α
may both function to afford some protection during times of increased oxidative stress and therefore their loss of sensitivity to insulin may serve a functional purpose in diabetes.

In this regard, Valle et al. (2005) propose that high levels of PGC-1α may protect against the accumulation of reactive oxygen species (ROS) formation, and by virtue, reduce the likelihood of inducing apoptotic cell death pathways. The authors support this claim via the observation that the PGC-1α mRNA levels were reduced with small interfering RNA (siRNA). In cells where PGC-1α was knocked-down, the cells exhibited reduced expression of mitochondrial detoxification proteins.

Whilst data from the literature suggests that FoxO1 promotes oxidative stress protection, and with consideration that the data described in this Chapter indicates that FoxO1 maintains a nucleic localisation via a reduced phosphorylation and thus maintains its transcriptional activity, it is clear that the suggested role that FoxO1 might have in protection from oxidative stress induction is insufficient to ameliorate the negative effects of oxidative stress on insulin sensitivity, if oxidative stress is the main antagonist in insulin resistance.

In conclusion, the data presented suggest that unlike healthy, insulin sensitive humans, patients with type 2 diabetes are unable to down regulate PGC-1α mRNA and downstream OxPhos genes in their skeletal muscles in response to insulin. This appears due to the inability for insulin signaling to phosphorylate FoxO1 and shuttle it from the nucleus to the cytosol. The exact
physiological consequence of this lack of responsiveness of OxPhos genes to insulin remains unclear, but could be related to cellular responses that are induced in an attempt to ameliorate the damaging effects of oxidative stress.
CHAPTER 3

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Investigation of FoxO1 mediated PGC-1α mRNA expression in Human primary skeletal muscle cell-culture following experimentally Induced insulin resistance.

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CHAPTER 3
INTRODUCTION

In the previous chapter, the role of FoxO1 on insulin mediated suppression of PGC-1α mRNA was examined in human biopsy specimens. In the present chapter, cell culture experiments were designed in order to determine if the actions of insulin on FoxO1 mediated PGC-1α mRNA expression were conserved in a better controlled cell culture skeletal muscle model. A cell culture approach was deemed appropriate as it was felt that this model would allow for the amelioration of the confounding variables involved in in-vivo experimentation, those being circadian oscillatory variation and the hormonal and metabolic changes in the environmental milieu that is associated with in-vivo physiology. Furthermore, I was unable to examine the nuclear abundance of total FoxO1 protein in human muscle (Chapter 2) due to insufficient tissue sample size, however, the use of the cell culture model described in this chapter would allow for assessment of nucleic FoxO1 abundance in-vitro.

The literature suggested that endogenous PGC-1α mRNA expression is low in commonly utilized, commercially available animal derived muscle culture systems (Handschin et al., 2003; St-Pierre et al., 2003). To address this issue without resorting to transfection studies utilising exogenous PGC-1α plasmid DNA, which it was felt, would not provide a true analysis of FoxO1’s role in insulin mediated suppression of PGC-1α mRNA expression, human primary skeletal muscle myotube cultures were propagated from biopsy samples.

Increased lipid storage within insulin sensitive tissues appears to be a central observation in the literature investigating the induction of insulin resistance in skeletal muscle in-vivo (Shulman
Fatty acids have been shown to induce insulin resistant characteristics in *in-vitro* cell culture conditions (Schmitz-Peiffer *et al*., 1999; Storz *et al*., 1999), however this isn’t observed in all cell types (Lundgren & Eriksson 2004; Kausch *et al*., 2003). Given that there is a strong association between lipid accretion in muscle and insulin resistance (Shulman 2000; Reynoso *et al*., 2003; Schmitz-Peiffer *et al*., 1999; Storz *et al*., 1999) both *in-vivo* and *in-vitro* and the work of Patti *et al*. (2003) and Mootha *et al*. (2003) suggests that *in-vivo* PGC-1α expression is reduced in type 2 diabetics (Patti *et al*., 2003; Mootha *et al*., 2003) and first degree relatives of type 2 diabetics patients (Patti *et al*., 2003), it seemed reasonable to investigate if the saturated fatty acid palmitate could induce repression in PGC-1α *in-vitro*.

Thus, the rationale for the experiments described in this chapter were: 1) to investigate if the saturated fatty acid palmitate could repress PGC-1α mRNA levels basally; and 2) investigate if palmitate treatment mirrors the perturbations imposed on the insulin signaling cascade observed in the previous chapter (Chapter 2) of this thesis with regard to reduced insulin stimulated FoxO1 phosphorylation and non-repression of PGC-1α mRNA.
METHODS

Primary human skeletal muscle cell culture

Muscle tissue from the *vastus lateralis* was obtained from young, healthy, insulin sensitive subjects via percutaneous needle biopsy and immediately transferred to ice-cold Ham F-10 media (Gibco, Brooklyn, NY, USA). The tissue was washed three times in ice-cold Ham-F10 media to remove any traces of blood, and placed over ice in a sterile Petri dish. The tissue was covered with ~ 3 ml of 0.05% Trypsin/EDTA and minced. The homogenate was collected, diluted in 15 ml of 0.05 % Trypsin/EDTA, agitated for 20 min and the supernatant collected and stored on ice. This procedure was repeated three times, the supernatants were pooled, filtered and then spun for 7 min at 1600 g at room temperature. The cell pellet was re-suspended in growth media (Alpha MEM with 10% FCS, 0.5% penicillin-streptomycin, 0.5% Amphotericin B) and placed in an uncoated 75 cm² cell culture flask and incubated for 30 min at 37°C (95% O₂/5% CO₂) to allow attachment of fibroblasts. The supernatant was collected and placed in another flask pre-coated with ECM and incubated as above for 24 h. Growth media was replenished every 48 hrs thereafter. Satellite cells appeared after ~ 7 days and were allowed to reach 60-70% confluence, at which point they were sub-cultured into separate dishes. When the myoblasts reached 70% confluence (approx 4-6 days), the growth media was replaced with differentiation media (2% HS, 0.5% penicillin-streptomycin, 0.5% Amphotericin B). Myoblasts were maintained in differentiation media until fully fused myotube cultures were obtained.
Cell treatments and analytical measures.

Myotube cultures were incubated in either vehicle ([VEH], 4% BSA [Sigma], 2% horse serum, 0.5% penicillin-streptomycin, 0.5% Amphotericin B cocktail) or palmitate ([PALM], 4% BSA [Sigma], 0.75 mM palmitic acid [Sigma], 2% horse serum, 0.5% penicillin-streptomycin, 0.5% Amphotericin B cocktail) media for 8 h. After this time, cells were serum starved for 4 h and experiments performed. For insulin treatment, the appropriate cultures were stimulated with 50 nM insulin for a period of 30 min for phospho-protein analysis or 8 h for mRNA analysis. For western blot analyses of total cell lysates, cells were lysed with ice-cold lysis buffer consisting of 50 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM Na$_3$VO$_4$, 5 mM EDTA, 0.5% Triton X-100, 10% glycerol (v: v), 2 µg.ml$^{-1}$ leupeptin, 100 µg.ml$^{-1}$ phenylmethylsulfonyl fluoride (PMSF), and 2 µg.ml$^{-1}$ aprotinin and snap frozen in liquid nitrogen. For mRNA analyses, myotube cultures were lysed with 400ul RNAbee (Tel-Test, Friendswood, TX) and the lysate snap frozen in liquid nitrogen and stored at -80°C until subsequent extraction.

RNA extraction and PCR analyses

RNA Extraction and Real-Time-Polymerase-Chain-Reaction analysis were procedures were undertaken as detailed in the methods section of Chapter 2 (RNA Extraction and Real-Time-Polymerase-Chain-Reaction analysis).

Western blot analyses of whole cell lysate

Preparation of whole cell lysate and Western blot analyses of whole cell lysate were undertaken as described in Chapter 2.
Nucleic protein isolation

Nuclear proteins were extracted from myotubes using a technique described previously (Chan et al., 2004). Briefly, myotubes were homogenised in 500 µl of pre-chilled Buffer A (250 mM sucrose, 10mM Tris pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM PMSF, and 2 ml/30 mg tissue protease inhibitor cocktail). The homogenate was spun at 500 g for 5 min at 4°C. The supernatant containing cytosolic materials (crude fraction) was then removed and stored at -80°C until required. Then 500 µl of pre-chilled Buffer B (50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50mM MgCl2, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM DTT, 1 mM PMSF, 2 ml/30 mg-1 tissue protease inhibitor cocktail) was added to re-suspend the remaining pellet. After 10 min incubation on ice with occasional mixing, the pellet mixture was then spun for 5 min at 3000 g at 4°C. The supernatant representing the nuclear fraction was extracted and stored at -80°C until required. Fraction purity was assessed by western blotting on Histone H1 as described previously (Chan et al., 2004).

2-Deoxyglucose uptake

Cells were serum starved overnight in α-MEM with 0.1% foetal bovine serum and treated with either vehicle or palmitic acid supplemented media. Following treatment, cells were equilibrated for 30 min in glucose free D-MEM and 0.1% BSA. Cells were then incubated in the presence or absence of insulin (50 nm) or 30 min after which the ³H-2-deoxyglucose solution was added for 30 min. Cytochylastin B was added to a well from each plate to determine basal glucose uptake. The reaction was stopped by the addition of ice cold PBS and 0.1mM Phloretin. Cells were washed two times in ice cold 1 x PBS then lysed in 0.1% SDS. 400ul of lysate was added to
scintillation vials containing 4 ml of scintillation fluid, $^3$H concentration was then determined in a beta spectrometer. Ten µl of the remaining lysate was used to determine total protein concentration via Bradford method using Pierce reagents (A: B: C; 50: 49: 1. Perbio, Rockford, IL) and BSA for standard curve.

**Statistical Analysis**

Data are presented as mean ± Standard Error of Mean (SEM). Statistical analysis consisted of a paired t-test for Figure 3.2 & 3.7, and two-way ANOVA (condition x treatment) for all other figures. Significance was accepted if $P \leq 0.05$. Where analyses revealed a significant interaction, a Newman Keuls post-doc test was used to locate specific differences. All statistical analyses were performed with the application of *Statistica* for Windows (Statsoft, Statistica®Tulsa OK).
RESULTS

2-Deoxyglucose uptake

2-deoxyglucose uptake was lower in insulin stimulated palmitate pre-treated myotubes compared with vehicle treated myotubes (Figure 3.1)

Basal mRNA expression

Consistent with the data in humans (Chapter 2), insulin stimulated mRNA expression of PGC-1α was lower (P<0.01) in palmitic acid treated (PALM) compared with vehicle (VEH) treated cells (Figure 3.2).

Basal and insulin stimulated intracellular protein phosphorylation

Consistent with the data obtained from human skeletal muscle, there was no difference in basal phosphorylated Akt when comparing VEH with PALM treated myotubes (Figure 3.3), insulin increased (P<0.05) Akt phosphorylation in VEH but not PALM treated myotubes (Figure 3.3). Basal phosphorylation of FoxO1 on (Ser$^{256}$) was higher in VEH compared with PALM myotubes (Figure 3.4). In-vitro, insulin increased (P<0.05) FoxO1 (Ser$^{256}$) phosphorylation in VEH but not PALM treated cells (Figure 3.4). Basal phosphorylation of FoxO1 (Thr$^{24}$) residue was not different when comparing groups in the basal state (Fig. 3.5) in-vitro. However, whilst insulin stimulation increased (P<0.05) FoxO1 (Thr$^{24}$) phosphorylation in VEH, it did not do so in PALM treated myotubes in-vitro (Figure 3.5). Insulin treatment decreased (P <0.05) the nuclear abundance (insulin induced cytosolic sequestration) of FoxO1 total protein in VEH, but not in PALM treated cells (Figure 3.6).
**Insulin stimulated mRNA expression**

The insulin stimulated mRNA expression of PGC-1α *in-vitro* is presented in Figure 3.7. The *in-vitro* mRNA abundance of the insulin stimulated expression (post stimulation) was expressed relative to basal (pre-stimulation) for both VEH and PALM, respectively. It was observed *in-vitro* that insulin decreased (P<0.05) the mRNA abundance of PGC-1α in VEH treated myotubes, but this was not the case in PALM treated cells.
Figure 3.1. *In-vitro* $^3$H-2-Deoxyglucose uptake in human primary skeletal muscle myotubes treated without and with palmitic acid
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Figure 3.5. Western blot analysis of FoxO1 (Thr^{24}) phosphorylation pre and post insulin, with and without palmitic acid treatment of human primary skeletal muscle myotubes. BASAL and INSULIN stimulated expression of phosphorylated FoxO1 (Thr^{24}) in vehicle (VEH) or palmitate (PALM) treated human primary skeletal muscle myotubes. *Difference (P<0.05) compared with VEH. †Difference (P<0.05) compared with basal. Data are mean ± SE. Phospho-FoxO1 (Thr24) expressed as a ratio of total FoxO1.
Figure 3.6. Nucleic abundance of FoxO1 protein pre a post insulin, with and without palmitic acid treatment of human primary skeletal muscle myotubes. BASAL and INSULIN stimulated nuclear abundance of total FoxO1 protein in human primary skeletal muscle myotubes treated with vehicle (VEH) or palmitate (PALM). *Difference (P<0.05) compared with VEH. †Difference (P<0.05) compared with basal. Data are mean ± SE.
Figure 3.7. PGC-1α gene expression in human primary skeletal muscle myotubes post insulin stimulation in presence of palmitic acid. Insulin stimulated (post insulin relative to pre-insulin, pre-insulin=1) mRNA expression of PGC-1α in primary human skeletal muscle myotubes treated with vehicle (VEH INSULIN) or palmitate (PALM INSULIN).

*Difference ($P<0.05$) compared with preclamp or pre-insulin. Data are mean ± SE.
DISCUSSION

The objective of this chapter was to investigate if the effects of insulin stimulation on PGC-1α gene expression were regulated in a similar way to those observed *in-vivo*, with the application of an *in-vitro* human primary skeletal muscle reductionism model of insulin resistance.

In order to try and ameliorate the confounding effects of species’ differences, human primary myoblast cultures were propagated from muscle biopsy specimens obtained from healthy non-insulin resistant subjects. Part of the rationale for using healthy subjects to obtain the muscle cultures, as opposed to insulin resistant subjects was that it was deemed appropriate to induce the insulin resistant state, in culture, with an endogenous compound. This approach was employed as it better compares with the *in-vivo* situation with regard to the onset of insulin resistance i.e. healthy cultures, in which the insulin signaling cascade was patent, were induced towards an insulin resistant state with the saturated fatty acid; palmitate. These considerations were particularly pertinent given the findings of Krutzfeldt *et al.*, (2000), who reported that that insulin action and signaling in cultured skeletal muscle cells from normoglycaemic lean insulin-resistant subjects was not different from that in cells from insulin-sensitive subjects. This suggests an important role of environmental factors in the development of insulin resistance in skeletal muscle.

A lipocentric (lipid induced) paradigm with regard to the aetiology of insulin resistance has emerged in the literature over the past few years. Moreover, this paradigm has become a predominant hypothesis to explain the onset of insulin resistance in skeletal muscle. In this
regard, an early study conducted in C2C12 myotubes (Schmitz-Peiffer et al., 1999) reported lipid induced inhibition of insulin-stimulated signal transduction and glucose metabolism. This study reported a reduction in the activation of the insulin stimulated signaling intermediate Akt following 18 h of palmitate pre-incubation. Interestingly, these authors concluded that palmitate treatment induced a down-regulation of insulin stimulated Akt activation independently of any changes in upstream signaling, namely IRS-1. Storz et al. (1999) reported a down-regulation of insulin stimulated Akt activation (phosphorylation), GLUT4 translocation and glucose uptake in muscle cells pre-incubated with palmitic acid. Interestingly these observations (in contrast to those of Schmitz-Peiffer et al. 1999), also reported perturbations in insulin signaling at the level of IRS-1 activation (phosphorylation). The data related to IRS-1 activation and palmitate treatment by Storz et al. (1999), were subsequently confirmed by Reynoso et al. (2003) in a murine model of lipid induced insulin resistance.

It is noteworthy that palmitate treatment does not always result in perturbation in insulin signaling and/or glucose uptake in-vitro (Kausch et al. 2003) and in-vivo (Lovejoy et al. 2002). Of note, this latter study (Lovejoy et al., 2002) investigated palmitate induced insulin resistance in-vivo and found that the treatments did not influence insulin sensitivity in lean individuals, but did have a negative impact on insulin sensitivity in overweight individuals. This again suggests that environmental factors play a significant role in the development of insulin resistance especially in the context of an already predisposing phenotype (i.e. overweight).

The finding detailed in Chapter 3, support and confirm those data reported in the literature that suggest a role for saturated fatty acid induction of insulin resistance. In terms of metabolic parameters, the data presented here clearly show that palmitate pre-treatment reduces insulin
stimulated glucose uptake, following a relatively acute pre-incubation. The data presented here consolidate and expand on the literature as the data clearly shows an effect of palmitate that result in changes in transcriptional regulation of the PGC-1α, the hierarchical gene responsible for mitochondrial biogenesis.

The observations that expand upon previous knowledge are those data that provide evidence of palmitate’s propensity to reduce PGC-1α gene expression basally (i.e. in the fed, non-insulin stimulated, state) *in-vitro*. This suggests that palmitate treatment is indeed a good means of experimentally inducing the insulin-resistant state, as the repression *in-vitro* compares well with the basal PGC-1α expression observed in type 2 diabetic subjects (Chapter 2), and those data reported in the literature (Mootha *et al.*, 2004, Patti *et al.*, 2003). As was the case in the healthy non-diabetic subjects, insulin repressed PGC-1α mRNA in control cultures, but failed to do so in palmitate treated myotubes. The reason for this non-repression of PGC-1α appeared to be related to an increased nucleic abundance of the Akt substrate FoxO1. A discussion of the physiological relevance of this observation constitutes a significant part of Chapter 7.

Of note, a recent publication provides evidence that the over-expression of PGC-1α (Muira *et al.*, 2006) results in degradation of muscle tissue and, moreover, this degradation is the result of adipocyte infiltration within muscle bed of the transgenic mice. It would appear that it is the regulation of PGC-1α at ‘normal’ levels is of key importance if normal muscle physiology is to be maintained since repression of PGC-1α is associated with diabetes and insulin resistance, whereas over-expression of PGC-1α mimics Luft’s disease, a disease associated with uncoupling of oxidative phosphorylation. In summary, the results from this chapter confirm the observations
presented in humans that show that insulin resistance is associated with a failure of insulin to suppress PGC-1α mRNA expression, likely due to an inability for FoxO1 to be phosphorylated and sequestered from the nucleus to the cytosol.
CHAPTER 4

CONTENTS

Investigation of FoxO1 mediated PGC-1α and atrophic mRNA expression in transgenic mice with muscle specific genetic ablation of Akt 2

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CHAPTER 4
INTRODUCTION

The activity of the signaling intermediate Akt is essential for the insulin stimulated negative regulation of FoxO1 induced gene expression. Akt 1 isoform and Akt 2 isoform knockout mice have been generated and described in the literature (Cho et al., 2001a; Cho et al., 2001b; Chen et al., 2001). The original report by Cho et al., (2001a) suggested that Akt 2 knockout mice are insulin resistant and exhibit a diabetes mellitus like syndrome. In contrast, whilst ablation of Akt1 resulted in similar growth retardation (Cho et al., 2001b, Chen et al., 2001) to that reported in Akt 2 null mice (Cho et al., 2001a), Akt 1 ablation did not result in a perturbation of insulin sensitivity or regulation of glucose homeostasis.

Given that the data described in Chapters 2 & 3 suggested that insulin stimulated Akt phosphorylation was reduced in: 1) the diabetic subjects from the in-vivo human clamp experiments (Chapter 2); and 2) in the palmitate treated in-vitro human primary skeletal muscle culture experiments (Chapter 3) it seemed reasonable to utilize the Akt 2 transgenic model described by Cho et al., (2001a) to further support the main findings described in Chapters 2 and 3. In this regard, it was hypothesized that Akt 2 transgenic mice may display similar attributes to both the diabetic subjects (from Chapter 2) and the palmitate treated human skeletal muscle myotubes with regard to the FoxO1 mediated regulation of PGC-1 alpha mRNA in response to insulin stimulation (Chapter 3).
Additionally, reports in the literature (Kamei et al., 2004) at the time of conducting initial experiments in the Akt 2 knockout experiments suggested that transgenic FoxO mice (FoxO over-expressed in muscle) shared similar phenotypic features with the Akt animals. In this regard, these lines of transgenic mice are both smaller in size and they both exhibit a certain degree of insulin resistance compared with their littermate controls (Cho et al., 2001a; Kamei et al 2004). It was deemed appropriate to examine the expression of genes associated with muscle atrophy (MAFbx and Murf1) in order to determine whether there were common effectors of this response. The rationale for this approach was one of wanting to set the stage for the work detailed in Chapter 5. It was also deemed appropriate to analyze a gene (Raptor) that is potentially repressed by FoxO (Jia et al., 2004). DAF 15 is repressed by DAF 16, the C.elegans orthologs of Raptor and FoxO1 respectively (Jia et al., 2004). Evidence to date suggests that Raptor is required for propagation of the translation/initiation pathway and as such its expression may correlate with tissue mass (Hara et al., 2002; Sarbassov et al., 2002).

Since the general consensus in the literature suggests that phosphorylation of FoxO1, and thus, repression of its transcriptional activity is Akt dependant we hypothesized that Akt 2 knockout animals would exhibit a reduced ability to suppress PGC-1α mRNA expression levels in the insulin stimulated state.
METHODS

Generation and experimental treatment of transgenic mice
Akt2 knockout mice were developed as previously described (Cho et al., 2001a). Akt2 -/- and wild type littermate control mice were fasted overnight and the following morning injected with 1 mU/g $^{-1}$ body mass insulin into the intraperitoneal space. Mice were then anesthetized (50 mg.kg $^{-1}$ pentobarbital) and gastrocnemius muscle was harvested and snap frozen in liquid nitrogen, 20 min post insulin injection.

Protein extraction and western blot analyses
Muscle homogenate preparation and western blot analyses were undertaken as described in Chapter 2.

Nucleic protein isolation
Nucleic protein isolation was undertaken as described in Chapter 3.

mRNA extraction and RT-PCR analyses
RNA extraction and real-time polymerase chain reaction analysis for mRNA abundance were undertaken as described in Chapter 2.
Statistical Analysis

Data are presented as mean ± Standard Error of Mean (SEM). Statistical analysis consisted of a paired t-test for figures 4.1, 4.2, 4.5, 4.6 & 4.7, and two-way ANOVA (condition x treatment) for all other figures. Significance was accepted if $P \leq 0.05$. Where analyses revealed a significant interaction, a Newman Keuls post-hoc test was used to locate specific differences. All statistical analyses were performed with the application of Statistica for Windows (Statsoft, Statistica® Tulsa OK).
RESULTS

Basal and insulin stimulated mRNA expression

In agreement with the in-vivo and in-vitro data, the basal mRNA expression of PGC-1α was lower (P<0.01) in Akt 2 -/- compared with WT mice (Figure 4.1). Moreover, insulin stimulation decreased (P<0.05) the mRNA expression of PGC-1α in WT, but failed to do so in Akt 2 -/- mice (Figure 4.2). The basal expression of these genes associated with atrophy (MAFbx and Murf-1) was determined in order to assess the basal effect of knocking out Akt on these parameters. The effect of insulin on expression of the atrogenes; MAFbx and Murf 1, and one of the upstream regulators of translation/initiation; Raptor, were also assessed at the mRNA level. As can be seen in Figure 4.5, basal expression of Murf-1 and MAFbx were significantly higher (p<0.05) in the Akt2 -/- mouse muscle. Concomitantly, basal Raptor mRNA expression was significantly lower in Akt2 -/- mouse muscle (Figure 4.7A). The insulin stimulated MAFbx mRNA expression was not different between WT and Akt2 -/- animals, however, Murf-1 mRNA was significantly reduced (P <0.05) in WT but not Akt2 -/- mice post insulin stimulation. Insulin stimulation induced a significant increase (P<0.05) in Raptor mRNA (figure 4.7B) in WT but failed to elicit increases in Raptor mRNA in muscles from Akt2 -/- mice.

Basal and insulin stimulated intracellular protein phosphorylation

As expected, insulin phosphorylated (P<0.05) Akt in wild type but not Akt2 -/- mice (Figure 4.2A). Likewise, insulin phosphorylated (P<0.05) FoxO1 (Ser^{256}) (Figure 4.2B) and FoxO1 (Thr^{24}) (Figure 4.3A) in wild type but not Akt2 -/- mice. Accordingly, insulin reduced (P<0.05)
the nucleic abundance of FoxO1 in wild type, but not Akt2 -/-, mice (Figure 4.3B). The protein abundance of Raptor was significantly lower (P<0.05) in Akt 2 -/- muscle upon insulin stimulation (Figure 5.8) when compared to WT mouse muscle, and there was a tendency for a reduction in basal protein expression. In WT mouse muscle, insulin elucidated a three-fold increase in Raptor protein, whereas Raptor abundance in Akt -/- mice did not change. As a proxy measure of Raptor activity, we measured the downstream kinase p70S6K (Figure 5.9). There was no difference in total p70S6K abundance basally or upon insulin stimulation (Figure 5.9), however, phosphorylation of p70S6K (Thr$^{389}$) was significantly increased in wild type muscle, but not Akt -/- muscle
Figure 4.1. Basal (prior to insulin stimulation) PGC-1α mRNA expression in muscle of wildtype and Akt 2 muscle specific knockout mice. Basal PGC-1α mRNA expression in wild-type (WT BASAL) and Akt knockout (KO BASAL) muscle. *Difference (P<0.05) compared with WT.
Figure 4.2. PGC-1α mRNA expression in muscle post insulin treatment of wildtype and Akt 2 muscle specific knockout mice. Insulin stimulated PGC-1α mRNA expression in wildtype (WT INSULIN) and Akt knockout (KO INSULIN) skeletal muscle. *Difference (P<0.05) compared with WT.
Figure 4.3 Akt (Ser\textsuperscript{473}) [A] & FoxO1 (Ser\textsuperscript{256}) [B] phosphorylation pre (basal) and post (insulin) insulin treatment of wildtype and Akt 2 muscle specific knockout mice. Phosphorylation of Akt (Ser473), FoxO1 (Ser256) in basal (BASAL) and insulin stimulated (INSULIN) gastrocnemius skeletal muscle obtained from wild-type (WT) and Akt2 knockout (Akt2 \(-/-\)) mice. *Difference \((P<0.05)\) compared with WT. †Difference \((P<0.05)\) compared with basal. Data are mean ± SE. Phospho-proteins (Phospho-Akt, FoxO1 Ser) expressed as a ratio of total-Akt and FoxO1 proteins respectively.
FIGURE 4.4. FoxO1 (Thr<sup>24</sup>) phosphorylation [A], and total nucleic abundance of FoxO1 [B] pre (basal) and post (insulin) insulin treatment of wildtype and Akt 2 muscle specific knockout mice. Phosphorylation of FoxO1 (Thr24) and FoxO1 nucleic abundance in basal (BASAL) and insulin stimulated (INSULIN) gastrocnemius skeletal muscle obtained from wild-type (WT) and Akt2 knockout (Akt2 −/−) mice. *Difference (P<0.05) compared with WT. †Difference (P<0.05) compared with basal. Data are mean ± SE. Phospho-proteins (FoxO1 [Thr24]) expressed as a ratio of total-FoxO1 proteins. Nucleic FoxO1 expressed as a ratio of Histone 1.
Figure 4.5. Basal Murf1 and Mafbx (atrogenes) mRNA expression in muscle of wildtype (WT) and Akt 2 muscle specific knockout mice (Akt2-/-). Murf-1 and Mafbx mRNA expression in wild-type (WT) and Akt 2 knockout (KO) muscle. *Difference (P<0.05) compared with WT (wild type values expressed as 1, and denoted by dotted line).
Figure 4.6. Insulin stimulated Murf1 and Mafbx (atrogenes) mRNA expression in muscles of wildtype (WT) & Akt 2 muscle specific knockout mice (KO). Murf-1 & Mafbx mRNA expression in wild-type (WT) and Akt 2 knockout (KO) muscle. *Difference ($P<0.05$) compared with WT (wildtype values expressed as 1, and denoted by dotted line).
Figure 4.7. Basal and Insulin stimulated Raptor mRNA expression in muscle of wildtype (WT) and Akt 2 muscle specific knockout mice (KO). Basal (A) & insulin stimulated (B) Raptor mRNA expression in wild-type (WT) and Akt 2 knockout (KO) muscle. *Difference (P<0.05) compared with WT.
Figure 4.8. Basal (BASAL) & insulin (INSULIN) stimulated Raptor protein expression in the skeletal muscle of WT and Akt 2 knockout (Akt -/-) mice. Total Raptor protein abundance in WT (black bars) and Akt -/- mice (white bars). There was a tendency for a reduced Raptor protein abundance in Akt -/- compared to wild-type mice. Insulin induced a significant ($P<0.05$) increase in Raptor protein abundance in WT but not Akt -/-. *Difference ($P<0.05$) compared with WT. †Difference ($P<0.05$) compared with basal.
Figure 4.9  p70 S6K (Thr^{389}) phosphorylation pre (BASAL) and post (INSULIN) insulin treatment of wildtype & Akt 2 muscle specific knockout mice. Phosphorylation of p70S6K (Thr^{389}) in basal (BASAL) and insulin stimulated (INSULIN) gastrocnemius skeletal muscle obtained from wild-type (WT) and Akt 2 knockout (Akt2 −/-) mice. *Difference (P<0.05) compared with WT. †Difference (P<0.05) compared with basal. Data are mean ± SE. Phospho-protein expressed as a ratio of total-p70S6K protein.
DISCUSSION

The objective of this chapter was to investigate if the effects of insulin stimulation on PGC-1α gene expression were directly regulated by FoxO1 in an Akt dependant manner. This was assessed using muscle specific, Akt 2 knockout (Akt 2-/-) mice.

Briefly, it was hypothesised that Akt 2 -/- animals would exhibit levels of PGC-1α mRNA expression both basally and upon insulin stimulation consistent with both of the diabetic models described in Chapters 2 and 3. It was further hypothesized that this non-repression of PGC-1α would be accompanied by a concomitant reduction in insulin stimulated FoxO1 phosphorylation. The data obtained mirrored very closely those observed in the type 2 diabetic muscles and the experimentally induced insulin resistant human, skeletal muscle myotubes. These data were somewhat expected, given the essential role that Akt has in propagating insulin signaling to its downstream effector FoxO1.

It is interesting that Akt 2 -/- animals are smaller than their wild-type control animals. Recently, Kamei et al., (2004) produced muscle specific FoxO1 over-expression mice and these animals were small, and have greater FoxO1 transcriptional activity (as evidenced by an increase in PGC-1α mRNA similar to the Akt 2-/- animals in this chapter. Given this similarity in the characteristics of these animals, the mRNA of the atrogenes Murf1 and MAFbx were measured in the basal and insulin stimulated state. Both Murf-1 and MAFbx have been reported to be associated with muscle atrophy and their expression is repressed in an insulin stimulated, FoxO dependant manner. Basally, both Murf-1 and MAFbx were significantly higher in Akt 2 -/-
animals, due to greater FoxO1 activity, as hypothesized. Murf 1 mRNA was repressed by insulin in wild type mice but not in the Akt 2-/- animals. There was no reduction in MAFbx mRNA post insulin stimulation in wild type mice when compared with Akt 2-/- mice. Previous work has identified MAFbx as being a bone fide target of FoxO1 transactivation (and this will be confirmed in the next chapter of this thesis Chapter 5). The data presented in this chapter, it can indicate that acute insulin stimulation does not have an effect on MAFbx expression irrespective of patent Akt signaling.

A report by Jia et al. (2004) suggested that the regulation of Raptor, a protein that complexes with mTOR to propagate signals downstream that allow translation/initiation to occur (Hara et al. 2002; Sarbassov et al., 2002), is under the control of FoxO in C. elegans. In the present study, evidence for a conserved response was observed with regard to FoxO’s regulation of Raptor. In the basal (non insulin stimulated) state, Raptor mRNA was reduced significantly within the context of increased FoxO activity (the Akt 2-/- animals), as Akt could no longer exert its inhibitory role on FoxO. It was of interest that, in the insulin stimulated state; Raptor mRNA and protein levels were increased significantly in WT, but not in the Akt 2-/-, animals. This suggests that insulin induced the rapid transduction of both Raptor mRNA and protein. Insulin has been reported to regulate aspects of the translation initiation pathway via its promotion of Raptors’ physical associated with mTOR. When insulin stimulated, the Raptor/mTOR complex propagates signals to downstream effectors of translation initiation. It would appear that insulin expedites this response to increase Raptor expression and Akt ablation impacts upon the ability of insulin to induce this response. Akt has been shown to regulate cell size via its hypertrophic properties, which, in the most part, are highlighted by its ability to increase translation/initiation pathways. It is also important to note that Akt negatively regulates FoxO1s transactivation of the atrophic
gene MAFbx. Thus, it could be suggested that Akt regulates cell size via its ability to: 1) suppress FoxO (ameliorating atrophic gene expression); and 2) by inducing translation/initiation that promotes growth.

The finding described in this chapter are largely confirmatory, but nonetheless important, given the nature of the previous data obtained in muscle from type 2 diabetic patients and experimentally induced insulin resistance in culture. The combined data of Chapters 2, 3 and 4 suggest that there is not only conservation across species regarding FoxO1’s role in the regulation of PGC-1 gene expression, but that the observations can be replicated both in-vivo and in-vitro. The work of this chapter also provided the impetus for direction for Chapter 5, as its appeared that there was a bone fide repression of Raptor mRNA in Akt 2/-/- animals and therefore within the context of increased FoxO activity.
CHAPTER 5

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Investigation of expression and regulation of proteins associated with growth in C2C12 myotube cultures expressing constitutively active FoxO1 and transgenic mice harboring muscle specific FoxO1 over-expression

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CHAPTER 5

INTRODUCTION

Loss of skeletal muscle tissue is a clinical manifestation of many conditions and diseases including aging, cancer, sepsis, HIV and diabetes (Price 2003), and important progress has been made in understanding the molecular regulation of both catabolism and anabolism within skeletal muscle (Hoffman & Nader 2004). The ubiquitin ligase MAFbx (atrogin-1) promotes skeletal muscle protein degradation in response to glucocorticoid (Stitt et al., 2004; Sandri et al., 2004), and contributes toward muscle atrophy via proteosomal degradation of MyoD (Tintignac et al 2005). MAFbx is a target gene of FoxO transcription factors (Stitt et al., 2004; Skurk et al., 2005). As discussed previously, FoxO1 is expressed in insulin sensitive tissues such as skeletal muscle, liver, and adipose tissue (Imae et al., 2003) and is an important target of insulin signaling downstream of Akt (see Chapters 2-4). Previous chapters described in detail how insulin negatively regulates FoxO1 transactivation of target genes, in such a way that upon insulin stimulation, Akt phosphorylates FoxO1, resulting in its exclusion from the nucleus. These data (Chapters 2-4) were consistent with many previous observations (Rena et al., 2001; Biggs et al., 1999; Takashi et al., 1999; Rena et al., 2001; Brunet et al., 1999). Importantly when FoxO1 is extruded from the nucleus it undergoes subsequent proteosomal degradation (Matsuzaki et al., 2003) following ubiquitination by Skp2 (Huang et al., 2005).

The decreased protein synthesis observed in skeletal muscle in various catabolic conditions is associated with defects in mRNA translation initiation (Cooney et al., 1997; Shah et al., 2000). The translation of mRNA is divided into three steps: initiation, elongation, and termination (Lang
et al., 2005) and is facilitated and regulated by eukaryotic initiation factors (Lang & Frost 2005). The stable heterotrimeric complex consisting of eIF4E, eIF4G and eIF4A, which form the active eIF4F molecule, plays a critical role in peptide chain orientation by regulating recruitment of capped mRNAs to the 43S pre-initiation complex (Pain, 1986). The eukaryotic initiation factor-4E (eIF4E) and its inhibitory protein, eukaryotic initiation factor-4E-binding protein-1 (eIF4E-BP1) have been suggested to be critical for the regulation of translation initiation (Lawrence & Abraham 1997). eIF4E-BP1 is a phosphoprotein that, in its non-phosphorylated state, forms a tight association with eIF4E and prevents its interaction with eIF4G, and recruitment to the 43S pre-initiation complex. When hypo-phosphorylated, as would be the case in the absence of nutrients or growth factors, eIF4E-BP1 association with eIF4E serves to repress translation (Schalm et al., 2003). In contrast, when phosphorylated, eIF4E-BP1 dissociates from eIF4E and allows the recruitment of capped mRNA and translation initiation (Rhoads 1999). This complex interaction is thought to represent a rate-limiting step in protein synthesis which can, in part, be ascribed to the fact that eIF4E is the least abundantly expressed subunit (Duncan et al., 1987). eIF4E-BP1 contains at least six phosphorylation sites, including two threonines (Thr37/46) that are hierarchical regulatory sites activated by mammalian Target of Rapamycin (mTOR) signaling (Fadden et al., 1997; Heesom et al., 1998; Gingras et al., 2001; Martin & Blenis 2002). In response to Akt activation, mTOR forms a complex with Raptor, a 150-kD polypeptide (Kim et al., 2002), and then phosphorylates eIF4E-BP1 (30). Raptor is critical for TOR signaling in-vivo (Kim et al., 2002) and is absolutely required for the mTOR-catalysed phosphorylation of eIF4E-BP1 in-vitro (Hara et al., 2002; Nojima et al., 2003).

Recent work in Drosophila (Hay & Sonnenberg 2004; Junger et al., 2003) and C. elegans (Teleman et al., 2005; Jia et al., 2004) suggests that orthologs of eIF4E-BP1 and Raptor are
targets of FoxO proteins. These observations raise the possibility that, in mammalian skeletal muscle, FoxO1 may not only contribute towards catabolic processes via activation of ubiquitin ligases, but also via repression of anabolic pathways. Using both C2C12 mouse myoblasts stably expressing FoxO1-ER fusion proteins that are rapidly activated by hydroxytamoxifen (4-OH TAM) treatment and transgenic mice that specifically over express constitutively active FoxO1 in skeletal muscle (FoxO1++/+ mice), I tested the hypothesis that FoxO1 inhibits mTOR signaling and protein synthesis. The data contained in this chapter show that FoxO1 increases total (but not phospho-) eIF4E-BP abundance, resulting in hypo-phosphorylation of eIF4E-BP. These observations occur in the face of a decreased abundance mTOR and Raptor, upstream regulators of eIF4E-BP phosphorylation, and concomitantly with reduced protein synthesis, as measured by [14 C] Phenylalanine incorporation.
C2C12 cells and cell culture

C2C12 skeletal muscle myoblasts stably expressing FoxO-ER fusion proteins were described previously (Bastie et al., 2005). In brief, C2C12 were stably transfected with the empty pBABE retrovirus or pBABE vectors expressing fusion proteins containing either a constitutively active form (where three Akt phosphorylation sites [Thr 24, Ser 256 and Ser 319] are replaced by alanines) or a transcriptionally inactive form (where His215 is replaced with arginine and reduces DNA binding) of human FoxO1 in-frame with a modified (tamoxifen-specific) version of the murine estrogen receptor-α ligand binding domain. Cells were selected with puromycin and colonies pooled for studies, as reported previously (Bastie et al., 2005). Fusion proteins are restricted to the cytoplasmic space until activation by treatment with 4-OH tamoxifen.

Cells were maintained in proliferation media consisting of Dulbecco’s modified Eagle’s Medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin cocktail at 37°C and 5% CO₂ until confluent. Confluent myoblast cultures were rinsed in 1X PBS, dissociated with 0.05% trypsin, pelleted (1600 rpm for 5 min), rinsed in warm 1X PBS and seeded into experimental plates pre-coated with 0.2% gelatin (100 mm plates for protein extraction and immunoprecipitation, 6-well plates for RNA extraction, and 12-well plates for protein synthesis experiments). When experimental cultures were 80% confluent, proliferation medium was changed to differentiation medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum and 1% penicillin/streptomycin cocktail. On day 4, fully fused myotubes cultures were supplemented with 10% FBS for 24 h to induce the fed state, ameliorate effects of endogenous FOXO proteins and drive protein synthesis. Following 24 h of
FBS treatment, cells were treated with 1 µM 4-hydroxytamoxifen in order to activate FoxO1-ER fusion proteins and promote their translocation to the nucleus or with vehicle (DMSO) for 0, 8, 12 and 24 h.

**Transgenic Animal Experiments**

The generation and phenotype of the FoxO1\(^{+/+}\) mice have been extensively reported previously (Kamei *et al.*, 2004). FoxO1\(^{+}\) and WT mice were anaesthetized via 2.5% Avertin (0.017 mL/g body weight, i.p.). *Gastrocnemius* muscles were then excised bilaterally using aseptic technique, weighed and stored at -80º C until further processing. All animals were housed in hepa-filtered cages, exposed to a 12:12 h light dark cycle (lights on at 0800 hours), and provided a standard rodent diet and water *ad libitum*. All surgical procedures were performed between 0800 and 0930 h.

**Western Blot analyses**

For Western blot analyses of total cell lysate from C2C12s, cells were lysed with ice-cold lysis buffer consisting of 50 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 5 mM EDTA, 0.5% Triton X-100, 10% glycerol (v: v), 2 µg.mL\(^{-1}\) leupeptin, 100 µg.mL\(^{-1}\) phenylmethylsulfonyl fluoride, and 2 µg.mL\(^{-1}\) aprotinin. *Gastrocnemius* muscle from the animals were homogenised in the same buffer as the C2C12 cultures with a Polytron homogenizer. Homogenates were spun at 16,000 g for 60 min at 4ºC and the supernatant was removed and rapidly frozen in liquid nitrogen. Protein concentration of the muscle lysates was subsequently determined (Pierce, Rockford, IL, USA). Lysates were solubilised in Laemmeli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 6%, (mTOR, Raptor) 12% (p70S6K1) and 18% (eIF4E-BP1, eIF4E),
polyacrylamide gels, transferred to a nitrocellulose membrane, blocked with 5% milk and
immunoblotted overnight with total-Raptor, total-eIF4E-BP1, phospho eIF4E-BP (Thr^{37/46}), total-
mTOR, phospho-mTOR (Ser^{2448}), total-p70S6K, phospho-S6K1 (Thr^{389}) antibodies (1:1000; Cell
Signaling, Beverley, MA) and total-eIF4E (BD Biosciences). After incubation with horseradish
peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences, Castle Hill, NSW,
Australia), the immunoreactive proteins were detected with enhanced chemiluminescence (Perkin
Elmer, Rowville, Victoria, Australia) and quantified by densitometry.

**C2C12 immunoprecipitation analysis**

Given that the dominant negative C2C12 FoxO1 cells (FoxO1-215) appeared to act as good
controls for comparison with the constitutively active FoxO1 cells (CA-FoxO1), I opted to only
utilise these cell lines for immunoprecipitation studies. Cell cultures were treated in exactly the
same way those for whole cell lysate analysis, but the experiments were only conducted over a 12
h period alone. Following 24 h of 10% FBS supplementation, cultures were treated for 0 or 12 h
with/without 4-OH TAM or DMSO (-); cells were lysed in buffer previously described (Hara et
al., 2002). This buffer has NP40 substituted for Triton X-100, and allows for assessment of
associated/bound Raptor/mTOR via immunoprecipitation. Briefly, buffer A constituents were as
follows: buffer A (20 mM Tris, 20 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 5 mM
EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 µg/ml aprotinin, 1
µM leupeptin [pH 7.4]) and 1% NP40. The cell lysates were left on ice for 10 min then
centrifuged at 10,000 g for 30 min at 4°C. 1000 µg/ml sample was incubated overnight at 4°C
with anti mTOR antibody (Cell Signaling). Protein A sepharose beads were added to the sample
and incubated for 4 hours at 4°C with gentle rocking. The protein A sepharose/sample complex
was spun in a centrifuge for 30 seconds and washed twice with buffer A and twice with buffer B (10 mM Hepes, 50 mM β-glycerophosphate, 50 mM NaCl [pH 7.4]) on ice. The pellet was re-suspended in 2X SDS sample buffer, mixed with a vortex, centrifuged for 1 minute and heated to 85°C for 5 minutes and placed on ice. Samples were resolved on 4% SDS-PAGE polyacrylamide gels, transferred to nitrocellulose membrane, blocked with 5% milk, washed 5 times with TBST and incubated overnight with anti-Raptor antibody (Cell Signalling). After overnight incubation in the primary antibody, membranes were incubated in horseradish peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences, Castle Hill, NSW, Australia), and the immunoreactive proteins were detected with enhanced chemiluminescence (Perkin Elmer, Rowville, Victoria, Australia) and quantified by densitometry.

Gene expression studies

Day 4 differentiated, myotube cell cultures were treated with 1 µM 4-OH TAM for 0, 8, 12, and 24 h or vehicle (DMSO). Animal tissues were homogenised with a polytron homogeniser. Following treatments (cell culture) and homogenisation (animal tissue), RNA was extracted via an on-column RNA isolation kit (Absolutely RNA Miniprep Kit, Stratagene, La Jolla, CA, USA) according to manufacturers' instructions. RNA samples were reverse transcribed using a thermal cycler (Perkin Elmer GeneAmp PCR 2400 Thermal Cycler, Rowville, Victoria, Australia) with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City CA, USA) in 40 µl reaction mixtures containing 1 x TaqMan RT Buffer, 5.5 mM MgCl₂, 500 µM 2’-deoxynucleoside 5’-triphosphate, 2.5 µM random hexamers, 0.4U/µl⁻¹ RNase inhibitor, 1.25 U/µl⁻¹ multiscribe reverse transcriptase. Control (reverse transcriptase negative) samples were also made and included in all PCR analyses to check for genomic DNA contamination.
**Protein synthesis studies**

Protein synthesis experiments were performed as described by Franch *et al.* (2003) with modifications. Briefly, day 4 myotube cultures were incubated in differentiation media supplemented with 0.6 mM phenylalanine (cold Phe) and 10% serum for 2 h prior to the addition of 0.5 μCi [\(^{14}\)C]-phenylalanine (hot Phe) per well for 2, 8, 12 & 24 h. The appropriate constructs (pBABE-empty vector, FoxO1-215 & CA-FoxO1) were activated (or not) with 1 μM 4-OH-TAM upon addition of [\(^{14}\)C]-phenylalanine. Following the time course, cells were washed x 3 with cold (1X) PBS and lysed in 600µl of 10% trichloracetic acid (TCA) and placed on ice for 60 min. The plates were thoroughly scraped and the lysate spun in a centrifuge at 16000 rpm for 15 min (4°C). Following centrifugation, the supernatant was discarded and the pellet washed x 3 with ethanol: ether (1:1 ratio). The pellet was solubilised overnight in 0.6 ml of 0.3 M NaOH at 4°C. Following overnight solubilisation, 0.5 mL of cell lysate was added to 4.0 ml scintillation solution (Starscint Perkin-Elmer, Boston, MA, USA), and the incorporated [\(^{14}\)C]-phenylalanine from the lysed pellet assessed via radioisotope scintillation. Counts were expressed against total protein.

**Statistical Analysis**

Data analyzed using a one way or two-way analysis of variance (ANOVA) with repeated measures (*Statistica®,* Tulsa, OK), with significance accepted with a P value of <0.05. If analyses revealed a significant interaction, a Newman Keuls post-hoc test was used to locate specific differences. Data are presented as means ± SEM.
RESULTS

eIF4E-BP1 and Raptor are transcriptional targets of FoxO1 in skeletal myotubes

To study the effects of FoxO1 on mTOR signaling in muscle cells, I employed C2C12 cells stably expressing either a constitutively active form of FoxO1 (CA-FoxO1), or a transcriptionally inactive form of FoxO1 (FoxO1-215) in-frame with a modified form of the estrogen receptor ligand binding domain that responds selectively to 4-OH TAM (Bastie et al., 2005). Previous studies with these cells have shown that fusion proteins are restricted to the cytoplasmic space in the absence of ligand and then rapidly translocate to the nucleus upon treatment with 4-OH TAM (Bastie et al., 2005). While FoxO proteins can prevent myocyte differentiation and fusion (Hribal et al., 2003), this model allowed us to maintain FoxO-ER fusion proteins in an inactive state during myoblast proliferations, differentiation and fusion, and then examine the effects of acutely activating the CA-FoxO1 fusion protein in mature myotubes by addition of 4-OH TAM [herein referred to as CA-FoxO1 (+) and CA-FoxO1 (-) indicative of the presence or absence of 4-OH TAM treatment, respectively]. As shown in Figure 5.1, activation of FoxO1 with 4-OH TAM treatment [CAFoxO1 (+)] resulted in marked elevation in the mRNA of the atrogene MAFbx at 8, 12, and 24 h, consistent with previous studies indicating that MAFbx is a direct target of FoxO action (Sandri et al., 2004; Stitt et al., 2004). I also noted marked elevation in the mRNA abundance of eIF4E-BP1 at 8, 12 and 24 h post activation (Figure 5.2). A progressive reduction in the mRNA expression of Raptor also was noted at later time points (12 and 24 h) (Figure 5.3). No changes were observed in the mRNA of eIF4E (Figure 5.4). These results show that eIF4E-BP1 and Raptor, like MAFbx, are transcriptionally regulated by activation of FoxO1 in muscle
cells. These changes were not observed in CA-FoxO cells in the absence of 4-OH TAM or in control C2C12 cells stably transfected with empty vector (pBabe) or cells expressing transcriptionally inactive FoxO (FOXO-215) irrespective of 4-OH TAM treatment.

**FoxO1 promotes hypo-phosphorylation of eIF4E-BP1 and increased binding of eIF4E-BP1 with eIF4E**

In the absence of insulin or growth factors, eIF4E-BP1 is hypophosphorylated and eIF4E-BP1 association with eIF4E serves to repress translation. When phosphorylated, eIF4E-BP1 dissociates from eIF4E and allows translation initiation (Schalm et al., 2003; Rhoads et al., 1999). To examine effects of FoxO1 activation on protein synthesis, myocytes were differentiated to myotubes via 4 day serum withdrawal, then stimulated with 10% serum to promote anabolism and treated with/without 4-OH TAM. No differences were observed in eIF4E protein abundance when comparing the conditions (Figure 5.5). Despite the fact that the myotubes were serum-treated, activation of CA-FoxO1 increased eIF4E-BP1 total protein, while phosphorylation at the regulatory sites (Thr\(^{37/46}\)) was decreased at 8 h and thereafter when comparing CA-FoxO1 (+) with other conditions (Figure 5.6). These findings were observed in the absence of any differences in either Akt total protein abundance or phosphorylation of Akt (Se\(^{473}\)) (Appendix 2). Next, to test whether the hypophosphorylation of eIF4E-BP1 was associated with increased eIF4E association to eIF4E-BP1, immunoprecipitation analyses were performed on cells expressing CA-FoxO1 or FoxO1-215 proteins treated with/without 4-OH TAM for 12 h. Consistent with our data showing that FoxO1 results in hypophosphorylation of eIF4E-BP1, CA-FoxO1 (+) cells displayed a markedly increased eIF4E-BP1 associated eIF4E after 12 h (Figure 5.7).
FoxO1 decreases Raptor and mTOR protein abundance, mTOR phosphorylation and mTOR associated Raptor

Since FoxO1 was associated with hypophosphorylation of eIF4E-BP1, I next examined its effect on components of mTOR signaling. A marked reduction in the abundance of both Raptor (23% decrease; Figure 5.8) and mTOR (12% decrease; Figure 5.9) protein was observed at 8 h and thereafter, despite cells being maintained in the presence of serum and nutrients, and in the absence of any changes in phosphorylation of Akt (Western blots analysis of Akt can be seen in Appendix 2). Changes in Raptor protein levels (Figure 5.8 at 8 h precede effects of FoxO1 activation on mRNA abundance (Figure 5.3), indicating that FoxO1 also may exert effects on raptor and mTOR protein expression at the post-transcriptional level. We next examined the phosphorylation of mTOR (Ser\(^{2448}\)) and showed that activation of FoxO1 markedly reduces mTOR phosphorylation after 12 h (Figure 5.9). In the absence of nutrients, a tight association between mTOR and Raptor prevents the access of mTOR to its targets (Hara et al., 2004), and the association of mTOR and Raptor is thought to be critical for effective signaling to downstream targets, including eIF4E-BP and p70S6K. Accordingly, mTOR associated Raptor was assessed via immunoprecipitation. As shown in Figure 5.10, activation of CA-FoxO1 results in a marked reduction in the formation of a complex between mTOR and Raptor that would be expected to limit signaling to downstream targets.

FoxO1 does not alter p70S6K abundance but decreases phosphorylation of p70S6K and impairs protein synthesis in skeletal muscle myotubes

In addition to eIF4E-BP1, p70S6K is also a major target of the Raptor/mTOR signaling complex (Hara et al., 2002). Since both the phosphorylation of mTOR and the association of mTOR with
Raptor were reduced by FoxO1, we next examined the effect of FoxO1 activation on p70S6K. We observed no differences in the total abundance of p70S6K protein when comparing CA-FoxO1 (+) with other conditions (Figure 5.11). However, phosphorylation of p70S6K on its regulatory residue (Thr^{389}) was markedly reduced in CA-FoxO1 (+) cells at 8, 12 and 24 h (Figure 5.11). This indicates that activation of FoxO1 not only induces changes in the expression of mTOR and Raptor, but also impacts upon the ability to phosphorylate and therefore propagate signaling to downstream targets. Since the phosphorylation of p70S6K and 4EBP-1 both play a critical role in promoting protein synthesis (Fingar et al., 2002), I next examined whether FoxO1 may impair protein anabolism. As shown in Figure 5.12, the incorporation of [14C] phenylalanine into protein is impaired in CA-FoxO1(+) cells at 8, 12 and 24 h. In contrast, I observed no change in ^{14}C incorporation in CA-FoxO1 myotubes treated with carrier alone or in control (pBABE) or FoxO1- 215 cells treated with 4-OH TAM.

**Effects of constitutively active FoxO1 on eIF4E-BP1, and mTOR signaling in-vitro were observed in FoxO1++/+ mice**

Kamei et al., (2004) have previously observed that muscle mass is markedly reduced in transgenic mice expressing constitutively active FoxO1 in skeletal muscle (FoxO1^++/+). While it is known that FoxO1 promotes catabolism, the *in-vitro* data presented in this chapter suggested that it also impairs anabolism via effects on eIF4E-BP1, and mTOR signaling. I was fortunate to obtain the transgenic mice from Professor Kamei and colleagues and next examined the muscles from FoxO1^++/+ mice relative to littermate control mice (herein refereed to as WT) to see if FoxO exerts similar anti-anabolic effects *in-vivo*. At the mRNA level I showed that like our CA-FoxO1 (+) myotubes, FoxO1^++/+ mice displayed increased MAFbx (Figure 5.13) and eIF4E-BP1
expression (Figure 5.14), but decreased Raptor (Figure 5.15) expression. Consistent with my in-vitro analyses, eIF4E mRNA abundance was not different when comparing FoxO1++/+ mice with WT (Figure 5.16). I next assessed the total and phosphoprotein abundance in FoxO++/+ and WT mice. As can be seen in Figure 5.17, the muscles from the transgenic mice were much smaller and paler in color, as reported previously (Kamei et al., 2004). The nuclear abundance of FoxO1 was markedly higher in the FoxO1++/+ mice (Figure 5.18). Consistent with my in-vitro data, the FoxO1++/+ mice exhibit decreased Raptor and mTOR protein abundance and decreased mTOR phosphorylation (Ser\textsuperscript{2448}) as well as increased eIF4E-BP1 protein abundance and decreased eIF4E-BP1 phosphorylation (Thr\textsuperscript{37,46}) in the presence of unchanged total or phosphorylated Akt (Ser\textsuperscript{473}). These changes were accompanied by a reduction in the phosphorylation of p70S6K on its regulatory residue (Ser\textsuperscript{389}). Together, these data suggest that the reduction in muscle mass associated with FoxO1++/+ mice is due to impaired anabolic signaling as well as up-regulated signaling through muscle specific E-3 ligases such as MAFbx (atrogin 1).
Figure 5.1. Atrogin-1 (MAFbx) mRNA expression in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. The mRNA abundance of MAFbx (Atrogin 1) in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes with a mutation of the ligand-binding domain of the estrogen receptor of the expression vector such that it specifically responds to hydroxytamoxifen (4-OH TAM). (+) indicates treatment with 4-OH TAM, (-) indicates treatment with vehicle for 0 h (filled bars), 8 h (dark gray bars), 12 h (light gray bars) and 24 h (open bars). * indicates difference (P<0.05) compared with 0 h.
Figure 5.2. eIF4E-BP1 (4E-BP1) mRNA expression in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. The mRNA abundance of eIF4E-BP1 in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes with a mutation of the ligand-binding domain of the estrogen receptor of the expression vector such that it specifically responds to hydroxytamoxifen (4-OH TAM). (+) indicates treatment with 4-OH TAM, (-) indicates treatment with vehicle for 0 h (filled bars), 8 h (dark gray bars), 12 h (light gray bars) and 24 h (open bars). * indicates difference (P<0.05) compared with 0 h.
Figure 5.3. Raptor mRNA expression in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. The mRNA abundance of Raptor in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes with a mutation of the ligand-binding domain of the estrogen receptor of the expression vector such that it specifically responds to hydroxytamoxifen (4-OH TAM). (+) indicates treatment with 4-OH TAM, (-) indicates treatment with vehicle for 0 h (filled bars), 8 h (dark gray bars), 12 h (light gray bars) and 24 h (open bars). * indicates difference (P<0.05) compared with 0 h.
Figure 5.4. eIF4E mRNA expression in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. The mRNA abundance of eIF4E in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes with a mutation of the ligand-binding domain of the estrogen receptor of the expression vector such that it specifically responds to hydroxytamoxifen (4-OH TAM). (+) indicates treatment with 4-OH TAM, (-) indicates treatment with vehicle for 0 h (filled bars), 8 h (dark gray bars), 12 h (light gray bars) and 24 h (open bars). No significant differences detected (P<0.05) compared with 0 h.
Figure 5.5. eIF4E total protein abundance in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. Total eIF4E protein in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes treated with 4-OH TAM (+) or without [vehicle] (-).
Figure 5.6. 4E-BP1 (eIF4E-BP1) total & phospho-protein abundance in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. Total and Phosphorylated (Thr37/46) eIF4E-BP1 in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) treated with (+) or without (-) 4-OH TAM. * Denotes significant difference (P <0.05) compared with 0h.
Figure 5.7. 4E-BP1 (eIF4E-BP1) associated (immuno-precipitated) eIF4E in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. eIF4E-BP1 associated eIF4E in dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) C2C12 cells treated with (+) or without (-) 4-OH TAM. * Denotes significant difference (P <0.05) compared with 0h.
Figure 5.8. Raptor total protein abundance in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. Total Raptor protein abundance in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes treated with (+) or without (-) 4-OH TAM. * Denotes significant difference (P <0.05) compared with 0h.
Figure 5.9. mTOR total and phospho protein abundance in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. Total and phosphorylated (Ser 2448) mTOR protein abundance in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes as described in treated with (+) or without (-) 4-OH TAM. * Denotes significant difference (P <0.05) compared with 0h.
Figure 5.10. mTOR associated (immunoprecipitated) Raptor abundance in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. mTOR associated Raptor abundance in dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) C2C12 cells treated with (+) or without (-) 4-OH TAM. * Denotes significant difference (P <0.05) compared with 0h.
Figure 5.11. p70 S6K1 total and phospho protein abundance in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. Total and phosphorylated (Thr 389) p70S6K protein abundance in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes treated with (+) or without (-) 4-OH TAM. * Denotes significant difference (P <0.05) compared with 0h.
Figure 5.12. $^{14}$C-Phenylalanine incorporation (measure of protein synthesis) in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. $^{14}$C phenylalanine incorporation in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes. (+) indicates treatment with 4-OH TAM, (-) indicates treatment with vehicle. * Denotes significant difference (P<0.05) compared with 0h.
Figure 5.13. MAFbx (Atrogin-1) mRNA expression in skeletal muscle of wildtype and FoxO ++/++ transgenic mice. The mRNA abundance of MAFbx in *gastrocnemius* muscles from transgenic mice that specifically overexpress FoxO1 in skeletal muscle (FoxO1 ++/+) and littermate control mice (WT). * indicates difference (P<0.05) compared with WT mice.
Figure 5.14. 4E-BP1 (eIF4E-bp1) mRNA expression in skeletal muscle of wildtype and FoxO ++/+ transgenic mice. The mRNA abundance eIF4E-BP1 in gastrocnemius muscles from transgenic mice that specifically overexpress FoxO1 in skeletal muscle (FoxO1 ++/+ ) and littermate control mice (WT). * indicates difference (P<0.05) compared with WT mice.
Figure 5.15  Raptor mRNA expression in skeletal muscle of wildtype and FoxO \textsuperscript{++/+} transgenic mice. The mRNA abundance of Raptor in \textit{gastrocnemius} muscles from transgenic mice that specifically overexpress FoxO1 in skeletal muscle (FoxO1 \textsuperscript{++/+}) and littermate control mice (WT). * indicates difference (P<0.05) compared with WT mice.
Figure 5.16. eIF4E mRNA mRNA expression in skeletal muscle of wildtype and FoxO ++/+ transgenic mice. The mRNA abundance of MAFbx in gastrocnemius muscles from transgenic mice that specifically overexpress FoxO1 in skeletal muscle (FoxO1 ++/+) and littermate control mice (WT). No significant differences detected (P<0.05) compared with WT mice.
Figure 5.17. Gross appearance (A) of wild-type and FoxO1 $$^{++/+}$$ transgenic mouse muscle. Nuclear FoxO1 abundance (relative to Histone 1) and total and or phosphorylation of mTOR, Raptor, eIF4E-BP1, p70S6K and Akt when comparing FoxO1 $$^{++/+}$$ with WT mice (B). Dissected gastrocnemius and soleus muscles (indicated by *) from (FoxO1 $$^{++/+}$$ mice) are smaller and paler than littermate control mice (WT) (A). Nuclear FoxO1 abundance (relative to Histone 1) and total and or phosphorylated mTOR, Raptor, eIF4E-BP1, p70S6K and Akt when comparing FoxO1 $$^{++/+}$$ with WT mice (B).
DISCUSSION

Skeletal muscle atrophy is a hallmark feature of various diseases including cancer, sepsis, HIV and diabetes (Price 2003). An understanding of the molecular, cellular events that regulate decreases in cell size is important if we are to target the atrophy observed in skeletal muscle that is associated with these diseases. At the present time, it is difficult to discern whether if skeletal muscle atrophy is a cause (aetiologically) or consequence (sequelae) of these syndromes.

The molecular signals that regulate the induction of translations/initiation complexes have already been determined. The main objective of this chapter was thus to determine if components of the mTOR pathway, and by virtue of association, components of the translation/initiation pathway, are to some extent regulated by FoxO proteins. Investigation and experimental testing of this hypothesis was valid given that it is now well known that FoxO proteins promote the induction of genes associated with ubiquitination (Stitt et al 2004; Sandri et al 2004).

FoxO proteins are conserved across species and cell type. Important reports in the literature have suggested that in lower order organisms (C. elegans & Drosophila) FoxO proteins regulate the relative expression and/or abundance of components of the mTOR pathway. They do this in two ways: 1) repression, and 2) by promotion of proteins involved in the translation/initiation pathways that either sit upstream or downstream of mTOR respectively.

Jia et al. (2004) were the first authors to provide evidence that FoxO (DAF 16) repressed the expression of Raptor (DAF 15). The data contained in this chapter confirmed a role for FoxO1 in
inhibition of translation/initiation. In this regard, the data suggested that Raptor abundance is repressed via forced expression of FoxO in-vitro and in-vivo in a mammalian system and confirmed previous findings in C. elegans (Jai et al., 2004). In addition, the findings suggest that eIF4E-BP1 abundance is increased via forced expression of FoxO1 in-vitro and in-vivo supports those data published by Junger et al. (2003) and Teleman et al. (2005a; 2005b) in Drosophila.

An interesting observation arising from this chapter is that related to data obtained for the incorporation of the $^{14}$C phenylalanine. Here I describe a situation where atrophic stimuli are imposed via activation of FoxO1 with a concomitant decrease in incorporation of $^{14}$C-phenyalanine into protein. The atrophy of skeletal muscle generally observed following disuse or denervation is brought about by decreases in protein synthesis, but within a context of normal rate of protein degradation (Ferrando et al., 1996). The model used in this chapter indicates that within the context of FoxO1 induced atrophy, protein synthesis is reduced in the absence of disuse, unloading or denervation. Furthermore, this decreased synthesis is observed within a context of degradation of proteins (mTOR and Raptor) whose activity is to promote protein synthesis. This is important because it suggests that FoxO1 may be an important therapeutic target in diseases where overt muscle wasting occurs in the absence of disuse and unloading.

Previous findings by Bastie et al. (2004) using a similar in-vitro model to the one described in this chapter and another study by Giannakou et al. (2004) suggested that the forced expression of FoxO in Drosophila increased the storage of lipid in the ‘fat body’ of these animals. The Drosophila fat body is a storage depot for lipid that is utilised during times of starvation. Bastie et al. (2004) showed that forced expression of FoxO in C2C12 murine skeletal muscle cells induces the expression of FAT/CD36 a lipid transporter whilst increasing the deposition of lipid
in these cells. Taken together, the data of Bastie et al. (2004) and those data presented in this chapter, further support the notion that the biological activity of FoxO proteins is conserved across species types. Schematic interpretation of these results figure 5.18.
Figure 5.18. Schematic representation of regulation of mTOR activity and protein anabolism by FoxO proteins.

Under conditions of nutrient and growth factor activity (left panel), Akt phosphorylates and inactivates FoxO proteins. Akt also phosphorylates tuberous sclerosis complex (TSC) proteins, allowing Rheb to phosphorylate and activate mTOR. mTOR phosphorylates eIF4EBP-1 (4EBP), allowing eIF4E to bind EIF4G and promote translation initiation of capped mRNAs. mTOR also phosphorylates p70S6K, which also promotes translation of protein. Transcriptionally active FoxO proteins stimulate the expression of eIF4E-BP1 (4EBP) which displaces eIF4G from eIF4E. FoxO proteins also suppress the expression of Raptor and mTOR, limiting the phosphorylation of 4EBP proteins and p70S6 kinase, and protein synthesis. Under conditions when nutrients are depleted and insulin and growth factor levels are low (right panel), Rheb is sequestered with TSC proteins, while FoxO proteins are active. FoxO proteins promote the expression of eIF4EBP-1 and suppresses the expression of Raptor and mTOR, resulting in reduced phosphorylation of p70S6K, sequestration of eIF4E in an inactive complex, and impaired anabolism.

Adapted from Southgate et al., (2006).
CHAPTER 6

CONTENTS

Effect of dexamethasone and TNF-α on the IGF-1 mediated localisation of FoxO1 in murine C2C12 myoblasts

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CHAPTER 6
INTRODUCTION

Increases in circulating Tumor Necrosis Factor-alpha (TNF-α also called cachectin), an endotoxin induced serum factor (Carswell et al., 1975) have been reported to be highly associated with type 2 diabetes and insulin resistance (Zinman et al., 1999; Saghizadeh et al., 1996; Kern et al., 2001).

The muscle wasting observed in AIDS (Chang et al., 1998), cachectic cancer patients (Baracos et al., 2000) as well as normal aging (Grounds 2002; Carter et al., 2001) are closely associated with the expression of TNF-α. TNF-α can interfere with muscle growth and regeneration, in part by disrupting both (Insulin-like-growth-factor-1) IGF-1 (Frost et al., 1997) and insulin signaling cascades (Uysal et al., 1997; Hirosumi et al., 2002).

Interestingly, Alikhani et al. (2005) recently reported that TNF-α induced nucleic localisation and subsequent activation of FoxO1 target genes in-vitro and in-vivo. Using primary dermal fibroblasts and mice inoculated with human recombinant TNF-α these authors discovered that FoxO1 functions as a ‘master switch’ that regulates gene expression necessary for TNF-α induced fibroblast apoptosis (Alikhani et al., 2005) Two recent reports (Stitt et al., 2004; Sandri et al., 2004) have provided evidence that the glucocorticoid, dexamethasone, induces the nucleic localisation of FoxO1 and this nucleic localisation results in the up regulation of MAFbx. Li et al. (2005) report that treatment of C2C12 myotubes with TNF-α induces the transcription of MAFbx (atrogin-1) in a p38 MAPK dependant manner. These authors did not attempt to
investigate the cellular localisation of FoxO1 in their experiments, but did suggested that in later studies that it would be wise to do so given the reports by Sandri et al. (2004) and Stitt et al. (2004) who provided evidence that MAFbx was a bone fide target of FoxO transactivation. Stitt et al. (2004), Sandri et al. (2004) and Alikhani et al. (2005) suggest that both glucocorticoid and pro-inflammatory cytokines mediate FoxO target gene expression.

Given the data detailed in Chapters 2, 3, and 4 suggested that FoxO1 exhibits a predominantly nucleic localisation in diabetic subjects (Chapter 2) and experimentally induced insulin resistance (Chapters 3 and 4), it was deemed appropriate to investigate other ‘diabetogenic’ compounds that might impact upon growth factor mediated re-localisation of FoxO1. Using exogenous compounds to impact upon growth factor mediated translocation of FoxO1 appeared a logical progression given the data from Chapter 3 suggested fatty acids can perturb this process. The main rationale for conducting the experiments detailed in this Chapter was to assess if TNF-α reduces the ability of IGF-1 to induce cytosolic sequestration of FoxO1 and compare this to regimes (starvation and glucocorticoid stimulation) that are known to impact upon FoxO localisation.
METHODS

Mouse C2C12 myoblasts were seeded and grown in proliferation media (DMEM, 10% FBS, 1% Penstrep) to confluence. When approximately 70% confluent, myoblasts were trypsinized and sub-cultured onto clambered glass slides. The glass slides were pre-coated with 0.2% gelatin in order to assist in myoblast attachment. When the subcultures had reached approximately 70% confluence, the myoblast cultures were transfected with eGFP-FoxO1 (a kind gift from Professor Terry Unterman, University of Chicago) plasmid DNA using lipofectamine according to the manufacture’s instructions. After the cells were incubated for 24 h in media containing the plasmid DNA, the media were replaced and the cells allowed to recover for 12 h. Following recovery, the cells were treated with TNF-α (20ng/ml [Sigma]) or dexamethasone (100 nM) for 8 h in the presence of 10% FBS. Following the 8 h treatment incubations, the appropriate cell cultures were stimulated with 10ng/ml R3-IGF-1 (a potent IGF-1 analogue [Sigma]) for 30 min.

Following cessation of cell treatments, myoblasts were fixed in 4% paraformaldehyde, washed 4 times with 1X PBS. Fixed culture slides were incubated with an aqueous fixative containing DAPI to stain the nuclei and viewed with a laser confocal microscope.

Isolation of nucleic fractions and western blot analysis of FoxO1 nucleic protein in wild-type C2C12s treated with dexamethasone/TNF-α/serum/IGF-1 was undertaken as described in Chapter 3.
RESULTS

Confocal microscopy images of C2C12 myoblasts (Figure 6.1, Panel [A]) indicate that in the starved state GFP-FoxO1 is predominantly nucleic, whereas in the fed state (serum fed), GFP-FoxO1 is predominantly cytosolic. Treatment of serum starved GFP-FoxO1 myoblasts with IGF-1 induced localisation of the GFP protein to the cytosol, a situation comparable to the fed state. These results suggest that serum treatment (the fed state) is sufficient to exclude GFP-FoxO1 from the nucleus and moreover, IGF-1 has the same propensity to result in cytosolic re-localisation following serum starvation. In non-transfected C2C12 myoblasts, endogenous FoxO1 is predominantly nucleic in the fed state, but treatment with IGF-1 following serum withdrawal results in nucleic exclusion (Fig 6.1, Panel [B]).

Confocal microscopy images of C2C12 myoblasts maintained in the fed state (serum fed) and then treated with dexamethasone (Figure 6.2 Panel [A]) for 8 h indicated that dexamethasone induces a ‘starved like’ effect, as GFP-FoxO1 becomes predominantly nucleic. This situation was not reversed by the addition of IGF-1, suggesting that dexamethasone is a potent inhibitor of FoxO1 cytosolic sequestration. In non-transfected C2C12 myoblasts, endogenous FoxO1 was predominantly nucleic in the fed state when treated with dexamethasone, and IGF-1 failed to induce cytosolic sequestration in the presence of serum and dexamethasone (Figure 6.2, Panel [B]).

Confocal microscopy images of C2C12 myoblasts maintained in the fed state (serum fed) and then treated with TNF-α (Figure 6.3 Panel [A]) for 8 h indicated that TNF-α induces a ‘starved
like’ effect similar to dexamethasone, as GFP-FoxO1 becomes predominantly nucleic. This situation is not reversed by the addition of IGF-1, suggesting that TNF-α is a potent inhibitor of FoxO1 cytosolic sequestration. In non-transfected C2C12 myoblasts, endogenous FoxO1 is predominantly nucleic in the fed state when treated with TNF-α, and moreover, IGF-1 failed to induce cytosolic sequestration in the presence of serum and TNF-α (Fig 6.3, Panel [B]).
Figure 6.1. (A) C2C12 myoblast transfected with wildtype GFP-FoxO1 then starved (serum free), fed (+ serum) or starved and treated with IGF-1 (Starved + IGF). (B) Nucleic abundance of FoxO1 in wildtype C2C12 myoblasts after serum starved conditions or serum starved conditions + IGF-1.
Figure 6.2. (A) C2C12 myoblast transfected with wildtype GFP-FoxO1 maintained in the fed state and treated with dexamethasone or dexamethasone + IGF-1. (B) Nucleic abundance of FoxO1 in wildtype C2C12 myoblasts maintained in the fed state and treated with dexamethasone or dexamethasone + IGF-1.
Figure 6.3. (A) C2C12 myoblast transfected with wildtype GFP-FoxO1 maintained in the fed state and treated with TNF-α or TNF-α + IGF-1. (B) Nucleic abundance of FoxO1 in wildtype C2C12 myoblasts maintained in the fed state and treated with TNF-α or TNF-α + IGF-1.
DISCUSSION

Imaging of C2C12 myoblasts using confocal microscopy, revealed that serum starvation induces a marked re-localisation of GFP-FoxO1 and the growth factor IGF-1 has the ability to re-localise GFP-FoxO1 to the cytosol to the same degree as serum. In agreement with both Stitt et al. (2004) and Sandri et al. (2004) the glucocorticoid, dexamethasone, induced a ‘starved-like’ effect on GFP-FoxO1 and stimulation with IGF-1 failed to re-localise GFP-FoxO1 to the cytosol. Alikhani et al. (2005) indicated that FoxO1 functions as a ‘master switch’ that regulates genes associated with fibroblast apoptosis in response to TNF-α stimulation. The confocal images presented support the Electromobility Shift Assay (EMSA) data of Alikhani et al. (2005) with regard to FoxO1 localisation after stimulation with TNF-α.

It is noteworthy that the nuclear localisation of endogenous FoxO1 as evidenced by western blot analysis of wild-type (non transfected) C2C12 myoblasts mirrors the response seen with the GFP-FoxO1 construct; i.e. when wild-type C2C12 myoblasts were treated with dexamethasone and TNF-α a starved like localisation was observed, indicating that FoxO1 was distributed predominantly in the nucleus. Similar to the results with GFP-FoxO1 myoblasts, IGF-1 failed to result in cytosolic sequestration of the protein suggesting that this is a bone fide response by FoxO1 to stimulation with dexamethasone and TNF-α and not simply an artifact associated with the GFP-FoxO1 utilisation.

Increases in glucocorticoid and TNF-α are known to induce muscle wasting and cachexia (Frost et al., 1997; Uysal et al., 1997; Hirosumi et al., 2002). Glucocorticoids are potent antagonists of
insulin action and, when in excess can promote insulin resistance and obesity (Brindley 1995). Increases in circulating TNF-α have been reported to be highly associated with Type 2 diabetes and insulin resistance (Zinman et al., 1999; Saghizadeh et al., 1996; Kern et al., 2001). Both glucocorticoids and TNF-α have been reported to promote insulin resistance via their ability to impact upon activation (phosphorylation) of Akt (Zinman et al., 1999; Saghizadeh et al., 1996; Frost et al., 1997; Uysal et al., 1997; Hirosumi et al., 2002). FoxO1 is a substrate of Akt and its transcriptional activity is negatively regulated via the activity of Akt (Biggs et al., 1999; Brunet et al., 1999; Guo et al., 1999; Kops et al., 1999; Nakae et al., 1999; Rena et al., 1999; Takaishi et al., 1999; Tang et al., 1999; Zhang et al., 2002). Given the results described here, it is reasonable to assume that the negative effects of dexamethasone and TNF-α on skeletal muscle function and insulin sensitivity could be mediated via their shared ability to promote FoxO1 nuclear localisation. Moreover, given the localisation of FoxO1 is nucleic in the presence of dexamethasone, and similarly with TNF-α stimulation, it seems reasonable to assume that both of these compounds would induce similar FoxO1 target genes. Increases in transcriptionally active FoxO1 have been associated with the induction of the E3-Ubiquitin ligase, MAFbx (Stitt et al 2004; Sandri et al., 2004). Thus it is tempting to speculate that the shared ability of these compounds to promote FoxO1 activity, mediates their negative effects on muscle function. This hypothesis remains to be tested and is beyond the scope of this thesis. However, the future directions (Chapter 7) discuss how this situation will be addressed.
CHAPTER 7

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CONCLUDING DISCUSSION AND FUTURE DIRECTIONS

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CHAPTER 7
CONCLUDING DISCUSSION & FUTURE DIRECTIONS

The regulation of PGC-1α and downstream OxPhos genes is complex and not fully elucidated, particularly in skeletal muscle. It was shown from the data described in Chapters 2 and 3, that in both human muscle biopsy specimens and primary human skeletal muscle cell culture, insulin reduces the expression of PGC-1α mRNA and down-stream OxPhos genes in a FoxO1 dependant manner in healthy, but not insulin resistant, skeletal muscle.

These data are concomitant with the observation that phosphorylation, and subsequent nuclear exclusion of FoxO1 is reduced in the skeletal muscle of type 2 diabetics and human primary skeletal muscle cultures treated with palmitic acid (experimentally induced insulin resistance), secondary to reduced insulin-stimulated Akt phosphorylation. Furthermore, the data presented in Chapters 2 and 3 demonstrated that the reduced phosphorylation and subsequent nuclear exclusion of FoxO1 were somewhat Akt dependent. The results presented in Chapters 2 and 3 regarding Akt’s role in negatively regulating (ameliorating) the insulin stimulated FoxO1 target gene transcriptional activity, were supported by those data presented in Chapter 4, that showed that effect of insulin on these processes was blunted in the skeletal muscle of transgenic mice where Akt 2 is genetically knocked out (Akt2 −/− mice).

The observation that PGC-1α and downstream genes were reduced in the skeletal muscle of patients with type 2 diabetes is consistent with previous studies (Patti et al., 2003; Mootha et al., 2003). At the time of writing, no previous studies have examined the mRNA expression of
COX4, FoxO1, or PSARL in the skeletal muscle of Type 2 diabetes. The mRNA analyses presented in Chapter 2 demonstrate that COX4 was reduced ($P<0.05$), while FoxO1 ($P=0.10$) and PSARL ($P=0.16$) mRNA tended to be lower in the Type 2 diabetic patient group. COX4, a nuclear gene encoding part of complex IV in mitochondrial respiration, was recently shown to be increased by PGC-1α over-expression in mice (Lin et al., 2002) and by aerobic training in elderly subjects (Short et al., 2003). In this latter study, however, this increase did not translate to increased insulin sensitivity (Short et al., 2003).

Only one previous preliminary study, (published only in abstract form), has examined the effect of palmitate treatment on OxPhos gene expression. Crunkhorn et al. (2004) showed that both PGC-1α and NRF-1 were markedly reduced by palmitate in the C2C12 muscle cell line. The results presented in Chapter 3 were consistent with this previous observation and suggested that even acute insulin resistance, as induced by 12 hours of palmitate treatment, reduced the mRNA of PGC-1α, the hierarchical regulator of OxPhos gene expression.

A major finding from the studies described in this thesis is that while insulin repressed PGC-1α and downstream OxPhos mRNA expression in healthy skeletal muscle, it did not do so in the muscles of patients with type 2 diabetes nor in experimentally induced insulin resistance. The effect of insulin on OxPhos gene expression in skeletal muscle is controversial, since insulin either increases PGC-1α in young and elderly subjects (Ling et al., 2004) or decreases PGC-1α in C2C12 cells (Crunkhorn et al., 2004). In addition, both ND1 and COX1 mRNA have been shown to be increased in patients by insulin stimulation (Huang et al., 1999), while the mRNA encoding COX4 and ND4 is increased by 8 h, but not by 4 h, of high dose insulin stimulation (plasma insulin ~350 pmol·l$^{-1}$) in healthy humans (Stump et al., 2003). Although not significant, 4 h low
dose insulin (plasma insulin ~50 pmol·l⁻¹) tended to repress COX3 and COX4 mRNA (Stump et al., 2003). With regard to data presented in this thesis (Chapters 2, 3 and 4), the results were very consistent irrespective of the model investigated when using PGC-1α mRNA as a readout. Notwithstanding the fact that cell culture experiments and rodent experiments are very different from the human in-vivo model, and palmitate treatment is only one model to induce cellular insulin resistance in-vitro, insulin significantly repressed PGC-1α mRNA expression in the controls of all models studied; those being healthy human muscle biopsy specimens, cultured human primary myotubes and muscle biopsy specimens from wild type mice. In addition, in the in-vivo study COX4 and NRF-1 were also repressed by insulin treatment. In contrast, in the insulin resistant state, insulin did not repress mRNA expression of these OxPhos genes either in-vivo or in-vitro.

One potential confounding variable related to the human in-vivo study was that the fasting glucose in type 2 (9.1 mM) was allowed to fall to 5.0 mM before we maintained euglycaemia at this concentration. Although we are unable to assess the effect of this lowered level of glycaemia in the patients with Type 2 diabetes, it cannot be ruled out that this possibly affected our measurements.

In a previous study that measured and reported a small increase in PGC-1α mRNA with insulin, no attempt was made to explain why insulin would promote expression of this gene (Ling et al., 2004). However, given that Akt phosphorylates FoxO1 to down-regulate PGC-1α promoter activity in both HEPG2 cells (Daitoku et al., 2003) and 293T cells (Matsuzaki et al., 2003) when insulin stimulated, this previous result is difficult to interpret. It must be noted, however, that whilst the results reported here demonstrate a repression of mitochondrial gene expression by
insulin, it is not implied that this would result in a decrease in mitochondrial function or respiration. With regard to the experiments detailed in Chapter 1, I was unable to measure indices of mitochondrial function such as CS, β-HAD, or MAPR after insulin treatment due to insufficient sample size. Therefore, it is not possible to determine from these data whether the repression of mRNA expression by insulin in CON or indeed the lack of repression in patients with Type 2 diabetes had any impact on mitochondrial function.

It is well known that insulin stimulation increases respiration, as measured by limb VO$_2$ (Kelley et al., 1999). In addition, Stump et al. (2003) have demonstrated marked increases in skeletal muscle mitochondrial ATP production rates (MAPR) with 4 h of insulin stimulation in the absence of any significant changes in mRNA expression of COX3, COX4, and ND4. Clearly, the physiological significance of FoxO1 regulation of PGC-1α mRNA expression by insulin and downstream physiological processes requires further research.

It may appear paradoxical that patients with type 2 diabetes have impaired mitochondrial function (Petersen et al., 2003; Patti et al., 2003; Mootha et al., 2003; Short et al., 2003; Huang et al., 1999; Stump et al., 2003; Kelley et al., 1999), but have a higher PGC1-α mRNA expression upon insulin stimulation may appear paradoxical. However, even though these patients are, in the basal state, relatively hyperinsulinaemic when compared with their healthy counterparts, this is not sufficient to chronically phosphorylate Akt (Figure 2.4) or indeed FoxO1 (Figures 2.5 and 2.6). Therefore, one would not expect FoxO1 to be a regulator of PGC-1α at any time other than when insulin is transiently increased in the immediate postprandial period. Rather, at most times, it is likely that other transcription factors for PGC-1α such as MEF2, NFAT, and CREB play a greater role than FoxO1 in regulating PGC-1α. Since inactivity would reduce the signals to these
transcription factors, it appears that these factors are overall quantitatively more important in regulating PGC-1α. It is also clear that these factors may also be influenced by fatty acids because in the basal state treating muscle cells in-vitro with palmitate reduces PGC-1α mRNA (Figure 3.2) but at the same time reduced FoxO1 phosphorylation at Ser^{256} (Figure 3.4). It also appears that these factors may play a role in regulating PGC-1α in the Akt2\(^{-/-}\) mice, since, in the basal state, FoxO1 phosphorylation or nuclear abundance was not different (Figs 4.2b, 4.3a & 4.3b respectively) and yet PGC-1α mRNA was reduced relative to littermate control mice (Figure 4.1).

At the time of writing, this was the first study to measure the effect of insulin on phosphorylation and subsequent nuclear exclusion of FoxO1 and the role of Akt in this process in the skeletal muscle of type 2 diabetics or experimental models of insulin resistance. Insulin-stimulated Akt phosphorylation was reduced in the muscle of type 2 diabetics and skeletal muscle myotubes rendered insulin resistant with palmitic acid. The reduced insulin-stimulated phosphorylation of FoxO1 on Ser^{256} in palmitate-treated myotubes (Figure 3.4) was significant in-vitro, and there was a strong tendency for this reduction in-vivo (figure 2.5). Insulin-stimulated phosphorylation of FoxO1 on Thr^{24} was consistent both in-vivo and in-vitro (Figs 2.6 & 3.5 respectively). This finding suggests that the localisation of FoxO1 in the nucleus is increased in insulin resistant muscle and the ability of insulin to reduce FoxO1 transactivation of PGC-1α via Akt-mediated phosphorylation is decreased. Unfortunately, due to tissue constraints, the nuclear abundance of FoxO1 could not be investigated in our in-vivo human studies. However, in-vitro, we observed a significant decrease in the nuclear abundance of FoxO1 in insulin-stimulated vehicle-treated myotubes, but not in insulin stimulated palmitate pretreated myotubes (Figure 3.6), suggesting
that when myotubes are experimentally rendered insulin resistant, FoxO1 remains predominantly nuclear and retains its inherent transactivation ability.

Based on in-vivo human experiments and the in-vitro cell culture experiments, I was unable to determine if impaired Akt phosphorylation was causally linked to the observation that FoxO1 phosphorylation and nuclear exclusion were also impaired in insulin resistance. Hence, experiments were performed in Akt2 −/− mice. In wild type (littermate) control mice insulin phosphorylated Akt (Figure 4.2a) and FoxO1 (Figures 4.2b and 4.3a) and resulted in reduced nuclear abundance of this protein (Figure 4.3b). This was in contrast with data obtained from the Akt2−/− mice where insulin failed to sequester FoxO1 to the cytoplasm, as evidenced by the maintenance of FoxO1 nucleic abundance in these animals when compared with wild type littermate control (Figure 4.3b). Hence, these data indicate that Akt plays a central role in the regulation of PGC-1α mRNA expression by FoxO1 with insulin stimulation (Figure 4.4).

Recently, Kamei et al. (2004) reported that PGC-1α mRNA levels are increased in muscle specific FoxO1 over-expression mice, suggesting that, as is seen in liver, FoxO1 promotes PGC-1α gene expression in muscle. This is consistent with the data presented here, insomuch as when we insulin-stimulated Type 2 diabetic muscle, experimentally induced insulin resistant human skeletal muscle myotubes and transgenic Akt 2−/− mice, an increased PGC-1α mRNA expression was observed. In this regard, although FoxO1 was not over expressed in the studies described in Chapter 2, 3 and 4, a decreased ability to phosphorylate and therefore negate FoxO1 transactivation of PGC-1α in the insulin resistant models was observed.

The importance of intact mTOR signaling and downstream targets in linking nutritional and hormonal cascades to the regulation of cell size is well established (Gingras et al., 2001; Zhang et
Importantly, recent evidence suggests that eIF4E-BP1, together with p70S6K, regulates animal growth and/or size (Fingar et al., 2002; Miron et al., 2002). Given the critical contribution that mTOR signaling affords to overall organismal growth and size and with consideration of the fact that evidence in the literature suggests FoxO proteins limit growth it was of interest to investigate if FoxO played a regulatory in the propagation of mTOR signaling. The data presented in Chapter 5 suggests that the induction of a constitutively active FoxO1 construct in C2C12 myotubes and constitutive expression of FoxO1 in the skeletal muscles of mice, results in increased abundance of eIF4E-BP1 mRNA and protein. The increases in eIF4E-BP1 protein and mRNA were concomitant with a reduction in the abundance of Raptor, an important mediator of mTOR pathway signaling, reduced phosphorylation of the downstream protein p70S6K, and associated with reduced protein synthesis as measured by incorporation of 14C phenylalanine into cells. These data provide compelling evidence that FoxO proteins, previously known to activate catabolic pathways via transcriptional activation of atrogenes such as MAFbx, also play an important role in negatively regulating anabolic pathways.

In this regard, Raptor acts as an essential scaffold protein with mTOR (Heesom et al., 1998; Gingras et al., 2001) and, in the presence of growth factor and nutrients, the Raptor/mTOR complex phosphorylates and removes eIF4E-BP1 inhibitory binding to eIF4E. The removal of the inhibitory binding of eIF4E-BP to eIF4E allows the formation of the eIF4F complex formation and subsequent propagation of translation (Hara et al., 2002; Nojima et al., 2003). Interestingly, the mTOR-catalyzed phosphorylation of eIF4E-BP1 in-vitro is entirely dependant on the presence of Raptor (Hara et al., 2002; Nojima et al., 2003), whereas the mTOR mediated phosphorylation of p70S6K (another downstream target of mTOR signaling essential for anabolism) in-vitro is less dependant on the presence of Raptor (Nojima et al 2003). The data
presented in Chapter 5 support Raptors’ proposed contribution towards p70S6K phosphorylation, in that when Raptor abundance is reduced, p70S6K phosphorylation is reduced concomitantly, but not ameliorated completely. Interestingly, Ohanna et al. (2005) suggested the presence of a pathway, independent of mTOR, which acts in concert with p70S6K to mediate Akt induced growth and hypothesized that inhibition of FoxO1 could fulfill such a function. While FoxO proteins can exert p70S6K-independent effects on growth, the data presented here provide evidence that FoxO proteins also may play an important role in regulating the phosphorylation (and, therefore, function) of p70S6K phosphorylation (Figures. 5.12 & 5.18). A corollary of this finding is that inhibition of FoxO1 (and thus catabolic processes) may be important in the ability to increase p70S6K phosphorylation/activation (and thus promote anabolism).

The data obtained in-vitro in genetically modified C2C12 myotubes were mirrored in FoxO1++/+ transgenic mice, suggesting that FoxO1 is a possible therapeutic target for clinical conditions where muscle wasting is indicated. As previously reported, FoxO1++/+ mice are characterized by markedly reduced muscles mass (Kamei et al., 2004). Given the well known effect of FoxO proteins on muscle specific E-3 ligases such as MAFbx, this skeletal muscle phenotype was ascribed to the up regulation of catabolic pathways. The data described in Chapter 5 demonstrate that FoxO1++/+ mice display markedly reduced mTOR signaling (Fig. 5.18), indicating that the skeletal muscle phenotype of these mice is also mediated, at least in part, by impaired anabolism. The exact physiological significance of the data presented here remains to be determined, but it is tempting to speculate that the role for FoxO1 is maybe to provide contextual growth inhibition. For example, when nutrients and insulin/growth factors are available, FoxO1 is transcriptionally inactive and growth pathways predominate (see schematic Figure 5.1). Conversely, when nutrients are depleted and insulin/growth factor levels are low, or under circumstances where
insulin signaling is impaired in the presence of adequate nutrients, such as insulin resistance (Southgate et al., 2005), transcriptionally active FoxO proteins may function both to promote the function of catabolic pathways that mobilize nutrient stores, including proteolysis, and also to suppress the function of anabolic pathways that promote growth, including protein synthesis. This concept is consistent with recent studies in the liver, where FoxO1 exerts positive effects on the activity of metabolic pathways that are adaptive for periods of nutrient restriction (amino acid catabolism, glycerol transport, gluconeogenesis) and negative effects on the expression of pathways involved in promoting anabolism in the fed state (glycolysis, the pentose phosphate shunt, lipogenesis and sterol synthesis) (Zhang et al., 2005). Together, these findings suggest that FoxO proteins play an important role in integrating the metabolic adaptation to changes in nutritional status in multiple target tissues and through effects on both anabolic and catabolic pathways.

**Conclusion**

In conclusion, there are few studies that have examined the relationship between PGC-1α expression and insulin-stimulated glucose transport and/or insulin sensitivity. The data that constitutes a significant part of this thesis indicates that insulin decreases the expression of genes involved in oxidative metabolism in healthy muscle, but this does not occur in patients with type 2 diabetes and in skeletal muscle cultures rendered insulin resistant. This is most likely due to a decrease in FoxO1 phosphorylation secondary to reduced Akt activity.
Recent data from other cell types, i.e. those other than muscle cells, suggest that PGC-1α gene expression is increased within a context of oxidative stress (Valle et al., 2005). This developing notion is intriguing as this could well be a rationale for non-repression of PGC-1α mRNA post insulin stimulation in our ‘diabetic’ models. In this regard, the observation described by the data presented in this thesis could well be ascribed to a protective effect afforded by increased PGC-1α mRNA in the insulin stimulated context as this would provide message for mitochondrial biogenesis. This notion is given credence when is it is considered that oxidative stress inducing stimuli are well known initiators of mitochondrial damage and dissemination.

In lower order organisms, FoxO1 has been to shown to limit organismal growth, a function that has been ascribed to FoxO’s role in the survival response initiated by growth factor withdrawal or nutrient deprivation respectively. Later studies from this thesis provide the first evidence that FoxO1 inhibits the function of protein anabolic pathways in skeletal muscle via increased expression and reduced phosphorylation of the translational repressor protein eIF4E-BP1 and impaired signaling through mTOR/Raptor complexes. FoxO1 may be an important therapeutic target for human diseases where anabolism is impaired.

This thesis highlights some interesting observations related to the physiological role that FoxO1 might play in mammalian skeletal muscle. FoxO proteins provide one of the major interfaces between the transcriptional output of insulin and other growth factors. The experimental data presented in Chapters 2, 3 and 4 indicate that FoxO1 cytosolic shuttling is perturbed and its transcriptional activity is increased in insulin resistant, diabetic models. Intact insulin signalling is known to decrease lifespan in lower order organisms whilst inducing increases in organismal size (growth). In contrast, down regulation of insulin signalling induces reductions in organismal
size, but those organisms that exhibit smaller size due to reduced insulin signalling also exhibit
greater longevity. Thus, as suggested by Antebi (2004), it appears that insulin signalling is pro-
aging, whilst inhibition of the pathway prolongs life. The data presented in Chapter 5 infers that
the increased FoxO activity imposed by the insulin resistance described in Chapters 2, 3 and 4
may well afford the cell increased longevity via the up regulation of growth inhibition pathways
i.e. survival pathways predominate at the expense of growth. It would appear that the role that
FoxO proteins play in relation to growth and longevity is dictated via their ability to be regulated.
It seems that neither absolute inhibition nor absolute promotion of FoxO1 transcriptional activity
favours long term probity. In this regard, if FoxO1 is predominantly inhibited, one would expect
the cell to lose its ability to induce genes associated with stress protection. Likewise, if FoxO1
were to be predominantly promoted, one would expect the cell to lose its ability to grow and
develop. Thus, it is the fine tuning or regulation of FoxO1 which dictates its physiological role in
the maintenance of cellular probity, growth and metabolism. Could it be then, that the insulin
resistance exhibited in peripheral tissues imposes a protective effect on tissues that exhibit
relative insulin insensitivity? This hypothesis requires further investigation. Further work
investigating FoxO1 role in lifespan extension in the context of insulin resistance forms a
significant part of the future directions section.

Future Directions: autophagy

Cellular homeostasis is maintained by a highly regulated balance between synthesis and
deregradation of intra-cellular components. For the most part, this balance is maintained via
intracellular signaling cascades in response to growth factor or nutrient availability. It appears
that similar intracellular processes are initiated in order to conserve cellular function, when
growth factors or nutrients are low. One such process that provides the cell with an ‘endogenous pool’ of nutrients during holistic nutrient deprivation is termed, “autophagy”. Autophagy describes an evolutionary conserved lysosomal pathway involved in the turnover of long-lived proteins and organelles (Klionsky & Ohsumi, 1999; Seglen & Bohley, 1992), and as such represents a transitory, tactical response to growth factor or nutrient deprivation (Yang et al., 2005). This notion is supported by the fact that autophagy has been shown to contribute toward the extension of life span induced by calorie restriction (Bergamini et al., 2004).

Autophagy is initiated with the formation of a multilayer-membrane bound vacuole (autophagosome) that sequesters fractions of the cytoplasm (Fengsrud et al., 1995). The autophagosomal membrane is derived from the pre-autophagosomal structure called the phagophore, which is a poorly characterized intracellular organelle (Fengsrud et al., 1995). The autophagosome is a double-membrane structure containing undigested cytoplasmic materials including organelles. The outer membrane of the autophagosome fuses with the lysosome membrane. Various hydrolytic enzymes are supplied to the autophagosome and the cytoplasm-derived contents are degraded together with the inner membrane of the autophagosome. This degrading structure is termed the autolysosome/autophagosome (Mizushima et al., 2004).

There is a potential link between autophagy and a number of diseases in humans. For example, cancer, cardiomyopathy and neurodegenerative disorders such as Alzheimer’s, Parkinson’s and Huntington’s diseases, amyotrophic lateral sclerosis and prion diseases are all associated with increased autophagic activity (Shintani & Klionsky 2004).
In summary, autophagy is characterized by sequestration of bulk cytoplasm and organelles in autophagic vesicles and delivery of them to the lysosome, where the materials in the vesicles are degraded and recycled to general nutrient stores to maintain essential cellular functions under starved conditions, a situation that mirrors insulin resistance and Type 2 diabetes (Klionsky et al., 1999).

**Mammalian Target of Rapamycin Signaling**

The mammalian target of rapamycin (mTOR) pathway is emerging as a major contributor in the regulation of cellular function. Mammalian Target of Rapamycin (mTOR) proteins are protein kinases that were first identified in *Saccharomyces cerevisiae* through mutants that confer resistance to growth inhibition induced by the immunosuppressive macrolide rapamycin (Kunz et al., 1993). Interestingly, in response to growth factor (e.g. insulin) activation, Raptor forms a complex with mTOR and phosphorylates eukaryotic Initiation Factor 4- Binding Protein 1 (eIF4E-BP1) (Hara et al., 2002). De-phosphorylation of eIF4E-BP1 is a limiting factor in protein synthesis, as a result of increased association with eIF4E. Raptor is indispensable for TOR signaling *in-vivo* (Kim et al., 2002), and is absolutely required for the mTOR-catalysed phosphorylation of eIF4E-BP1 *in-vitro* (Hara et al., 2002; Nojima et al., 2003).

**Insulin, mTOR and autophagy**

Insulin, one the most post potent growth factors, has been shown to negatively regulate autophagic vacuole formation in hepatic (liver) tissue *ex-vivo* (Kanazawa et al., 2004). Moreover, Mizushima et al., 2004 reported a marked induction of autophagy in the muscle fibers of transgenic animals in response to nutrient starvation (a situation that induces hypoinsulinaemia).
Negative regulation of autophagy by insulin, is mediated via the activation of the PI-3K/PDK-1/Akt pathway which is thought to propagate through to the mammalian target of rapamycin (mTOR) pathway (Scott et al., 2004). The importance of mTOR activation in the amelioration of autophagy is highlighted by the data of Blommart et al., (1995) & Noda & Ohsumi, (1998). These authors showed that inactivation of mTOR by mutation or by treatment with rapamycin induced autophagy despite the presence of ample nutrients, indicating that mTOR acts to suppress autophagy under non-starved conditions, and as such represents a requisite molecule for autaphagic vacuole formation. Thus, under favorable conditions, activation of mTOR promotes protein synthesis and inhibits protein degradation (nutrient transport turnover and autophagy). Upon unfavorable conditions, mTOR is inactive, leading to a reduction in protein synthesis and an up regulation of protein degradation. Therefore, it appears that TOR maintains a balance between protein synthesis and degradation such that the cell can rapidly adjust mass accumulation to a level appropriate to the nutrient supply (Schmelze & Hall 2000).

**FOXO Transcription Factors**

FOXO transcription factors are the mammalian orthologs of DAF-16, the *Caenorhabditis elegans* (*C. elegans*) winged helix Forkhead transcription factors (Daitoku et al., 2003). FOXO1 is expressed in insulin sensitive tissues such as skeletal muscle (Southgate et al., 2005), liver, and adipose tissue (Imae et al., 2003). In these tissues, FOXO factors play a key role in transmitting insulin signaling downstream of acute transforming retrovirus thymoma (Akt). In this context, insulin negatively regulates FOXO trans-activation of target genes, in such a way that upon insulin stimulation, Akt phosphorylates FOXO proteins, resulting in their cytosolic sequestration
(nucleic exclusion) (Rena et al., 1999; Biggs et al., 1999; Takaishi et al., 1999) and subsequent proteosomal degradation (Matsuzaki et al., 2003) by Skp2 (Huang et al., 2005).

**FOXO & mTOR**

We have recently shown (Southgate et al., 2006) that components of the mTOR pathway are regulated by FOXO1 (See data from Chapter 5). By using inducible, constitutively active FOXO1 constructs in C2C12 myotubes and transgenic animals, we have shown that FOXO promotes the degradation of Raptor an essential scaffold protein, that associates with mTOR to propagate mTOR’s growth response. Concomitantly, FOXO1 increases the protein and mRNA abundance of eukaryotic Initiation Factor- Binding Protein 1 (eIF4E-BP1), a molecule that inhibits initiation of translation by inhibiting mRNA processing.

In previously published work (Southgate et al., 2005 [Data from Chapters 2, 3 and 4]), treatment of human primary skeletal muscle myotubes with palmitic acid (a saturated fatty acid which experimentally induced insulin resistance) reduced the propensity for insulin to re-localise FOXO1 to the cytosol resulting in the non-suppression of the FOXO target gene, PGC-1α.

In Chapter 6, a similar response in C2C12 was observed in myoblast cells, where IGF-1 (a growth factor with similar biological activity to insulin) failed to re-localise GFP-FOXO1 in the presence of Tumor Necrosis Factor-alpha (TNF-α) and the glucocorticoid dexamethasone.

Lee et al., (2003) and Giannakou et al., (2004) showed in C. elegans and Drosophila respectively suggest that in lower organisms FoxO orthologs (Daf-16 and dFOXO respectively) play a key
role in longevity and lifespan extension. Taken together, the data by Lee et al., (2003), Giannakou et al., (2004), Klonisky et al., (1999), and those data presented in Chapter 5 and those data presented in Chapter 6 tentatively suggest that FoxO may well be involved in the formation of autophagic vacuole formation. Future work will seek to determine whether FoxO1’s role in lifespan extension is mediated via its propensity to reduce mTOR signaling, and thereby, induce autophagic vacuole formation.
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**APPENDIX 1**

**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Implant Probe</th>
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<tr>
<td>PKCγ</td>
<td>TCAAGCAAGGACAGAATTCATT</td>
<td>GATGGTGCTTCAATGATTG</td>
<td>GATGGTGCTTCAATGATTG</td>
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<td>ERα</td>
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<td>CGTCCCTGTGACCTGTTCC</td>
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<td>HAM</td>
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<td>PARL</td>
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<tr>
<td>SDF</td>
<td>TCAACCTCAACGAGAGAACCT</td>
<td>CGTCCCTGTGACCTGTTCC</td>
<td>CGTCCCTGTGACCTGTTCC</td>
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**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (yrs)</th>
<th>Body Weight (kg)</th>
<th>Fasting blood glucose (mmol.L⁻¹)</th>
<th>Fasting plasma insulin (pmol.L⁻¹)</th>
<th>Fasting plasma FFA (mmol.L⁻¹)</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>41.9 ± 2.9</td>
<td>86.8 ± 1.7</td>
<td>4.5 ± 0.1</td>
<td>62.7 ± 7.6</td>
<td>0.9 ± 0.1</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>TYPE 2</td>
<td>43.7 ± 2.7</td>
<td>109.1 ± 9.6*</td>
<td>9.1 ± 1.4*</td>
<td>193.8 ± 60.2*</td>
<td>1.1 ± 0.1*</td>
<td>8.4 ± 0.6*</td>
</tr>
</tbody>
</table>

Relationship between glucose infusion rate (GIR) and citrate synthase maximal activity (top), relationship between mitochondrial transcription factor A (TFAM) and citrate synthase maximal activity (middle) and relationship between nuclear respiratory factor-1 (NRF-1) and citrate synthase maximal activity (bottom) in patients with type 2 diabetes (open symbols) and control subjects (closed symbols) A decrease in the CT value of 1 denotes a halving in copy number (n=7 Type 2, n=9 Control).
**APPENDIX 2**

<table>
<thead>
<tr>
<th></th>
<th>pBABE</th>
<th>FoxO1-215</th>
<th>CA-FoxO1</th>
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<tbody>
<tr>
<td><strong>TAM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 H</td>
<td></td>
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<td></td>
</tr>
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<td>8 H</td>
<td></td>
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<td>12 H</td>
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<td></td>
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<tr>
<td>24 H</td>
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</table>

Total and phospho Akt protein abundance in murine C2C12 skeletal muscle myotubes stably expressing various FoxO1 constructs. Total and phosphorylated (Ser 473) Akt protein abundance in C2C12 wildtype (pBABE), dominant negative (FoxO1-215) or constitutively active (CA-FoxO1) myotubes treated with (+) or without (-) 4-OH TAM. No significant differences detected (P <0.05) compared with 0h.