BIOMECHANICAL FORCES UPREGULATE MYOGENIC GENE INDUCTION IN THE PRESENCE OR ABSENCE OF INFLAMMATION - A POSSIBLE ROLE OF IGFR1-PI3K-AKT PATHWAY

DISSERTATION

Presented in Partial Fulfillment for the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

by
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* * * * *

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ABSTRACT

C2C12 myoblasts proliferate in response to mitogens and, upon mitogen withdrawal, differentiate into multinucleated myotubes. Over the past decade, several studies have unraveled important mechanisms by which the four myogenic regulatory factors, MRF’s (Myod1, Myf5, myogenin, and MRF4/Myf6) control the specification and the differentiation of the muscle lineage. The members of bHLH transcription factors act synergistically with the myocyte enhancer binding factor-2 (MEF2) family of co-factors to induce synthesis of muscle restricted target genes. It is well established that Myod1 and Myf5 are required for commitment to the myogenic lineage, whereas myogenin plays a critical role in the expression of the terminal muscle phenotype previously established by Myod1 and Myf5, and MRF4 partly subserves both roles.

In this study, we first induced C2C12 myoblasts to differentiate by transferring from growth medium (GM) to differentiation medium (DM) and characterizing the phenotype of C2C12 cells and examining the expression patterns of crucial myogenic factors and its targets genes. Later, by means of an in vitro model system, cyclic equibiaxial stretching and treatment with a proinflammatory cytokine, TNF-α, we have examined the effects of tensile forces imposed on C2C12 myoblasts.

The expression pattern of Myod1 showed a considerable presence in the undifferentiated myoblast and during the early stages of differentiation with a modest increase over the final days of myotube formation. Concomitantly, expression of the Mef2a transcription factor and the cell cycle inhibitor, Cdkn1a, were increased, marking the onset of myogenesis and cell cycle withdrawal. The expression of myogenin was little to none during the first 2 days with levels rising rapidly during the next 3 days of differentiation. As expected, myosin heavy chain isoforms and α-tropomyosin showed a rapid increase following the expression of myogenin and, by day 4, multinucleated myotubes had formed, staining positively for myosin heavy chain protein.
TNF-α inhibits skeletal muscle differentiation via activation of the NF-κB signal transduction pathway and its subsequent induction of Nos2a and NO production, leading to MYOD1 protein loss. Therefore, we used Nos2a mRNA expression as a biomarker to optimize the dose and magnitude of CTS required for the inhibition of inflammatory responses. The results demonstrate that C2C12 myoblasts respond to mechanical signals in a magnitude-dependent manner, inhibiting rhTNF-α-induced Nos2a expression to various degrees in a magnitude-dependent manner.

Next we examined salient members of the bHLH and MEF2 family of proteins to delineate the mechanisms of CTS-mediated regulation of skeletal muscle phenotype in the presence or absence of inflammation. At low physiological levels, CTS activates one of the earliest events in muscle cell differentiation by augmenting rapid and significantly greater expression, synthesis, and nuclear translocation of Myod1. Further, the nuclear Myod1 and myogenin levels are upregulated by CTS even in the presence of TNF-α, and mRNA expression of these genes is significantly increased. The function of bHLH and MEF2 transcription factors, in context of the myogenic differentiation, is to upregulate induction of skeletal muscle structural proteins. CTS markedly upregulates the transcriptional activation of Myh1, Myh2 and Myh4, and synthesis of MYHC and TPM1 proteins.

It is well accepted that muscle cells are responsive to biomechanical signals such as those experienced during exercise and can upregulate skeletal muscle hypertrophy. Mechanical signals have been shown to phosphorylate and activate AKT and its downstream target mTOR resulting in increased proteins synthesis in myotubes. Similarly, mechanical stretch activates epidermal growth factor receptor and angiotensin II type 1 receptor, resulting in AKT phosphorylation. The effects of TNF-α alone or in addition to CTS on IGFR1-PI3K-AKT pathway and the early events leading to AKT activation were examined next. We found that GSK3β was phosphorylated by CTS as opposed to the unstretched groups. A delay in activity, as compared to AKT phosphorylation, possibly could be a result of the intermediate steps that are involved before GSK3β phosphorylation. Further, our results show that after blocking the PI3K activity, using LY294002, the CTS mediated phosphorylation of AKT is blocked at the...
Ser 473. Finally by using different inhibitors, such as AG1024 that blocks the actions of IGFR1 and IR kinases and pertussis toxin that blocks the effects of G-protein coupled receptor, we examined if mechanotransduction occurs at the receptor level by activating multiple substrates including growth factor, insulin and G- coupled receptors.

While numerous studies have examined the mechanoresponsiveness of muscle tissues in vivo, only a few studies have elucidated the mechanisms of stretch-mediated upregulation of myogenesis in vitro. Elucidation of such intracellular mechanisms is critical because it provides pieces of missing information to the response of muscle tissues during exercise and other mechanical activities. Biomechanical signal mediated myogenesis is likely to be beneficial if the augmented myogenesis could be reproduced in vivo.

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Dedicated to my family for their
unconditional love and unwavering support
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<td>Alpha Skeletal Muscle Actin</td>
</tr>
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<td>ACTB</td>
<td>Beta Actin</td>
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<td>AKT/PKB</td>
<td>Thymoma Viral Proto-oncogene 1 / Protein Kinase B</td>
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<td>ANOVA</td>
<td>Analysis Of Variance</td>
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<td>bHLH</td>
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<td>CDKN1A</td>
<td>Cyclin Dependant Kinase Inhibitor 1A</td>
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<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<tr>
<td>IKK</td>
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<td>ILK</td>
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<td>Insulin Receptor Substrate</td>
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<tr>
<td>MAFbx</td>
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<td>MADS box transcription enhancer factor 2</td>
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<td>MRF</td>
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<td>SMD</td>
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<td>STDEV</td>
<td>Standard deviation</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor alpha</td>
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<td>Tumor Necrosis Factor Receptor 1</td>
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<td>TPM1</td>
<td>Tropomyosin 1</td>
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CHAPTER 1

Introduction

1.1 Overview

Muscle is a unique tissue that is exquisitely tailored for biomechanical force generation and movement. Skeletal muscle differentiation is a well established process in which specific molecular and cellular events occur in a precisely defined spatial and temporal manner. In chronic inflammatory conditions, such as sepsis, diabetes and infections, multiple proinflammatory cytokines like TNF-α and IFN-γ cause activation of NF-κB, resulting in the loss of myogenic proteins and leading to the disruption of the differentiation process and ultimate muscle degeneration (Ladner et al., 2004; Guttridge, 2004). The effects of biomechanical forces on skeletal and cardiac muscle in particular, have been studied quite extensively and the effects of mechanical stretch on the differentiation process are a subject of controversy. Pro-differentiation studies have shown that biomechanical signals promote nuclear translocation of myogenic transcription factors and increase the expression of myosin heavy chain proteins (Rauch and Loughna, 2004; Sakiyama et al., 2005). Moreover, mechanical signals have been shown to phosphorylate and activate AKT and its downstream target mTOR resulting in increased proteins synthesis in myotubes (Hornberger et al., 2004). Similarly, mechanical stretch activates epidermal growth factor receptor and angiotensin II type 1 receptor, leading to AKT phosphorylation and its activation (Kippenberger et al., 2004). Contrarily, Kumar et al., 2004, have shown that cyclic mechanical strain inhibits skeletal myogenesis through the activation of focal adhesion kinase, Rac-1 GTPase and NF-κB transcription factor. Despite these contradictory claims, the effects of biomechanical signals on skeletal myogenesis during chronic inflammatory conditions remain largely unexplored. The primary research goal of this dissertation is to identify the effects of
mechanical forces on skeletal muscle differentiation during inflammatory conditions and dissect the actions of these forces on the regenerative IGFR1-PI3K-AKT pathway.

1.2 Skeletal muscle differentiation

Skeletal muscle proliferation and differentiation is a highly regulated multi-step process during which myoblasts irreversibly exit from the cell cycle and fuse to form multinucleated myotubes. The entire process is under the control of several basic Helix Loop Helix (bHLH) family of transcription factors and the MADS box transcription enhancer factor 2 (MEF2) family of proteins (Berkes and Tapscott, 2005; Molkentin and Olson, 1996). The bHLH family, also known as MRF’s (myogenic regulatory factors), includes Myod1, myogenin (Myog), Myf5, and MRF4 (Myf6). The transcription of muscle specific genes is activated by the binding of Myod1 to a consensus sequence called the E-box, present in the regulatory region of these muscle specific genes. One of the early targets is myogenin, an early marker for myoblast commitment to differentiation. Following the upregulation of myogenin, the cyclin dependent protein kinase inhibitor, Cdkn1a / p21 is induced, resulting in an irreversible exit from the cell cycle and commitment to the differentiation program (Fujio and Guo, 1999; Lawlor and Rotwein, 2000). Evidence indicates that the Myod1 and MEF2 interact with each other synergistically to induce muscle restricted-target gene expression (Wang et al., 2001; Wilson-Rawls et al., 1999).

1.2.1 Myogenic regulatory factors

The MRF’s are critical for the determination and terminal differentiation of skeletal muscle. In the two decades since their discovery, in vivo studies have elucidated the specific roles of Myod1 and its relatives Myf5, myogenin, and MRF4, while cell culture studies have uncovered the basic mechanisms by which they function in transcription. Commitment to the myogenic lineage requires the upregulation of the myogenic regulatory factor family (MRF’s). This is demonstrated by the total loss of skeletal muscle in Myod1:Myf5 double knockout mice (Kablar et al., 1998). Proliferating myoblasts withdraw from the cell cycle to become terminally differentiated myocytes.
that express the “late” MRFs, Myogenin and MRF4, and subsequently muscle-specific genes such as myosin heavy chain (MYHC) and α-tropomyosin (TPM1).

Taken together, these studies suggest that Myod1 and Myf5 are required for commitment to the myogenic lineage, whereas myogenin plays a critical role in the expression of the terminal muscle phenotype, previously established by Myod1 and Myf5, and MRF4 partly subserves both roles. Thus, Myod1 and Myf5, and to an extent, MRF4, are considered “commitment” factors, whereas myogenin is a “differentiation” factor, and MRF4 has aspects of both functions (Figure 1.1). Although the expression patterns and general functions of each of the MRF’s are clearly distinct from one another, the specific target genes of each factor in vivo are not known.

1.2.2 MEF2 family of myogenic transcription factors

The MRF’s are assisted by the MEF2 family of transcription factors in order to mediate expression of muscle-specific genes (Black and Olson, 1998). MEF2 proteins belong to the MADS (MCM1, agamous, deficiens, serum response factor) box-containing transcription factor family. The MEF2 family consists of four members, MEF2A-D, each of which is encoded on a separate gene. While the expression of MRF’s is restricted to muscle, MEF2 genes are expressed widely during development. MEF2 on its own, does not possess the intrinsic ability to recapitulate the myogenic differentiation program, nevertheless, Mef2 and Myod1 interact directly in vitro and activate transfected reporters driven by E boxes and Mef2 binding sites (Molkentin et al., 1995). The E boxes and Mef2 binding sites are often located within close proximity in the promoters, providing further support for a cooperative interaction between Myod1 and Mef2 (Wasserman and Fickett, 1998).

1.2.3 Myogenic target genes

The MRF’s, together with Mef2 family proteins coordinate the activities of a host of co-activators and co-repressors, resulting in a tight control of gene expression during myogenesis. The events occurring at muscle-specific promoters have been dissected in detail, uncovering a multitude of functional and direct interactions between MRF’s,
signaling proteins, chromatin modifying factors, and other transcriptional regulators. Myod1 activation leads to robust expression of several well-characterized target genes such as myogenin, M-cadherin, myosin heavy and light chains, and muscle creatinine kinase. In addition to these muscle-specific genes, a study by Otten et al., 1997, has shown that Myod1 upregulates expression of the cyclin-dependent kinase inhibitor \textit{Cdkn1a/p21}, causing an irreversible exit of the differentiating cells from the cell cycle.

The genes that are expressed during myogenesis have been characterized by many groups. These studies show that Myod1 regulates genes expressed at different times during myogenesis, and promoter-specific Myod1 binding is a major regulatory mechanism for specific patterns of gene expression (Bergstrom et al., 2002). Further analysis by Delgado et al., 2003, reveals the existence of 12 groups of coordinately regulated genes that are expressed prior to the transcriptional induction of myogenin. Although clusters of genes are expressed at different times following Myod1 induction, chromatin immunoprecipitation experiments indicate that Myod1 directly binds to the regulatory elements of genes expressed throughout the differentiation program. Moreover a study by Penn et al., 2004, shows a feed-forward mechanism wherein some genes are activated immediately by Myod1 and these genes cooperate with Myod1 to express later Myod1 targets.

1.3 Chronic muscle inflammation and muscle atrophy

Chronic low-grade inflammation results in muscle wasting in disorders such as cachexia, sepsis, type 2 diabetes, and atherosclerosis. In all of these disorders there is an increase in systemic levels of proinflammatory cytokines TNF-\(\alpha\), IFN-\(\gamma\), IL-1\(\beta\), and IL-6. An increase in these cytokines limits the ability of muscle to regenerate by causing downregulation of myogenic transcription factors (Myod1, myogenin, MEF2), and breakdown of muscle proteins such as myosin heavy chains (MyHClIa, MyHClIib), troponin (TnI, TnT), and \(\alpha\)-tropomyosin (TPM1) (Acharrya et al., 2004; Guttridge, 2004). Muscle wasting seen in such catabolic states appears to be mediated by activation of ubiquitin and proteasome-dependent pathways through increases in the expression of relevant proteins, including ubiquitin E2-conjugating enzyme and subunits of the
proteasome (Lecker et al., 1999). Moreover, multiple different perturbations can cause skeletal muscle atrophy by means of NF-κB and AP-1 activation (Wyke and Tisdale, 2005). NF-κB activation has been shown to induce transcriptional upregulation of E3 ubiquitin-ligase MuRF1. Similarly, MAFbx is also upregulated by p38 MAPkinase resulting in muscle atrophy. Both of these kinases are inhibited by the FOXO family of transcription factors which are one of the AKT targets (Figure 1.3) (Glass, 2005). Collectively, TNF-α and IFN-γ-induced inhibition of myogenic protein synthesis and the induction of proteases and inflammatory mediators result in progressive loss of muscle function.

1.4 Role of NF-κB in muscle atrophy

NF-κB consists of rel A (p65), rel B, c-rel, p50 and p52 proteins. The p50 and p52 are cleaved from p105 and p102 inhibitory proteins. NF-κB is a ubiquitous rapid response transcription factor involved in immune and inflammatory reactions and mediates expression of cytokines, and several proteins involved in proinflammatory reactions. TNF-α receptor complex activation (Sheikh and Huang, 2003; Jin and El-Deiry, 2005) is followed by a series of phosphorylation reactions of proteins in the receptor complexes, and leads to cytokine specific activation of TNF-receptor-Associated Factors-2 (TRAF-2). This in turn, recruits Receptor Interacting Kinase (RIP) that phosphorylates MEKK-3. MEKK-3 phosphorylates IKK-α / IKK-β for their activation. NF-κB in the cytosol is sequestered by inhibitory proteins, I-κBα and I-κBβ (Viatour et al., 2005; O'Connor et al., 2005). IKK-α and IKK-β phosphorylate I-κBα/β, which is rapidly degraded by the 26S proteosomes, allowing nuclear translocation of NF-κB. The I-κB family contains several proteins, including I-κBα, I-κBβ, p105, Bcl-3, p100, I-κBγ and I-κBε (Jimi and Ghosh, 2005). However, I-κBα and I-κBβ are the major proteins that bind NF-κB and inhibit its translocation to the nucleus. The inducible form of NF-κB is a heterodimer composed of NF-κB1 (p50) and Rel A (p65). Activation of various subunits of NF-κB and I-κB is believed to regulate induction / inhibition of various proinflammatory genes (Jimi and Ghosh, 2005; Xiao, 2004) (Figure 1.2). The ability of
TNF-α to promote muscle loss and prevent differentiation has been extensively studied. Either on its own or in combination with other cytokines like IFN-γ, TNF-α can induce the breakdown of myotubes through the loss of myosin heavy chain protein (Guttridge et al., 2000). TNF-α mediated inhibition of Myod1 synthesis by activating the NF-κB pathway is well documented in the literature (Guttridge et al., 2000; Ladner et al., 2003). Coletti et al., 2002, have shown a novel pathway by which TNF-α inhibits muscle differentiation through activation of caspases by an NF-κB independent pathway. Moreover, previous studies, albeit not extensive, have shown that TNF-α could activate the AP1 transcription factor to inhibit myogenesis through the modulation of Myod1 activity (Bengal et al., 1992; Chen et al., 2006). Taken together, it seems likely that TNF-α activation of NF-κB is one of the intracellular signaling mechanisms mediating muscle atrophy during chronic inflammation.

1.5 NOS2A and myogenesis.

Although it is well established that TNF-α inhibits skeletal muscle differentiation via activation of the NF-κB signal transduction pathway (Guttridge et al., 2000) the effects of subsequent induction of NOS2A and NO production on skeletal muscle differentiation is quite controversial. Lee et al., 1997, have shown that NF-κB dependent expression of Nos2a is required for myoblast fusion in chick embryonic cells, while Kaliman et al., 1999, have shown that IGF-II induces NF-κB and NOS2A downstream of PI3K action that is critical for myogenesis. Similar studies by Piao et al., 2005, shows that NADPH oxidase (NOX2) stimulates muscle differentiation downstream of the PI3K / p38 MAPK pathway by activating the NF-κB / NOS2A pathway via the generation of free radicals.

On the contrary, Di Marco et al., 2005, have shown how various cytokines like TNF-α and IFN-γ can cause activation of inducible nitric oxide gene, NOS2A and downregulate MYOD1 protein in myoblasts and myotubes. The fold increase in the transcription rate of the Nos2a gene by cytokines is different than the final mRNA expression. The human Nos2a mRNA has a destabilizing sequence which is an AU-rich
element (ARE) consisting of repeats of a pentamer in its 3' UTR. The mouse homolog is known as miNOS-ARE. HuR, an mRNA binding protein, binds with ARE’s and stabilizes the mRNA. Stable Nos2a mRNA leads to NOS2A protein synthesis and the end result is increased NO production, superoxides and peroxynitrite free radical generation. Subsequently, peroxynitrite released from the cell can act in an autocrine fashion causing destabilization of the Myod1 mRNA message or breakdown of the final MYOD1 protein.

### 1.6 AKT and skeletal muscle differentiation

Insulin-like growth factors (IGF-I and IGF-II) are potent stimulators of muscle differentiation through the induction of myogenin and MEF2. Studies on signaling through the IGF receptor have revealed that insulin receptor substrate-1 (IRS-1) is tyrosine phosphorylated at multiple sites and acts as a docking site for phosphatidylinositol 3-kinase (PI3K). PI3K is the next major downstream signaling molecule which converts PIP2 to PIP3 resulting in AKT recruitment and its subsequent activation (Jiang et al., 1999; Nader, 2005). The serine / threonine protein kinase B (PKB), also known as AKT (v-AKT murine thymoma viral oncogene homolog), is phosphorylated by PI3K, and is essential for IGF-dependent differentiation (Jiang et al., 1998; Wilson et al., 2004). All of the AKT isoforms (AKT1, AKT2, and AKT3) are activated by growth factors in a PI3K-dependent manner.

AKT’s have been implicated in several cellular processes, such as cell survival, apoptosis, glucose uptake in myocytes and adipocytes, proliferation and differentiation (Datta et al., 1999; Yang et al., 2004). Earlier studies have shown that ectopic expression of activated AKT1 can promote extensive skeletal myoblast differentiation in different myoblast cell lines even in the absence of IGF-1, and can reverse the inhibitory effects of PI3K inhibitors like Wortmannin and LY294002 on myogenic differentiation (Rommel et al., 1999; Jiang et al., 1998). Of the three isoforms, AKT2 appears to be most intricately involved with muscle cell differentiation, as evidenced by: (i) microinjection studies of specific antibodies implicated AKT2, but not AKT1 with muscle differentiation; (ii) AKT1 is expressed constitutively whereas AKT2 expression is specifically induced during SMD; (iii) both the mRNA and protein levels of AKT1 are not changed whereas
AKT2 levels are elevated during muscle differentiation; and (iv) Myod1 transcriptionally regulates AKT2 by binding to the AKT2 promoter region, and AKT2 in turn triggers MYOD1-MEF2 transcriptional activity, resulting in increased myogenin expression (Altomare et al., 1998; Calera and Pilch 1998; Vandromme et al., 2001). Collectively, these observations strongly suggest that AKT2, but not AKT1, plays a specific role in myogenesis under physiological conditions.

1.7 Upstream events leading to AKT activation

Following is a brief description of upstream events involved in the activation of AKT pathways that may be regulated by biomechanical signaling. The serine / threonine protein kinase AKT is a major downstream target of PI3K and is an essential component of muscle cell differentiation. AKT has 3 different distinct domains. The pleckstrin homology domain (PH) is involved with binding to the PIP3, the regulatory domain with sites for phosphorylation leading to activation, and the catalytic domain, the kinase active region which it shares with other members of the AGC superfamily of kinases (protein kinase A / G / C) (Figure 1.3). AKT is activated by dual regulatory mechanisms that require both its membrane relocalization from the cytosol to plasma membrane and c-terminal phosphorylation at Thr308 / Ser473 (Hemmings, 1997).

In general, activation of AKT by growth factors (e.g., IGF-1) is mediated by PI3K. PI(3,4)P2 and PI(3,4,5)P3 are generated mainly by D3 phosphorylation of PI(4)P2 and PI(4,5)P3, respectively with activated class Ia or Ib PI3K. PI(3,4)P2 could be also generated by 5-dephosphorylation of PI(3,4,5)P3 by Src homology 2 (SH-2)-containing 5-phosphatase, SHIP. On the other hand, PTEN (phosphatase and tensin homolog), is a protein phosphatase that dephosphorylates PIP3 at the D3 position and acts as a negative regulator of PI3K/AKT signaling during myogenesis (Wan and Helman, 2003). PI(3,4)P2 and PI(3,4,5)P3 are capable of binding to the plekstrin homology (PH) domain of AKT and other kinases like PDK1 (Matsui et al., 2005). The binding of the plekstrin homology (PH) domain of AKT to PI(3,4)P2 and PI(3,4,5)P3 on the plasma membrane releases the autoinhibitory role of this domain, allowing phosphoinositide-dependent kinase-1 (PDK1) to phosphorylate AKT on Thr308. Thr308 phosphorylation partially
activates AKT while full activation requires phosphorylation at Ser473 which remains as yet unsolved, although the kinase responsible is referred to as PDK2 (Stokoe et al., 1997). Other possible activation mechanisms at the Ser473 involves autophosphorylation by AKT itself (Toker et al., 2000) and PDK1 phosphorylating both the Thr308 and Ser473 (Balendran et al., 1999). Furthermore, PI3K activity could be irreversibly inhibited by the fungal metabolite Wortmannin or the synthetic compound LY294002 (Figure 1.3).

1.8 AKT downstream targets.

Activated AKT phosphorylates and inhibits a host of other proteins that affect cell growth, cell cycle entry, and cell survival (Datta et al., 1999; Sen et al., 2003). The major downstream targets of AKT include GSK-3β, mTOR, FOXO, and IKK-α (Chen et al., 2005; Kumar and Madison, 2005). **GSK-3β**, a serine / threonine protein kinase which is constitutively active in unstimulated muscle cells phosphorylates many proteins including glycogen synthase, eIF2B, c-Myc, and cyclin D, keeping them inactive or promoting their degradation. Phosphorylation of GSK-3β suppresses its activity resulting in the activation of pathways that are normally repressed by GSK-3β (Fang et al., 2005; Vyas et al., 2002; Seimi et al., 2004). A second target of AKT is the serine / threonine kinase **mTOR**, (mammalian target of Rapamycin). Phosphorylation of mTOR by AKT results in an increase in protein translation by two mechanisms, (i) mTOR phosphorylates and activates p70S6K, a positive regulator of protein translation, (ii) mTOR phosphorylates and inhibits 4E-BP1 (PHAS-1), a negative regulator of protein initiation factor eIF-4E (Aoki et al., 2006; Bodine et al., 2001; Rommel et al., 2001).

The **FOXO** subfamily of Forkhead transcription factors consists of FOXO1A (FKHR), FOXO3A (FKHRL-1), and FOXO4 (MLLT7/AFX1), which are all inactivated by AKT. Phosphorylation of FOXO members by AKT leads to their retention in the cytoplasm. This failed nuclear translocation results in the inhibition of FOXO dependent transcriptional programs (Arden, 2004; Burgering and Medema, 2003; Kandarian and Jackman, 2006). These transcription factors are implicated in regulating diverse cellular functions including differentiation, proliferation and cell survival. In skeletal muscle, FOXO transcription factors promote atrophy by regulating the atrophy-related genes
(“atrogene”), MuRF-1/RNF28 and MAFbx/FBXO32 (Skurk et al., 2005; Stitt et al., 2004; Sandri et al., 2004). These atrogene products are E3 ubiquitin ligases that mediate protein ubiquitin conjugation (ubiquitination) and their subsequent targeting for proteosomal degradation. AKT activation has been shown to enhance the degradation of I-κB and co-operate with other factors to induce NF-κB (RelB/p52) activation. This ability to regulate NF-κB activity may be through direct interaction with IKKs, since AKT can associate with the IKK complex in vivo (Ouyang et al., 2006; Tanaka et al., 2005; Gustin et al., 2004). The molecular details of AKT-dependent regulation of the IKK complex are largely unknown. However, AKT has been shown to phosphorylate and activate IKKα at a critical regulatory site, Thr23 (Ozes et al., 1999; Chen et al., 2002). This mechanism is implicated in the survival signaling cascade from AKT to NF-κB resulting in the expression of pro-survival genes like Bcl-xL and caspase inhibitors such as BIRC2/c-IAP1 and BIRC3/c-IAP2. These findings support the premise that AKT can promote survival and enhance anabolic protein mechanisms by: (i) downregulating GSK-3β activity; (ii) preventing nuclear translocation of FOXO transcription factors; (iii) upregulating mTOR activity resulting in increased protein synthesis; and (iv) activating pro-survival genes through interaction with NF-κB signaling pathway.

1.9 Role of satellite cells in myogenesis and repair.

Satellite cells in skeletal muscle originate either from the pluripotent stem cells derived from the vasculature or residual myoblasts. They play a key role in repair and regeneration following injury to myoblasts. They lie beneath the basal lamina and express M-cadherin (M-cad) and also express myogenic factors including Myod1, myogenin and Myf5. During embryonic differentiation, mononucleated myoblasts first proliferate and then fuse to form myotubes that later get innervated and develop into muscle fibers. Following fusion, the myotubes enter post-mitotic state with no further divisions in the myotubes or the fibers. The extra nuclei required for growth are provided by satellite cells fusing with muscle fibers at the terminal region, which is the region responsible for longitudinal growth of muscle fibers. When muscle fibers sustain damage extra nuclei are needed for the repair process to prevent cell death. Loss of muscle fibers could result in a
decrease in muscle mass and a functional deficit. The extra set of genes required for protein synthesis during repair is normally derived from satellite cells.

1.10 IGF’s and repair.

IGF stimulation has been well documented to activate the PI3K-AKT pathway responsible for muscle differentiation, as well as protein synthesis and prevention of muscle atrophy (Sacheck et al., 2004; Stitt et al., 2004). The role of IGF-I and -II in regulating growth and development of various tissues has been known for many years. More recently, the paracrine / autocrine regulation of these hormones and their activity during skeletal muscle development and repair have become apparent. In vitro, IGF-I and IGF-II are able to alter MRF’s expression and promote proliferation and differentiation / fusion of myoblasts (Engert et al., 1996). The IGF gene could be spliced into different forms and the most commonly recognized ones are the IGF-IEa and MGF (mechano growth factor). MGF and IGF-IEa splice variants yield the same mature peptide, which is derived from the highly conserved exons 3 and 4 of the IGF-I gene. The functions of IGF-IEa are myoblast fusion and myotube formation, while MGF (also known as IGF-IEb) is responsible for myoblast proliferation and secondary myotube formation by generating satellite cells. The expression patterns are different between the different IGF splices with MGF peaking during 1-4 days of muscle injury, initiating proliferation and differentiation, while IGF-IEa, peaking at 7-10 days, is responsible for myotube formation (Cheema et al., 2005).

1.11 Biomechanics and muscle tissues.

The effects of biomechanical forces on skeletal muscle have been studied quite extensively but the mechanisms through which mechanical signals are transduced to chemical signals remain largely unidentified. Moreover the effects of these signals on skeletal muscle differentiation are conflicting based on the model system used. However, several findings have suggested that mechanical signal transduction in muscle may occur through signaling pathways that are downstream of IGF-I signaling, as validated by observations that muscle releases IGF-I on mechanical stimulation which is a potent
agent for promoting muscle growth and affecting phenotype, and that IGF-I can function in an autocrine fashion. Pro-differentiation studies have shown that biomechanical signals promote nuclear translocation of myogenic transcription factors which increases the expression of myosin heavy chain proteins (Rauch and Loughna, 2004; Sakiyama et al., 2005). Accumulating evidence shows that at least two signaling pathways downstream of IGF-I binding can influence muscle growth and adaptation. Signaling via the calcineurin / nuclear factor of activated T-cell pathway has been shown to have a powerful influence on promoting the slow / type I phenotype in muscle but can also increase muscle mass (Tidball, 2005).

Further, mechanical signals have been shown to lead to phosphorylation and activation of AKT and its downstream target mTOR, resulting in increased proteins synthesis in myotubes (Hornberger et al., 2004). Signaling via the AKT / mTOR pathway can also increase muscle growth, and recent findings show that activation of this pathway can occur as a response to mechanical stimulation applied directly to muscle cells, independent of signals derived from other cells. Similarly, mechanical stretch activates epidermal growth factor receptor and angiotensin II type 1 receptor, resulting in AKT phosphorylation and its activation (Kippenberger et al., 2004). Moreover, Cheema et al., 2005, have shown the effects of mechanical signals on IGF1 splice variants that both MGF and IGF-1Ea expression is increased with ramp loading, while cyclic stretch increases MGF with a simultaneous decrease in IGF-1Ea expression. In addition to the aforementioned regenerative effects, mechanical signals have been known to be anti-inflammatory by preventing the activation of NF-κB in chondrocytes (Deschner et al., 2003). Contrarily, Kumar et al., 2004, have shown that cyclic mechanical strain inhibits skeletal myogenesis through the activation of focal adhesion kinase, Rac-1 GTPase and NF-κB transcription factors.

1.12 Purpose of the present study

Here we hypothesized that biomechanical signals abrogate the actions of proinflammatory cytokines and regulate myogenesis by upregulating the expression of bHLH and MEF2 family of myogenic transcription factors, which eventually lead to
increased myoblast differentiation and myotube formation. Hence, we set out to determine the biomechanical signal-dependent spatial and temporal expression of myogenic transcription factors and muscle structural proteins in C2C12 myoblasts, in vitro. Additionally, the effects of biomechanical signals on the expression of differentiation-associated genes were assessed in the presence of a proinflammatory molecule, TNF-α.

**Our hypothesis**

“Biomechanical signals act on skeletal muscle cells in a magnitude and frequency dependent manner. At appropriate magnitudes and frequencies, these signals serve as potent regenerative signals that induce cell proliferation and differentiation in healthy muscle, as well as mitigate the morbidity caused by inflammatory mediators.”

Experiments documented herein are aimed at examining the cellular and molecular actions of biomechanical signals in the presence or absence of inflammation. This work was performed in an in vitro cell culture system using C2C12 myoblasts and provides us new insights into the dynamics of mechanical stretch-induced modulations of intracellular responses. Chapter 1 is a general introduction to the proposed body of research in this dissertation. Main topics include an elaborate description of (i) the skeletal muscle differentiation process, with emphasis on the interplay between the myogenic transcription factors, (ii) the effects of chronic inflammation on muscle atrophy, with a special focus on NF-κB signaling pathway, (iii) the activation and targets of PI3K-AKT signaling pathway, and (iv) the various theories regarding the potential mechanisms of actions of mechanical forces on muscle tissues. Chapter 2 examines the expression profiles of myogenic transcription factors and muscle structural proteins during the differentiation process, followed by optimization of the magnitudes and frequencies of biomechanical forces applied to achieve maximal anti-inflammatory effect in an in vitro cell culture model system. Chapters 3 is a continuation of chapter 2 which elucidates the effects of the optimal biomechanical force on the expression patterns of myogenic factors in the presence or absence of inflammation. Data in chapters 2 and 3
have been published recently (Chandran et al., 2007). Chapter 4 examines the actions of biomechanical forces in the activation mechanisms in IGFR1-PI3K-AKT pathway, and its downstream target, GSK3β and, finally, the mechanotransduction at the receptor level. Chapter 5 is the summary of findings and general discussion of events during myogenesis following the application of biomechanical forces.
Figure 1.1 Skeletal muscle differentiation processes. Skeletal muscle proliferation and differentiation are highly regulated multi-step processes during which myoblasts irreversibly exit from the cell cycle and fuse to form multinucleated myotubes. The entire process is under the control of several of the basic Helix Loop Helix (bHLH) family of transcription factors and MADS box transcription enhancer factor 2 (MEF2) family of proteins. The bHLH family includes Myod1, myogenin (Myog), Myf5, and MRF4 (Myf6); while the MEF2 family consists of four members, MEF2A-D. The members of the Myod1 and MEF2 families interact with each other synergistically to induce muscle-restricted target gene expression such as that of myosin heavy chains, myosin light chains, α-tropomyosin, troponin I and T.
Figure 1.2 The role of NF-κB mediated muscle atrophy. TNF-α receptor complex activation is followed by a series of events involving TNF-Receptor-Associated Factor-2 (TRAF-2), Receptor Interacting Kinase (RIP) and MEKK-3, leading to IKK-α / IKK-β phosphorylation. NF-κB in the cytosol is sequestered by inhibitory proteins, I-κBα and I-κBβ. IKK-α and IKK-β phosphorylate I-κBα/β, which is rapidly degraded by 26S proteosomes, allowing nuclear translocation of NF-κB, leading to (1) expression of various proinflammatory genes such as NOS2A, COX-2, etc. leading to Myod1 mRNA loss and (2) directly binding the Myod1 promoter regions and preventing its expression.
Figure 1.3 IGFR1-PI3K-AKT pathway. Activation of IGFR1 by Insulin-like Growth Factors (IGF-I and IGF-II) results in the recruitment of insulin receptor substrate-1 (IRS-1) which is tyrosine phosphorylated at multiple sites and acts as a docking site for Phosphatidyl Inositol 3-Kinase (PI3K). PI3K is the next major downstream signaling molecule which phosphorylates PI(4)P2 and PI(4,5)P3 to PI(3,4)P2 and PI(3,4,5)P3. On the other hand, PTEN (phosphatase and tensin homolog) is a protein phosphatase that dephosphorylates PIP3 at the D3 position and acts as a negative regulator of PI3K-AKT signaling during myogenesis. The major downstream targets of AKT include GSK-3β, mTOR, FOXO, and IKK-α. (1) The FOXO subfamily of forkhead transcription factors which consists of FOXO1A (FKHR), FOXO3A (FKHRL-1), and FOXO4 (MLLT7/AFX1) are all inactivated by AKT. Phosphorylation of FOXO family members by AKT leads to their retention in the cytoplasm. This failed nuclear translocation results in the inhibition of FOXO dependent transcriptional programs. In skeletal muscle, FOXO transcription factors promote atrophy by regulating the atrophy-related genes
(“atrogenes”), MuRF1/RNF28 and MAFbx /FBXO32. (2) A second target of AKT is the serine/threonine kinase mTOR, (mammalian target of Rapamycin). Phosphorylation of mTOR by AKT results in an increase in protein translation by two mechanisms, (i) mTOR phosphorylates and activates p70S6K, a positive regulator of protein translation, (ii) mTOR phosphorylates and inhibits 4E-BP1 (PHAS-1), a negative regulator of protein initiation factor eIF-4E. (3) GSK-3β, a serine / threonine protein kinase which is constitutively active in unstimulated muscle cells phosphorylates many proteins, including glycogen synthase, eIF2B, c-Myc, and cyclin D, keeping them inactive or promoting their degradation. (4) AKT activation has been shown to enhance the degradation of I-κB and to co-operate with other factors to induce NF-κB (RelB/p52) activation.
Figure 1.4 Hypothesized actions of biomechanical signals. CTS abrogates the actions of TNF-α-mediated inhibition of skeletal muscle differentiation and simultaneously upregulates skeletal muscle differentiation (SMD). Insulin-like Growth Factors (IGF-I) stimulates muscle differentiation by activating the PI3K-PIP3-AKT pathway triggering Myod1-MEF2 transcriptional activity. Expression and synergistic interaction of bHLH transcription factors, MYOD1 and MYOG, and the MEF2 family of proteins regulate the sequential steps in myogenesis, resulting in the induction of muscle restricted target genes such as Myosin Heavy Chain (MYHC), Myosin Light Chains (MYLC) and α-Tropomyosin (TPM1). On the contrary, TNF-α induces expression of proinflammatory molecules like NOS2A, TNF-α, and TNFR1 via the NF-κB pathway and, in parallel, inhibits MYOD1 and MYOG expression and/or protein loss, consequently blocking the expression of muscle specific proteins. CTS (1) abrogates the TNF-α induced expression of NOS2A synthesis and, (2) upregulates MYOD1 induction by activating the AKT pathway.
CHAPTER 2
Characterization of C2C12 myoblasts and optimization of biomechanical signals on C2C12 cells

2.1 Introduction
Throughout life, muscle is constantly exposed to mechanical forces. Muscle cells are equipped with an inherent capacity to generate, perceive, and respond to biomechanical stimuli (Vandenburgh, 1992; Tidball, 2005). Many lines of investigations provide evidence that biomechanical forces regulate muscle cell function and a moderate amount of mechanical loading is essential for muscle homeostasis (Goldspink, 1998; Cheema et al., 2005). Signals generated by mechanical forces are converted into molecular events that in turn regulate multiple anabolic and catabolic processes like proliferation, organogenesis, repair, regeneration, fatigue, and muscle pathologies (Guttridge et al., 2000; Henderson and Carter, 2002). For example, skeletal muscle hypertrophy in response to exercise, muscle atrophy with immobilization, and satellite cell proliferation following mobilization of injured or inflamed muscle, all point to the mechanosensing and mechanoresponsiveness of muscle cells (Aikawa et al., 2001; Cheema et al., 2005; Powell et al., 2002). However, the mechanisms of action of biomechanical signals that regulate gene expression during skeletal muscle hypertrophy and differentiation, during inflamed conditions, are less understood.

Muscle development is a complex process in which undifferentiated myoblasts exit the cell cycle and undergo fusion to form myotubes. In vivo, skeletal muscles are populated with satellite cells that serve as predecessors of myoblastic cells, and undergo differentiation into myotubes in a highly specific spatial and temporal sequence of molecular events. However, the majority of investigations have utilized cell lines of myoblastic phenotype such as murine C2C12 cells, to experimentally delineate the
multistep process of muscle cell differentiation (Dodou and Xu, 2003; Fujio and Guo, 1999). Expression of the bHLH family of transcription factors and the MADS box transcription enhancer factor 2 (MEF2) family of proteins are required for the regulation of sequential steps in myogenesis. The first muscle specific transcription factor expressed in this process is **Myod1**, which binds to an E-box located in the consensus sequences of the regulatory region of muscle specific genes (Berkes and Tapscott, 2005; Molkentin and Olson, 1996). **MYOD1** induces **Myog**, another early target gene recognized as a biomarker indicating the commitment of myoblastic cells to differentiation (Naidu et al., 1995; Rawls et al., 1998). Thereafter, expression of proteins belonging to MEF2 family and their synergistic interaction with MYOD1 results in the induction of muscle restricted target genes (Wang et al., 2001; Wilson-Rawls et al., 1999). Concurrently, induction of the **Cdkn1a** mRNA expression and CDKN1A protein synthesis leads to the irreversible exit of cells from the cell cycle and their commitment to the differentiation program (Fujio and Guo, 1999, Lawlor and Rotwein, 2000). These events are followed by transcriptional upregulation of skeletal muscle-specific genes such as **Myh1**, **Myh2** and **Myh4** which are responsible for the synthesis of myosin heavy chains, as well as **Tpm1** (α-tropomyosin), **Tnni** (troponin I) and **Tnnt** (troponin T) and other genes required for muscle assembly and function (Lawrence et al., 1989; Lyons et al., 1990; Miller, 1990). In parallel, differentiating myoblastic cells in close proximity fuse to form multinuclear myotubes. The entire process of myogenesis is carefully controlled at each step and any disturbances critically modulate its outcome.

Muscle inflammation is shown to initiate degradation of intrinsic muscle proteins as well as inhibit myogenesis by suppressing expression of differentiation-associated molecules and eventually myotube formation in C2C12 cells *in vitro* (Guttridge, 2004). Treatment of C2C12 myoblast-like cells with a proinflammatory cytokine, TNF-α, leads to the loss of **MYOD1**, **MYOG**, **CDKN1A**, **MYHC**, and **TPM1** proteins, resulting in the eventual inhibition of myotube formation (Acharrya et al., 2004; Guttridge, 2004). TNF-α inhibits skeletal muscle differentiation via induction of **Nos2a** and NO production in an NF-κB dependent manner, which subsequently leads to **Myod1** mRNA degeneration (Dimarco et al., 2005; Guttridge et al., 2000). Furthermore, several genetic approaches have
demonstrated that TNF-α negatively regulates the process of myogenesis in vitro (Guttridge et al., 2000; Ladner et al., 2003). Conversely, mechanical loading of chronically or acutely inflamed muscles has been shown to regain muscle strength and function in vivo by mechanisms as yet little understood.

To study mechanotransduction / mechanosensory responses of muscle cells, it is vital to apply clearly defined mechanical stimuli. Such an application can be accomplished through the use of in-vitro stretch devices. These devices are used in a cell culture environment and the forces delivered to the culture membrane can be controlled and well defined. In-vitro stretch devices are classified as uniaxial or multiaxial (Figure 2.11). The major difference between these uniaxial and multiaxial stretch devices is the type of mechanical forces delivered to the elastic culture membrane. A vacuum operated flexcell stretch device can apply both the stretch regimens by using different loading posts. Unrestricted airflow through the stretch plates diminishes the potential of developing internal pressure gradients and the rate of change in the membrane is a true reflection of the pressure change in the system.

In a uniaxial stretch application, the tension is applied in a single direction and the applied tension is uniformly distributed with a minor degree of compression along the transverse axis. This is very similar to deformation experienced by a single myoblast when stretched uniaxially. The strains experienced are longitudinal and transverse strains. The change in surface area can be calculated by finite element analysis and indicated by ∆SA, and is less than the strain applied due to the compression along the transverse axis. During multiaxial stretch applications, the cells experience radial and circumferential strains and it is distributed non-uniformly with no degree of compression. There is a tendency for increased radial strains and decreased circumferential strains peripherally compared to the center. Infact, the radial strains at the periphery could reach twice the strain values in other regions. The change in surface area can be calculated by spherical deflection geometry indicated by ∆SA, which is greater than the strain applied and there is no degree of compression along any axis (Figure 2.11).

Here we hypothesized that biomechanical signals regulate myogenesis despite the presence of inflammatory molecules, and upregulate expression of the bHLH and MEF
family of myogenic transcription factors, which eventually lead to increased myoblastic differentiation and resultant myotube formation. Hence, we determined the biomechanical signal-dependent spatial and temporal expression of myogenic transcription factors and muscle structural proteins in C2C12 myoblasts, *in vitro*. Additionally, the effects of biomechanical signals on the expression of differentiation-associated genes were assessed in the presence of a proinflammatory molecule TNF-α. These biomechanical signal induced events act as potent anti-inflammatory signals that suppress TNF-α-induced *Nos2a* mRNA and protein expression in C2C12 cells. We show that biomechanical forces upregulate myogenic gene expression as well as promote differentiation by increased synthesis of myogenic proteins in an environment primed for inflammation. Here we examine the expression profiles of myogenic transcription factors and muscle structural proteins during the differentiation process followed by optimization of the magnitudes and frequencies of biomechanical forces applied to achieve maximal anti-inflammatory effect in an in-vitro cell culture model system. The effects of the optimal biomechanical force on the expression patterns of myogenic factors and its proteins in the presence or absence of inflammation is presented in Chapter 3.

2.2 MATERIAL AND METHODS

**Cell culture.** Murine C2C12 cells with a myoblast-like phenotype were propagated in growth medium (GM) containing Dulbecco’s modified Eagle’s medium with high glucose (DMEM-H), 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) (Invitrogen, CA). Cells were maintained at subconfluent densities and passaged every 3-4 days. To induce myoblast differentiation, cells were grown to 60–70% confluence in GM, washed once in phosphate-buffered saline (PBS), and subsequently cultured in differentiation medium (DM) containing DMEM-H supplemented with 2% horse serum (Invitrogen, CA), penicillin (100 U/ml), streptomycin (100 µg/ml), and 100 ng/ml insulin. Under these conditions, C2C12 cells formed myotubes within 3 to 5 days.
Application of Cyclic Tensile Strain (CTS). C2C12 cells were seeded in GM at 3x10^5 cells/well on Bioflex Collagen Type I coated 6-well plates (Flexcell International, NC) and grown to 70% confluence (2-3 days). The medium was replaced with DM and cells were subsequently subjected to CTS at an optimal magnitude and frequency using a Flexcell 4000T/FlexLink Cell Culture System (Flexcell International) (Figure 2.10) in the presence or absence of recombinant human tumor necrosis factor-α (rhTNF-α; 5 ng/ml) (Table 2.1). The effects of CTS were examined following a 24, 48, or 72 h exposure to CTS. Control (untreated) cells and cells exposed to rhTNF-α alone in each assay were cultured in DM on Bioflex Collagen Type I coated 6-well plates, but not exposed to CTS.

RNA purification and real-time polymerase chain reaction. Total RNA was isolated from cells using the RNeasy Mini kit (Invitrogen, CA), subjected to DNase I digestion (Ambion, TX), and stored in DNase-/RNase-/Protease-free water at -80°C. The concentration, purity and integrity of RNA were assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE) or Experion automated electrophoresis system (Bio-Rad Laboratories, CA). One µg of total RNA was reverse transcribed with 0.5 µg of oligo d(T)12-18, 1X First Strand Synthesis Buffer, 5 mM DTT, 200U/µl of Superscript III reverse transcriptase, and 40U/µl of RNaseOUT inhibitor (Invitrogen, CA) for 60 min at 55 °C. Real-time RT-PCR was performed using TaqMan gene expression assays as recommended by the manufacturer (Applied Biosystems, CA), and data acquired using an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, CA). The following pre-validated TaqMan assays were obtained from Applied Biosystems: NOS2A (Mm00440485_m1), Myod1 (Mm00440387_m1), Myog (Mm00446194_m1), Myh1 (Mm01332500_gH) and Cdkn1a (Mm00432448_m1) (Table 2.2). Custom TaqMan assays were developed as necessary using Primer Express software (Applied Biosystems, CA,). SYBR Green based real-time PCR primers were designed using OLIGO software (Molecular Biology Insights, CO). iQ SYBR Green PCR Supermix (Bio-Rad Laboratories, CA) containing assays were performed at optimized annealing temperatures as determined empirically for each primer set. Primers were
designed to span exon/intron junctions to diminish the chances of spurious genomic DNA amplification. The SYBR Green real-time primer sequences used were as follows: Mef2a sense, 5'-CAG GCT TCA GCC TGG CAG CAG-3', antisense 5'-GCT GGA GCT GCT CAG ACT GTC CAC-3'; Myh2 sense 5'-AGC AGA AGA GGA AGA GAA GCA GGA AG-3', antisense 5'-CTT CAG CTC CTC CGC CAT CAT G-3'; Myh4 sense 5'-CAC CTG GAG ATG AAC AAC-3', antisense 5'-GTC CTG TAG AAC AGC CGA CG-3'; Tpm1 sense 5'-ATC ATC AGG AGA GGA GCA GGA AG-3', antisense 5'-CTT CTT TGG CAT GGG CCA CTT TC-3'; Rps18 sense 5'-GGA AAA TAG CCT TCG CCA TCA TC-3', antisense 5'-GCC AGT GGT CTT GGT GTG AC-3' (Table 2.3). The data obtained by real time RT-PCR were analyzed by the comparative threshold (C_T) cycle method. In this method, the amount of the experimental target, normalized to a housekeeping gene transcript (Rps18), and relative to a calibrator (either control untreated sample or rhTNF-α-stimulated cells), is given by the estimation 2^{-\Delta\Delta C_T}, where \Delta\Delta C_T = \Delta C_T (sample) - \Delta C_T (calibrator), and \Delta C_T is the C_T of the target gene subtracted from the C_T of Rps18.

**Western blot analysis.** Immediately after termination of the experiment, Bioflex plates were kept on ice, washed 3 times with ice cold PBS and subjected to lyses with RIPA buffer (Santa Cruz Biotech, CA) containing protease inhibitors (Roche Diagnostics, IN). The expression of each protein was analyzed by subjecting 20 µg of whole cell lysate to SDS-10% polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred to a nitrocellulose membrane (New England Nuclear, MA), and reacted with blocking buffer containing PBS-T (PBS with 0.02% Tween-20) and 2% nonfat milk for 60 min. The blots were then reacted overnight at 4°C with monoclonal / polyclonal primary antibody recognizing a specific protein in blocking buffer at a predetermined dilution. The antibodies used were mouse anti-NOS2A IgG, (BD Transduction, CA), mouse monoclonal anti-β-Actin IgG (Sigma, MO). Subsequently, blots were washed 3 times with PBS-T and incubated in dark with IRDye 680 or IRDye 800CW conjugated goat anti rabbit or goat anti mouse IgG (Li-Cor Biosciences, NE) for 60 min at room temperature. Thereafter, membranes were washed 3 times with PBS-T and imaged under
ODYSSEY imager (LI-COR Inc, NE), and quantitatively analyzed using ODYSSEY application software, version 2.1. Results were expressed as the mean ± SD from 3 independent experiments.

**Statistical Analysis.** Results were expressed as the mean ± standard deviation from three independent experiments performed in triplicates. Statistical analysis was performed using two-way ANOVA and post-hoc Tukey using the SPSS statistical package (v.13.0). A $p < 0.05$ was deemed significant and represented as, * indicating significant differences between control and TNF-α groups, § significant differences between TNF-α and CTS / TNF-α groups and ¶ significant differences between control and CTS groups.

**2.3 RESULTS**

**Temporal expression pattern of myogenic transcription factors.** The myogenic regulatory factors (MRF’s) are critical for the determination and terminal differentiation of skeletal muscle. Commitment to the myogenic lineage requires the upregulation of *Myod1* and *Myf5*, basic helix-loop-helix transcriptional activators of the myogenic regulatory factor family (MRF). The expression pattern of bHLH and mef2 family of transcription factors was studied during the differentiation process to understand the expression profiles of these crucial myogenic genes so that the application of mechanical forces could be timed ideally. The induction of *Myod1* showed a modest increase during the first 72h time point, while the expression of *Myog* was marked with 40 fold induction during the 96h of differentiation (Figure 2.2, 2.3). Earlier studies suggest that Myod1 and Myf5 are required for commitment to the myogenic lineage, whereas myogenin plays a critical role in the expression of the terminal muscle phenotype previously established by Myod1 / Myf5, and MRF4 partly subserves both roles. The MRF’s are assisted by the myocyte enhancer factor 2 (Mef2) family of transcription factors in order to mediate expression of muscle-specific genes (Black and Olson, 1998). While expression of MRF’s is restricted to muscle, Met2 genes are expressed widely during development. The expression of *Mef2a* showed a moderate increase during the differentiation process.
peaking around 72h (Figure 2.4). Although Mef2 on its own lacks the intrinsic ability, it interacts with Myod1 \textit{in vitro} to drive the myogenic differentiation program (Molkentin et al., 1995).

**Temporal profiling of muscle structural proteins.** The MRF’s, together with Mef2 family proteins and other general and muscle-specific factors, coordinate the activities of a host of co-activators and co-repressors, resulting in tight control of gene expression during myogenesis. Myod1 activation leads to robust expression of several well-characterized target genes such as myogenin, M-cadherin, myosin heavy and light chains, and muscle creatine kinase. In addition to these muscle-specific genes, studies by Otten et al., 1997, have shown that Myod1 up-regulates expression of the cyclin-dependent kinase inhibitor, Cdkn1a / p21, causing an irreversible exit of the differentiating cells from the cell cycle. Here first, we examine the expression of cyclin-dependent kinase inhibitor Cdkn1a / p21, which has steady increase during the 96h of differentiation facilitating the exit from cell cycle and possibly fusion (Figure 2.5). As for the expression of myosin heavy chain isoform and tropomyosin1, there was a huge amount of induction during the late phases of differentiation with little to no expression during the first 48h followed by massive increase at 72 and 96h of differentiation (Figure 2.6 to 2.9).

**TNF-\(\alpha\)-induced Nos2a expression is dose dependent.** The next step involved determining the optimal dose of TNF-\(\alpha\) that could be abrogated by mechanical forces. Nos2a mRNA expression was analyzed over a dose range 0, 1, 5, 10 and 20 ng/ml of TNF-\(\alpha\). The expression pattern showed a ramp effect from 1 through 10 ng/ml of TNF-\(\alpha\) concentration and was sustained at 20 ng/ml (Figure 2.12). A dose of 5 ng/ml, which induced a 15-fold increase, was deemed optimal for further experiments.

**CTS regulates TNF-\(\alpha\)-induced Nos2a expression in a magnitude-dependent manner.** Most myopathies involve inflammation induced by proinflammatory cytokines like TNF-\(\alpha\) that initiate muscle degradation through the activation of multiple proinflammatory genes including Nos2a (Di Marco et al., 2005). Therefore, we next examined the effects
of rhTNF-α on C2C12 cells, where Nos2a mRNA induction was used as a marker to estimate the effects of CTS. Since the dose-response curve showed a robust Nos2a induction by 5 ng/ml of rhTNF-α (Figure 2.12), this concentration of rhTNF-α was used in all the following experiments. Although exposure of C2C12 cells to CTS resulted in the suppression of TNF-α-induced Nos2a mRNA expression and NOS2A protein synthesis between the magnitudes of 3% and 18%, the maximal suppression was observed at magnitudes of 3% elongation (Figure 2.13, 2.14).

**CTS regulates TNF-α-induced Nos2a expression in a frequency-dependent manner.** The next step involved optimizing the frequency applied to attain maximal suppression of inflammation. Determination of the effects of various frequencies of CTS (0.25, 0.05, 0.01 or 0.0016 Hz) revealed that frequencies of 0.25 to 0.05 Hz maximally attenuated rhTNF-α-induced Nos2a mRNA and protein expression (Figure 2.15). Therefore, in subsequent experiments CTS at a magnitude of 3% at 0.05 Hz was used to examine the effects of CTS on myogenic differentiation in C2C12 cells.

**CTS downregulates TNF-α and TNFR1 expression.** It is well established that TNF-α is a potent activator of NF-κB pathway which leads to TNF-α expression itself, resulting in a positive feedback loop between the transcription factor and the cytokine (Baud et al., 2001). So we examined whether CTS also regulated expression of other proinflammatory genes, such as TNF-α and TNFR1. Similar to Nos2a mRNA expression, CTS alone down regulated TNF-α and TNFR1 in C2C12 cells. As shown in Figure 2.16, within 4 h of TNF-α treatment, CTS also downregulated TNF-α-dependent TNF-α and TNFR1 expression significantly.

**2.4 Discussion**

C2C12 myoblasts proliferate in response to mitogens and upon mitogen withdrawal differentiate into multinucleated myotubes (Weintraub, 1993). Over the past decade, several studies have unraveled important mechanisms by which the four MRF’s
(Myod1, Myf5, myogenin, and MRF4/Myf6) control the specification and the differentiation of the muscle lineage. Early studies led to the hypothesis that these factors were redundant and could functionally replace one another (Arnold and Braun, 1996). However, recent experiments using *in vivo* and *in vitro* models have demonstrated that, in fact, different aspects of the myogenic program are controlled by different factors *in vivo*, suggesting that these factors play distinct roles during myogenesis (Chanoine et al., 2004).

In this study, we induced C2C12 myoblasts to differentiate by transfer from growth medium (GM) to differentiation medium (DM) and characterized the phenotype of C2C12 cells and examined the expression patterns of crucial myogenic factors and their targets genes. Later, by means of an *in vitro* model system, cyclic equibiaxial stretching, and a proinflammatory cytokine, TNF-α, we have emulated the effects of tensile forces imposed on satellite cells during muscle movement *in vivo*. One of the earliest events in muscle cell differentiation is the transcriptional activation and increased synthesis of the bHLH family of transcription factors, MYOD1, MYOG, MYF5 and MYF6. These transcription factors by acting at multiple points regulate the determination of skeletal muscle phenotype. The activity of the MRF’s during proliferation and differentiation of muscle precursor cells has clearly been demonstrated to be dependent on specific cell-cycle control mechanisms as well as distinct interactions with other regulatory molecules, such as the ubiquitously expressed E proteins and several other transcription factors. During this process, bHLH transcription factors act synergistically with the myocyte enhancer binding factor-2 (MEF2) family of co-factors to induce synthesis of muscle restricted target genes (Wang et al., 2001; Wilson-Rawls et al., 1999).

C2C12 cells differentiated into well defined myotubes over a period of 4 days and stained positively for myosin heavy chain protein (*Figure 2.1*). It is well established that Myod1 and Myf5 are required for commitment to the myogenic lineage, whereas myogenin plays a critical role in the expression of the terminal muscle phenotype previously established by Myod1 and Myf5, and MRF4 partly subserves both roles. Thus, Myod1 and Myf5 can be considered “commitment” factors or “early MRF’s”, whereas
myogenin is a “differentiation” factor or “late MRF”, and MRF4 has aspects of both functions. The expression pattern of Myod1 showed a considerable presence in the undifferentiated myoblast and during the early stages of differentiation with a modest increase over the final days of myotube formation. Concomitantly, expression of the mef2a transcription factor and the cell cycle inhibitor Cdkn1a were increased, marking the onset of myogenesis and cell cycle withdrawal. While the expression of myog showed little to none during the first 2 days, with levels rising rapidly during the next 3 days of differentiation. The induction of myogenin is a key event in later stage of differentiation, causing expression of myogenic target genes. As expected, myosin heavy chain isoforms and α-tropomyosin showed a rapid increase following the expression of myog and by day 4, multinucleated myotubes had formed, staining positively for myosin heavy chain protein (Figure 2.1 to 2.9).

First, we used Nos2a mRNA expression as a biomarker to optimize the dose and magnitude of CTS required for the inhibition of inflammatory responses. The results demonstrate that C2C12 myoblast-like cells respond to biomechanical signals in a magnitude-dependent manner, inhibiting rhTNF-α-induced Nos2a expression to various degrees between the magnitudes of 3% to 18% of strain. However, the maximal inhibition of rhTNF-α actions was observed at the magnitudes of 3% and 6%. These magnitudes of strain are similar to those used in earlier studies for in vitro and ex vivo muscle stimulation (Hornberger et al., 2005). Furthermore, in chondrocytes, CTS at these magnitudes also inhibits expression of several proinflammatory genes controlled by NF-κB transcriptional activity, such as, Nos2a, Ptgs2 (Cox2), Mmp1 and Il1b (IL-1β) (Agarwal et al., 2004; Madhavan et al., 2006). Since TNF-α actions on muscle cells are mediated via the NF-κB pathway, it is likely that CTS may also inhibit expression of other proinflammatory genes in addition to Nos2a.

It is well established that TNF-α is a potent activator of the NF-κB pathway which leads to TNF-α expression, itself resulting in a positive feedback loop between the transcription factor and the cytokine (Baud et al., 2001). So, we further confirmed whether CTS also regulated expression of other proinflammatory genes, such as TNF-α.
and TNFR1. Similar to Nos2a mRNA expression, CTS alone downregulated TNF-α and TNFR1 in C2C12 cells in the presence or absence of inflammation. The data from the experiments, thus far, presents a viable model system for examining the actions of biomechanical forces on myogenic factors and its target genes along with a potential to modulate muscle differentiation in vitro.
### Table 2.1
Experimental Design (II)

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<td>-</td>
<td>+</td>
</tr>
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<td>-</td>
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<td>Primer/Probe</td>
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**Table 2.2**

Taqman Primer and probe sequences for Real-time PCR (II)
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<td>Anti-sense 5'-GCC AGT GGT CTT GGT GTG CTG AC-3'</td>
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<tr>
<td>Mef2a</td>
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<td>Anti-sense 5'-GCT GGA GCT GCT CAG ACT GTC CAC-3'</td>
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<td>Myh2</td>
<td>Sense 5'-AGC AGA CGG AGA GGA GCA GGA AG-3'</td>
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<td>Anti-sense 5'-CTT CAG CTC CTC CGC CAT CAT G-3'</td>
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<td>Myh4</td>
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<td>Anti-sense 5'-GTC CTG CAG CCT CAG CAC GTT-3'</td>
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<td>Sense 5'-GCT GAC CCT CTG CTC TAC GAA TC-3'</td>
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<td>Anti-sense 5'-CTA CTT CCA GCG TGT CCT CGT G-3'</td>
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**Table 2.3**

SYBR green Primer sequences for Real-time PCR (II)
Figure 2.1 Characterization of C2C12 myoblasts. Undifferentiated myoblasts (A) and differentiated myotubes (B) after 4 days in differentiation medium were stained for myosin heavy chain (MYHC, red) using MF-20 antibody and F-actin (green) stained with fluorescein phallodin.
Figure 2.2 *Myod1* mRNA profiling. mRNA expression profiles for myogenic transcription factors *Myod1*, after 24, 48, 72 and 96h differentiation shows a marginal increase in expression. Undiff - Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
Figure 2.3 Myogenin mRNA profiling. mRNA expression profiles for myogenic transcription factors Myog, after 24, 48, 72 and 96h differentiation shows a gradual increase in expression. Undiff- Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
**Figure 2.4 Me2A mRNA profiling.** mRNA expression profiles for myogenic transcription factors mef2a, after 24, 48, 72 and 96h differentiation shows a gradual increase in expression. Undiff- Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
Figure 2.5 *Cdkn1a* mRNA profiling. mRNA expression profiles for cyclin dependant kinase inhibitor, *cdkn1a*, after 24, 48, 72 and 96h differentiation shows a gradual increase in expression. Undiff- Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
Figure 2.6 *Myh1* mRNA temporal profiling. mRNA expression profiles for muscle structural protein, *myh1*, after 24, 48, 72 and 96h differentiation shows a rapid increase in expression during the final stages. Undiff- Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
Figure 2.7 *Myh2* mRNA temporal profiling. mRNA expression profiles for muscle structural protein, *myh2*, after 24, 48, 72 and 96h differentiation shows a rapid increase in expression during the final stages. Undiff- Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
Figure 2.8 Myh4 mRNA temporal profiling. mRNA expression profiles for muscle structural protein, myh4, after 24, 48, 72 and 96h differentiation shows a rapid increase in expression during the final stages. Undiff- Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
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Figure 2.9 *Tpm1* mRNA temporal profiling. mRNA expression profiles for muscle structural protein, *Tpm1*, after 24, 48, 72 and 96h differentiation shows a rapid increase in expression during the final stages. Undiff- Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
Figure 2.10 Application of Cyclic Tensile Strain (CTS). C2C12 cells were seeded in GM at 3x10^5 cells/well on Bioflex Collagen Type I coated 6-well plates (Flexcell International, NC) and grown to 70% confluence (2-3 days) (Top panel). The medium was replaced with growth medium or Differentiation medium, and cells were subsequently subjected to CTS at an optimal magnitude and frequency using a Flexcell 4000T/FlexLink Cell Culture System (Flexcell International) (Bottom panel) in the presence or absence of recombinant human tumor necrosis factor-α (5 ng/ml) (Experimental design; Table 2.1)
Schematic of uniaxial vs. multiaxial strain application. Gray areas are the loading posts; blue and gray areas are the total surface of the membrane. Multi-axial stretch applies strain along both of the axes resulting in radial and circumferential strain, while uni-axial stretch applies strain along the X-axis with a minor compression along the Y-axis, resulting in longitudinal and transverse strain components. In a multiaxial stretch device, a decreasing gradient of circumferential strain and an increasing gradient of radial strain occur towards the periphery, compared to the center.
Figure 2.12 *Nos2a* mRNA expression is dose dependant. C2C12 cells grown on Bioflex plates were exposed to various concentrations of TNF-α (0, 1, 5, 10, 20 ng/ml) for 4h and the expression of *Nos2a* mRNA expression was analyzed by Real Time (RT)-PCR. Data represent mean and SD of three separate experiments performed in triplicates. TNF-α, Tumor Necrosis Factor - α, Rps18, Ribosomal protein S 18.
Figure 2.13 Effects of CTS of different magnitudes on Nos2a mRNA expression. C2C12 cells grown on Bioflex plates were exposed to CTS at various magnitudes (3, 6, 9, 12, and 18%) for 4h, in the presence or absence of TNF-α. C2C12 cells were either exposed for 4 h to CTS and/or TNF-α to analyze Nos2a mRNA expression by Real Time (RT)-PCR. Data represent mean and SD of three separate experiments performed in triplicates. * indicates $p<0.05$ between control and TNF-α treated cells, and § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells. CTS, cyclic tensile strain.
Figure 2.14 Effects of CTS of different magnitudes on NOS2a protein synthesis. C2C12 cells grown on Bioflex plates were exposed to CTS at various magnitudes (3, 6, 9, 12, 18%) for 4h, in the presence or absence of TNF-α. C2C12 cells were either exposed for 4 h to CTS and/or TNF-α to analyze protein levels by Western blot analysis. Data represent mean and SD of three separate experiments performed in triplicates. * indicates $p<0.05$ between control and TNF-α treated cells, and § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells. CTS, cyclic tensile strain, RFU, Relative Fluorescence Unit.
Figure 2.15 Effects of CTS of various frequencies on *Nos2a* mRNA expression. C2C12 cells grown on Bioflex plates were exposed to CTS for 4h at various frequencies (0.25, 0.05, 0.01, 0.001 Hz), in the presence or absence of TNF-α. C2C12 cells were either exposed for 4h to CTS and/or TNF-α to analyze *Nos2a* mRNA expression by Real Time (RT)-PCR. Data represent mean and SD of three separate experiments performed in triplicates. * indicates $p<0.05$ between control and TNF-α treated cells, and § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells. CTS, cyclic tensile strain.
Figure 2.16 Effects of CTS on TNF-α and TNFR1 mRNA expression. C2C12 cells grown on Bioflex plates were exposed to CTS for 4h, in the presence or absence of TNF-α. C2C12 cells were either exposed for 4h to CTS and/or TNF-α to analyze regulation of TNF-α and TNF-R1 mRNA expression by 3% CTS at 0.05 Hz assessed by Real Time PCR. Data represent mean and SD of three separate experiments performed in triplicates. * indicates $p<0.05$ between control and TNF-α treated cells, and § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells. CTS, cyclic tensile strain.
CHAPTER 3

Biomechanical signals upregulate myogenesis in the presence or absence of TNF-α

3.1 Introduction

Results from Chapter 2 indicated that the skeletal muscle differentiation model is a viable model system for examining the actions of biomechanical forces on myogenic factors and its target genes along with a potential to modulate myogenic differentiation in the presence of an inflammatory mediator, TNF-α, in vitro. Muscle inflammation is shown to initiate degradation of intrinsic muscle proteins as well as inhibit myogenesis by suppressing expression of differentiation associated molecules and eventually myotube formation in C2C12 cells, in vitro (Guttridge, 2004). Treatment of C2C12 myoblast-like cells with a proinflammatory cytokine, tumor necrosis factor (TNF)-α, leads to the loss of MYOD1, MYOG, CDKN1A, MYHC, and TPM1 proteins, resulting in the eventual inhibition of myotube formation (Acharrya et al., 2004; Guttridge, 2004). It is also known that TNF-α inhibits skeletal muscle differentiation via induction of NOS2A and NO production in an NF-κB dependent manner, which subsequently leads to Myod1 mRNA degeneration (Di marco et al., 2005; Guttridge et al., 2000). Data presented in chapter 2 shows that biomechanical signal induced events act as potent anti-inflammatory signals that suppress TNF-α-induced NOS2A mRNA and protein expression in C2C12 cells. Next, we investigated the expression patterns of myogenic factors and its protein synthesis with the application of CTS and/or TNF-α. The mechanisms of TNF-α mediated skeletal muscle atrophy has been very clearly elucidated in the literature and our findings yielded similar results. Hence, we set out to determine the biomechanical signal-dependent spatial and temporal expression of myogenic transcription factors and muscle structural proteins in C2C12 myoblasts, in vitro.
Additionally, the effects of biomechanical signals on the expression of differentiation-associated genes were assessed in the presence of a proinflammatory molecule TNF-α. We show that biomechanical forces upregulate myogenic gene expression as well as promote the progression of differentiation by increased synthesis of myogenic proteins in an environment primed for inflammation.

3.2 MATERIAL AND METHODS

**Cell culture.** Same as described in Chapter 2.

**Application of Cyclic Tensile Strain (CTS).** Same as described in Chapter 2.

**RNA purification and real-time polymerase chain reaction.** Same as described in Chapter 2.

**Western blot analysis.** Same as described in Chapter 2. The antibodies used were rabbit anti-MyoD IgG, rabbit anti-myogenin IgG, rabbit anti-MEF2A IgG, rabbit anti p21 IgG (Santa Cruz Biotechnology, CA) and mouse monoclonal anti-β-Actin IgG (Sigma, MO). Monoclonal mouse anti-myosin heavy chain IgG (MF-20) and mouse anti-α-tropomyosin (CH1) were obtained from Developmental Studies Hybridoma Bank, IA.

**Immunofluorescence.** Immediately following each experiment, C2C12 cells to be analyzed by immunofluorescence were washed with ice-cold PBS and fixed using 2% paraformaldehyde (pH 8.0) for 20 min, and permeabilized with 0.2% Triton X-100 for 10 min. Thereafter, the flexible silicone elastomer membranes from the BioFlex plates were excised and divided into six equal pie-shaped pieces. The membranes were reacted with blocking buffer (Vector Labs, CA) and incubated overnight with primary antibodies as appropriate (described above) at 4 °C. The membranes were washed three times with PBS-T and then incubated with CY3-conjugated goat anti-rabbit or goat anti-mouse IgG for 45 min at room temperature. Subsequently, cells were counterstained with FITC-conjugated phalloidin (Invitrogen, CA) to visualize F-actin and DAPI (Sigma, MO) to
visualize nuclei. The membranes were subsequently mounted using Vectashield Mounting Medium (Vector Labs, CA), examined under epifluorescence using a Zeiss Axioplan microscope, and immunofluorescence images captured with Zeiss imaging software. The number of MYHC positive cells was enumerated in four different areas of 4.84 x10^4 μm², fluorescence estimated with the use of Axiovision software, and the mean and standard deviation (SD) calculated.

**Statistical Analysis.** Results were expressed as the mean ± standard deviation from three independent experiments performed in triplicates. Statistical analysis was performed using two-way ANOVA and post-hoc Tukey using the SPSS statistical package (v.13.0). A p < 0.05 was deemed significant and represented as, * indicating significant differences between control and TNF-α groups, § significant differences between TNF-α and CTS / TNF-α groups and ¶ significant differences between control and CTS groups.

### 3.3 RESULTS

**CTS upregulates Myod1 induction.** During myogenesis, Myod1 is the first muscle-specific transcription factor upregulated in differentiating myoblasts. Therefore, to assess transcription factors regulated by CTS, we first examined the expression of Myod1 mRNA expression and MYOD1 protein synthesis in differentiating C2C12 cells. C2C12 myoblasts were either untreated (control) or exposed to rhTNF-α, CTS, or CTS+rhTNF-α. Within 24 h of initiating differentiation, C2C12 cells exhibited an increased Myod1 mRNA expression (Figure 3.1), and this expression was more than 4.2-fold increased in response to CTS. TNF-α exposure suppressed more than 96% of the Myod1 mRNA expression induced by DM. More importantly, concomitant exposure of cells with CTS during treatment with rhTNF-α resulted in a complete abrogation of TNF-α-mediated inhibition of Myod1 mRNA expression (Figure 3.1). Myod1 mRNA expression was highest during the first 24 h, and during the ensuing 24 h it gradually decreased in CTS treated cells, but remained at the same levels under other treatments. Further analysis after 72 h of CTS or CTS and TNF-α treatment revealed that its levels did not change
significantly. Western blot analysis revealed that MYOD1 is maintained in control cells at similar levels. However, the presence of rhTNF-α suppresses its expression over the entire 72 h period. Contrarily, in both C2C12 cells exposed to CTS alone or CTS+rhTNF-α, MYOD1 synthesis was at least 4-fold greater in comparison to control cells, suggesting that CTS alone not only upregulated MYOD1 synthesis, it markedly abrogated rhTNF-α-induced inhibition of MYOD1 synthesis (Figure 3.2). Intracellular localization of MYOD1 by immunofluorescence staining demonstrated that the majority of MYOD1 was concentrated in the nuclei of control cells (Figure 3.3). As expected, the presence of MYOD1 in rhTNF-α treated cells was negligible in the cytoplasm or nuclei (Figure 3.3). More interesting was the fact that CTS-induced upregulation of Myod1 was paralleled by its nearly complete nuclear translocation in cells exposed to CTS alone as well as in cells exposed to CTS+rhTNF-α concomitantly (Figure 3.3).

**CTS upregulates Myog expression and abrogates rhTNF-α-mediated suppression of Myog expression.** Nuclear translocation of MYOD1 results in the transcriptional activation of Myog gene expression (Berkes and Tapscott, 2005). To determine whether the upregulation and nuclear translocation of MYOD1 by CTS was functionally significant, we next performed differential gene expression analysis of Myog by real-time RT-PCR, in the absence or presence of rhTNF-α treatment. As shown in Figure 3.4, Myog mRNA expression in C2C12 cells subjected to CTS increased 3.8-fold during the first 24 h, and continued to increase in both cells exposed to CTS and controls, during the ensuing 24 and 48 h. Not surprisingly, rhTNF-α exposure inhibited Myog mRNA expression significantly. MYOG protein induction assessed by Western blot analysis revealed that, in comparison to control cells, CTS induced a 5.1-, 3.2- and 1.8-fold increase in MYOG accretion at 24, 48, and 72 h, respectively (Figure 3.5). Intracellular localization of MYOG by immunofluorescence analysis revealed that, following its synthesis, more than 95% of MYOG is translocated to the nucleus (Figure 3.6). Consistent with the inhibition of mRNA expression, rhTNF-α inhibited MYOG synthesis during the first 48 h, however, some synthesis of MYOG was observed in rhTNF-α
treated cells at 72 h (Figure 3.5 and 3.6). However, CTS markedly abrogated the rhTNF-α-dependent inhibition of MYOG synthesis and allowed Myog mRNA expression, above control levels at all time points tested (Figure 3.5 and 3.6). In addition, the elevated presence of MYOG in the nucleus and its absence in the cytoplasmic compartment suggested that upregulation of MYOG synthesis by CTS may be paralleled by its transcriptional activity (Figure 3.6).

**CTS upregulates Mef2a expression.** The MEF2 family of proteins bind to MYOD1 as co-transcription factors and facilitate muscle-restricted gene expression. Therefore, we further determined whether CTS-mediated augmentation of myogenesis involves Mef2a upregulation. Not surprisingly, CTS upregulated Mef2a mRNA and MEF2A protein expression (Figure 3.7). As expected, rhTNF-α treatment suppressed both Mef2a expression and MEF2A synthesis. However, CTS inhibited the rhTNF-α-mediated downregulation to allow Mef2a mRNA expression and MEF2A protein synthesis above control levels (Figure 3.7 and 3.8).

**CTS upregulates Cdkn1a expression.** During myogenesis, a myoblasts must exit from the cell cycle to commit itself to myogenic differentiation. In this process, following induction of MYOG, the cyclin dependent kinase inhibitor, CDKN1A, facilitates cell cycle exit and terminal differentiation (Fujio et al., 1999; Lawlor and Rotwein, 2000). Examination of the expression and synthesis of Cdkn1a demonstrated that Cdkn1a mRNA expression is not upregulated in response to CTS, but rhTNF-α suppresses its expression significantly during the initial 24 h of treatment (Figure 3.9). The mRNA levels for Cdkn1a did not change significantly during 24 to 72 h of treatment. Nevertheless, a marginal upregulation (1.5- to 1.8-fold) of CDKN1A protein synthesis was observed in response to CTS at 48 h. rhTNF-α exposure consistently inhibited greater than 60% of CDKN1A synthesis in comparison to control cells. CTS also counteracted rhTNF-α-induced suppression of CDKN1A, and a minimal upregulation of its synthesis was observed in cells subjected to simultaneous exposure of CTS+rhTNF-α at all time points tested (Figure 3.10).
CTS upregulates skeletal muscle structural proteins. In a temporal analysis of differentiation, the ultimate consequence of the upregulation of myogenic transcription factors and CDKN1A is increased synthesis of muscle-restricted target proteins like, MYHC, TPM1, and TNNI/TNNT. Hence, to determine the functional consequence of CTS-mediated upregulation of bHLH and MEF2 family transcription factors, we next examined the mRNA expression and protein synthesis of the various isoforms of MYHC (Myh1, Myh2 and Myh4) and Tpm1. In C2C12 cells maintained in DM, the expression of Myh1, Myh2, and Myh4 mRNA during the first 24 h of differentiation was negligible, while it dramatically increased in the ensuing 24 h, exhibiting a 14-, 73-, and 132-fold increase in Myh1, Myh2 and Myh4 mRNA, respectively (Figure 2.6, 2.7 and 2.8). Strikingly, in comparison to controls cells maintained in DM, CTS further induced a 3.8-, 2.8- and 1.5-fold increase in mRNA expression for Myh1, Myh2, and Myh4 mRNA, respectively (Figure 3.11). In parallel, 72 h after initiation of differentiation, CTS augmented a 3.2-fold increase in MYHC synthesis above that observed in control cells (Figure 3.12). rhTNF-α treatment inhibited 89% of the Myh1, 81% of Myh2, and 92% of Myh4 mRNA expression (Figure 3.11). This was also reflected by a 64% reduction of the MYHC synthesis in cells treated with rhTNF-α. Simultaneous exposure of cells to CTS abrogated rhTNF-α induced inhibition of Myh1, Myh2 and Myh4 mRNA expression. Nevertheless, the levels of Myhc mRNA were not higher than those present in control cells maintained in DM. In parallel, Western blot analysis using the MF-20 monoclonal antibody confirmed the results of above experiments, in that simultaneous exposure of CTS during rhTNF-α treatment neutralized the effects of rhTNF-α, albeit the presence of MYHC protein did not exceed above those expressed by control C2C12 levels (Figure 3.11). As shown in Figure 3.15 and 3.16, CTS+rhTNF-α regulated Tpm1 mRNA expression and TPM1 protein synthesis in a manner similar to the MYHC proteins.

Enumeration of MYHC positive cells by immunofluorescence demonstrated that rhTNF-α significantly suppressed MYHC expression in C2C12 cells. However, cells simultaneously exposed to CTS+rhTNF-α exhibited a complete reversal of rhTNF-α induced suppression of MYHC expression to control levels (Figure 3.14). The exposure
of CTS alone yielded 2.6-fold greater MYHC positive cells as compared to controls during a 72 h period of differentiation. C2C12 cells transform into myotubes in DM within 72 to 96h (Berkes and Tapscott, 2005; Molkentin and Olson, 1996). Consequently, we investigated whether the expression of muscle target proteins is paralleled by phenotypic changes in C2C12 cells in response to CTS and/or rhTNF-α. Consistent with our hypothesis, immunofluorescence analysis detected a 2.7-fold increase in MYHC positive multinucleate myotubes formed by the fusion of cells in cultures exposed to CTS, as compared to control cells (Figure 3.13). Not surprisingly, cells treated with rhTNF-α stained poorly for MYHC, and lacked multinucleate cell formation (Figure 3.13). C2C12 cells treated concurrently with CTS+rhTNF-α exhibited MYHC positive cells and multinucleate myotube formation that were equal in numbers to control cells maintained in DM, further confirming that CTS abrogates the rhTNF-α−induced suppression of phenotypic changes in C2C12 cells.

3.4 Discussion

The results demonstrate that biomechanical signals stimulate rapid, sustained, and temporally controlled activation of bHLH and MEF2 family transcription factors in C2C12 cells to drive myogenic differentiation. It is well accepted that muscle cells are responsive to biomechanical signals such as those experienced during exercise and these signals can upregulate skeletal muscle hypertrophy (Aikawa et al., 2001; Cheema and Brown, 2005). During muscle hypertrophy, the binding of MYOD1 to the E-box consensus sequences in the regulatory region of muscle-specific genes initiates transcription of several bHLH and MEF2 family proteins and their ultimate cooperative interactions regulate myogenic differentiation (Dodou et al., Wang et al., 2001; Wilson-Rawls et al., 1999). Contrarily, during skeletal muscle atrophy these signals are blocked leading to a loss of skeletal muscle function (Guttridge, 2004).

By means of an in vitro model system, utilizing C2C12 myoblast-like cells, cyclic equibiaxial stretching, and a proinflammatory cytokine, TNF-α, we have emulated the effects of tensile forces imposed on satellite cells during muscle movement in vivo. TNF-α inhibits skeletal muscle differentiation via activation of the NF-κB signal transduction
pathway and induces the expression of \textit{Nos2a} and other proinflammatory cytokines. Therefore, we used \textit{Nos2a} mRNA expression as a marker to optimize the dose and magnitude of CTS required for the inhibition of inflammatory responses. The results demonstrate that C2C12 myoblast-like cells respond to mechanical signals in a magnitude-dependent manner, inhibiting rhTNF-\(\alpha\)-induced \textit{Nos2a} expression to various degrees between the magnitudes of 3\% and 18\% of strain. However, the maximal inhibition of rhTNF-\(\alpha\) actions was observed at the magnitudes of 3\% and 6\%. One of the earliest events in muscle cell differentiation is the transcriptional activation and increased synthesis of the bHLH family transcription factors, MYOD1, MYOG, MYF5 and MYF6. These transcription factors, by acting at multiple points, regulate skeletal muscle phenotype. During this process, bHLH transcription factors act synergistically with the myocyte enhancer binding factor-2 (MEF2) family of co-factors to induce synthesis of muscle restricted target genes (Wang et al., 2001; Wilson-Rawls et al., 1999). We have examined salient members of the bHLH and MEF2 family of proteins to delineate the mechanisms of CTS-mediated regulation of skeletal muscle phenotype. CTS, as an extracellular signal appears to be involved in regenerative responses of skeletal muscle cells (Hornberger et al., 2005). At low physiological levels, it activates one of the earliest events in muscle cell differentiation by augmenting rapid and significantly greater expression, synthesis, and nuclear translocation of MYOD1. Furthermore, we have observed that nuclear Myod1 and myogenin levels are upregulated by CTS even in the presence of TNF-\(\alpha\), and that mRNA expression of these genes is significantly increased. This suggests CTS-dependent upregulation of their nuclear translocation is paralleled by an increase in their transcriptional activity, even in the presence of TNF-\(\alpha\). Muscle cell growth is controlled by two distinct mechanisms. One involves insulin-like growth factor (IGF)-induced activation of phosphoinositol-3-kinase (PI3K) and its subsequent activation of mTOR (mammalian target of rapamycin) pathway, and the other that is independent of PI3K activation, involving calcineurin signaling (Guttridge, 2004). Multiaxial stretch has been shown to activate PI3K-independent mTOR signaling in differentiating C2C12 cells (Hornberger et al., 2005). Whether the mechanically induced transcriptional activation of Myod1 is PI3K-dependent or independent is as yet not clear.
In either case, eventual transcriptional activation of Myod1 is an absolute requirement for muscle cell differentiation. Therefore, CTS-mediated augmentation of *Myod1* expression at levels that are several fold greater than controls, suggests that biomechanical signals have greater capacity to induce skeletal muscle differentiation.

Subsequent to MYOD1 activation, expression of *Myog* is the credible molecular marker for myoblast commitment to differentiation. In differentiating C2C12 cells, *Myog* expression gradually increases over 24 to 72h of differentiation. CTS induces marked upregulation of *Myog* expression that parallels DM-induced differentiation. The fact that myogenin expression corresponds to commitment of cells to myogenic differentiation further indicates that the actions of CTS are myogenic. In parallel to the upregulation of *Myog*, CTS induces upregulation of two proteins, MEF2A and CDKN1A. Interestingly, while we observed CDNK1A upregulation at all time points tested, we did not observed similar changes in *Cdnk1a* mRNA expression. Whether this is due to increased stability of mRNA or increased translation is unknown. Nevertheless, by augmenting transcriptional activation of *Mef2a*, CTS upregulates transcriptional activity of *Myod1* and hence the synthesis of skeletal muscle structural proteins (Rawls et al., 1998; Wilson-Rwals et al., 1999). Synthesis and activation of CDKN1A is essential for the irreversible exit of C2C12 cells from the cell cycle and commitment to the differentiation program. Interestingly, CTS does not appear to upregulate transcriptional activity of *Cdkn1a* above control levels, however, its synthesis is upregulated in cells exposed to CTS, suggesting a likely post-transcriptional control of this protein by CTS.

The function of bHLH and MEF2 transcription factors is to upregulate induction of skeletal muscle structural proteins. A consistent upregulation of muscle structural proteins in response to CTS, suggests that the transcription factors upregulated by CTS are functionally active. One of the most abundant proteins in a mature myotube is MYHC. Of the various MYHC isoforms, we examined the expression of *Myh1* (MYH IIx), *Myh2* (MYH IIa) and *Myh4* (MYH IIb) for CTS effects. CTS markedly upregulates the transcriptional activation of *Myh1*, *Myh2* and *Myh4*, and synthesis of MYHC and TPM1. Our findings have further revealed that CTS, within 72 h, augments more than a 2.6 fold increase in MYHC positive cells and nearly a 2.7 fold increase in myotube
formation, as compared to C2C12 cells maintained in DM. Collectively, these observations underscore the importance of biomechanical signals in muscle regeneration, and suggest that these extracellular signals are converted into biochemical events that can initiate and sustain the differentiation program that converts myoblasts into myotubes.

Muscle pathologies involving inflammation are the major cause of muscle weakness and loss of function. Accumulated evidence suggests that, exercise or physical therapies provide increased functionality to inflamed muscle, and its lack results in muscle atrophy (Goldspink, 2003). To investigate the role played by CTS in preventing muscle inflammation, we examined the actions of CTS on C2C12 cell differentiation in the presence of TNF-α. TNF-α, is a mediator of muscle inflammation. It downregulates the myogenic transcriptional program by activating NF-κB and its subsequent binding to the promoter regions of Myod1 results in reduced synthesis and activity of MYOD1 (Acharrya et al., 2004; Guttridge, 2004). In our studies, TNF-α suppressed promyogenic actions of DM at all steps, from the induction and nuclear translocations of MYOD1 and MYOG, to synthesis of MEF2A and CDKN1A. Thus, TNF-α repressed the DM-induced exit of C2C12 cells from the cell cycle and commitment to the differentiation. CTS counteracted the actions of TNF-α and allowed expression of bHLH and MEF2 transcription factors, as well as CDKN1A, despite the presence of inflammatory levels of TNF-α. Nevertheless, the levels of these transcription factors were similar or higher than those observed in control cells grown in DM. Since expression of Cdkn1a is one of the key events in the differentiation process that leads to a post-mitotic state and fusion of cells to form multinucleated myotubes, CTS actions may be critical in removing the TNF-α-dependent inhibition of cells to exit from the cell cycle.

By three distinct ways, we examined the consequences of exposure of cells to CTS in the presence of TNF-α, i.e., MYHC expression, number of MYHC positive cells, and the number of myotubes formed. Our findings are consistent with earlier reports that TNF-α severely impairs the ability of C2C12 cells to synthesize MYHC and TPM1 (Acharrya et al., 2004; Guttridge, 2004). Furthermore, our findings provide additional evidence that TNF-α inhibits C2C12 cell fusion and myotube formation even in the
presence of low levels of MYHC in the cells. Contrarily, CTS rescues C2C12 cells from TNF-α mediated inhibition of MYHC synthesis, as indicated by the levels of MYHC in the cells and the number of MYHC positive cells. Interestingly, CTS also upregulates myotube formation in the presence of TNF-α. Thus, it can be concluded that CTS is able to activate synthesis of skeletal muscle target genes and myotube formation, and may thus prevent TNF-α-induced muscle decay in C2C12 myogenic cells.

Although stretch induced upregulation of myogenic regulatory factors has been shown in whole muscle models (Lowe and Alway, 1999; Marsh et al., 1998) and culture models (Rauch and Loughna, 2006), our findings are the first to demonstrate that CTS is a potent myogenic signal that dramatically upregulates myogenesis despite the presence of a strong proinflammatory environment. Signals generated by CTS are converted into molecular events that dynamically augment activation and induction of myogenic transcription factors, skeletal muscle structural proteins, and eventual cell fusion to form multinucleate myotubes. Signals generated by CTS also act as potent inhibitors of the intracellular actions of proinflammatory cytokines like TNF-α on one hand, and induce muscle cell differentiation under a proinflammatory environment, on the other. Thus, biomechanical signals play an essential role in muscle regeneration and pathological muscle repair. In view of the fact that non steroidal anti-inflammatory drugs and steroids provide limited success in inflammatory myopathies, use of appropriate biomechanical signals may provide a promising mechanism for the development of therapeutic approaches.
<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Rps18</td>
<td>Sense 5’-Tgg Gcg Gcg Gaa Aat Ag-3’</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5’-Tgc Ttt Cct Caa Cac Cac Atg-3’</td>
</tr>
<tr>
<td></td>
<td>Probe 5’-Cac Tgc Cat Taa Ggg Cgt Ggg-3’</td>
</tr>
<tr>
<td>Myod1</td>
<td>Applied biosystems (Mm00440387_m1)</td>
</tr>
<tr>
<td>Myog</td>
<td>Applied biosystems (Mm00446194_m1)</td>
</tr>
<tr>
<td>Myh1</td>
<td>Applied biosystems (Mm01332500_gH)</td>
</tr>
<tr>
<td>Cdkn1a</td>
<td>Applied biosystems (Mm00432448_m1)</td>
</tr>
</tbody>
</table>

**Table 3.1**

Taqman primer and probe sequences for Real-time PCR (III)
<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| Rps18       | Sense 5'-ATC ATC GAG AGC GAC CTG GAA CG-3'  
Anti-sense 5'-GCC AGT GGT CTG GGT GTG CTG AC-3' |
| Mef2a       | Sense 5'-CAG GCT TCA GCC TGG CAG CAG-3',  
Anti-sense 5'-GCT GGA GCT GCT CAG ACT GTC CAC-3' |
| Myh2        | Sense 5'-AGC AGA CGG AGA GGA GCA GGA AG-3'  
Anti-sense 5'-CTT CAG CTC CTC CGC CAT CAT G-3' |
| Myh4        | Sense 5'-CAC CTG GAG CGG ATG AAG AAG AAC-3'  
Anti-sense 5'-GTC CTG CAG CCT CAG CAC GTT-3' |
| Tpm1        | Sense 5'-ATC ATC GAG AGC GAC CTG GAA CG-3'  
Anti-sense 5'-CTT TGG CAT GGG CCA CTT TC-3' |
| TNF-α       | Sense 5'-GTT CTA TGG CCC AGA CCC TCA CAC-3'  
Anti-sense 5'-CGT AGT CGG GGC AGC CTT GTC-3' |
| TNFR1       | Sense 5'-GCT GAC CCT CTG CTC TAC GAA TC-3'  
Anti-sense 5'-CTA CTT CCA GCG TGT CCT CGT G-3' |

**Table 3.2**  
SYBR green Primer sequences for Real-time PCR (III)
Figure 3.1 Myod1 gene expression is upregulated by CTS. C2C12 myoblasts grown on Bioflex plates in the presence or absence of TNF-α were subjected to CTS for various time intervals and analyzed for (A) Myod1 mRNA expression by RT-PCR. Data exhibit mean and SD of 3 separate experiments, * indicates $p<0.05$ between control and TNF-α treated cells, § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells, and ¶ indicates $p<0.05$ between control and CTS treated cells. DM, differentiation medium.
Figure 3.2 Effects of CTS on MYOD1 protein synthesis. C2C12 myoblasts, grown on Bioflex plates in the presence or absence of TNF-α, were subjected to CTS for various time intervals and analyzed for MYOD1 protein levels by Western blots. Data is a representation from one of three separate experiments. RFU, relative fluorescence unit.
Figure 3.3 Localization and synthesis of MYOD1 protein following CTS. C2C12 myoblasts grown on Bioflex plates in the presence or absence of TNF-α were subjected to CTS for various time intervals and analyzed for the presence of MYOD1 in cells by immunofluorescence. Data is a representation of one of three separate experiments, white arrows point to the presence of nuclear MYOD1 in cells and red arrows indicate the minimal presence of MYOD1 in the nuclei. The bars represent 50 μ. GM, growth medium, DM, differentiation medium.
Figure 3.4 *Myog* gene expression is upregulated by CTS. C2C12 cells in DM were subjected to CTS at a magnitude of 3% and 0.05 Hz for various time intervals in the presence or absence of TNF-α. The expression of *Myog* mRNA was analyzed by RT-PCR and data exhibit mean and SD of 3 separate experiments. * indicates $p<0.05$ between control and TNF-α treated cells, § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells, and ¶ indicates $p<0.05$ between control and CTS treated cells. DM, differentiation medium.
Figure 3.5 Effects of CTS on MYOG protein synthesis. C2C12 cells in DM were subjected to CTS at a magnitude of 3% and 0.05 Hz for various time intervals in the presence or absence of TNF-α. The expression of MYOG synthesis was analyzed by Western blots (B) and data represents one of three separate experiments with similar results. RFU, relative fluorescence unit.
**Figure 3.6 Localization and synthesis of MYOG protein following CTS.** C2C12 cells in DM were subjected to CTS at a magnitude of 3% and 0.05 Hz for various time intervals in the presence or absence of TNF-α. The intracellular localization of MYOG by immunofluorescence and data represents one of three separate experiments with similar results. White arrows point to the presence of nuclear MYOG red arrows point to lack of MYOG in cells. The bars represent 50 μ. GM, growth medium, DM, differentiation medium.
Figure 3.7 *Mef2a* gene expression following the application of CTS. C2C12 cells exposed to CTS in the presence or absence of TNF-α for various time intervals. The mRNA expression for *mef2a* was assessed by RT-PCR and data represent mean and SD of 3 separate experiments performed. DM, differentiation medium.
Figure 3.8 MEF2A protein synthesis following the application of CTS. C2C12 cells exposed to CTS in the presence or absence of TNF-α for various time intervals. MEF2A synthesis was assessed by Western blot analysis. Data represents one of three separate experiments with similar results. RFU, relative fluorescence unit.
Figure 3.9 *Cdkn1a* gene expression following the application of CTS. C2C12 cells exposed to CTS in the presence or absence of TNF-α for various time intervals. The mRNA expression for *Cdkn1a* was assessed by RT-PCR. Data represent mean and SD of 3 separate experiments performed. * indicates $p<0.05$ between control and TNF-α treated cells. DM, differentiation medium.
Figure 3.10 CDKN1A protein synthesis following the application of CTS. C2C12 cells exposed to CTS in the presence or absence of TNF-α for various time intervals. The CDKN1A protein level was assessed by western blot analysis. Data represents one of three separate experiments with similar results. RFU, relative fluorescence unit.
Figure 3.11 CTS upregulates gene expression of MYHC isoforms. The relative expression of Myh1, Myh2 and Myh4 mRNA in cells maintained in DM and exposed to CTS in the presence or absence of TNF-α at 72h. Data represents mean and SD from 3 different experiments performed in triplicates. * indicates $p<0.05$ between control and TNF-α treated cells, § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells, and ¶ indicates $p<0.05$ between control and CTS treated cells.
Figure 3.12 MYHC protein synthesis after CTS application. Cells were exposed to CTS and/or TNF-α and the synthesis of MYHC protein was detected by anti-myosin antibody, MF-20 at 72h. Data represents 1 out of 3 experiments with similar results. RFU, relative fluorescence unit.
Figure 3.13 MYHC staining following the application of TNFα and/or CTS. Cells were exposed to CTS and/or TNF-α and immunostained at 72h. Analysis of MYHC protein by immunostaining of C2C12 cells for MYHC in red (a, c, e, g, i), F-actin staining in green (phalloidin-FITC), and nuclear staining in blue (DAPI) (b, d, f, h, j) showing MYHC positive and multinucleate myotubes in response to CTS. Data represents 1 out of 3 experiments with similar results. GM, growth medium, DM, differentiation medium, my, myotubes, sn, single nucleated myoblasts, mn, multinucleated myotubes.
Figure 3.14 Increased myotube formations with the application of CTS. Cells were exposed to CTS and/or TNF-α. The immunostaining from Figure 3.13 was enumerated for the total number of MYHC positive cells in 4 different areas of size $4.84 \times 10^4 \, \mu m^2$. * indicates $p<0.05$ between control and TNF-α treated cells, § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells, and ¶ indicates $p<0.05$ between control and CTS treated cells.
Figure 3.15 *Tpm1* gene expression is upregulated by CTS. Cells were exposed to CTS and/or TNF-α and the relative expression of *Tpm1* mRNA was analyzed using RT-PCR. Data represents mean and SD from 3 different experiments performed in triplicates. ¶ indicates $p<0.05$ between control and CTS treated cells.
Figure 3.16 TPM1 protein synthesis following CTS application. Cells were exposed to CTS and/or TNF-α and TPM1 protein synthesis was analyzed by western blot analysis. The data represents 1 out of 3 experiments with similar results. RFU, relative fluorescence unit.
CTS abrogates the actions of TNF-α-mediated inhibition of skeletal muscle differentiation and simultaneously upregulates skeletal muscle differentiation (SMD). Insulin-like growth factors (IGF-I) stimulates muscle differentiation by activating PI3K-PIP3-AKT pathway, triggering Myod1-MEF2 transactivational capacity. Expression and synergistic interaction of bHLH transcription factors, MYOD1 / MYOG, and MEF2 family of proteins regulate sequential steps in myogenesis, resulting in the induction of muscle restricted target genes such as myosin heavy chain (MYHC), myosin light chains (MYLC) and α-Tropomyosin (TPM1). On the contrary, TNF-α induces expression of pro-inflammatory molecules like Nos2a, TNF-α, and TNFR1 via NF-κB pathway and in parallel inhibits Myod1 and Myog expression and/or protein loss, consequently blocking...
the expression of muscle specific proteins. CTS abrogates the TNF-α induced expression of NOS2A synthesis (1), upregulates Myod1 by activating the AKT pathway (2). Thus mechanical signals, by inhibiting inflammation and upregulating skeletal muscle differentiation act as potent reparative signals during inflammation.
CHAPTER 4

Activation of the IGFR1 - PI3K - AKT pathway in C2C12 myoblasts by mechanical signals

4.1 INTRODUCTION

The serine/threonine protein kinase B (PKB), also known as AKT (v-AKT murine thymoma viral oncogene homolog), is phosphorylated by PI3K, and is essential for IGF-dependent differentiation (Jiang et al., 1998; Wilson et al., 2004). All of the AKT isoforms (AKT1, AKT2, and AKT3) are activated by growth factors in a PI3K-dependent manner. AKT’s have been implicated in several cellular processes, such as cell survival, apoptosis, glucose uptake in myocytes and adipocytes, proliferation and differentiation (Datta et al., 1999; Yang et al., 2004). Earlier studies have shown that ectopic expression of activated AKT1 can promote extensive SMD in different myoblast cell lines, even in the absence of IGF-I, and can reverse the inhibitory effects of PI3K inhibitors like Wortmannin and LY294002 on myogenic differentiation (Rommel et al., 1999; Jiang et al., 1998). AKT has 3 different distinct domains (Figure 4.1). The plecstrin homology domain (PH) is involved with binding to the PIP3, the regulatory domain with sites for phosphorylation and its activation and the catalytic domain, the kinase active region which it shares with other AGC kinases (protein kinase A/G/C) (Figure 4.2). AKT is activated by dual regulatory mechanisms that require both its membrane relocalization from the cytosol to plasma membrane and c-terminal phosphorylation at Thr308 / Ser473 (Hemmings, 1997).

Insulin-like growth factors (IGF-I and IGF-II) are potent stimulators of muscle differentiation through the induction of myogenin and MEF2. Studies on signaling through the IGF receptor have revealed that insulin receptor substrate-1 (IRS-1) is tyrosine phosphorylated at multiple sites and acts as a docking site for
phosphatidylinositol 3-kinase (PI3K). In general, activation of AKT by growth factors (IGF-1) is mediated by PI3K. PI(3,4)P2 and PI(3,4,5)P3 are generated mainly by D3 phosphorylation of PI(4)P2 and PI(4,5)P3, respectively with activated class Ia or Ib PI3-Kinase. PI(3,4)P2 could be also generated by 5-dephosphorylation of PI(3,4,5)P3 by Src homology 2 (SH-2)-containing 5-phosphatase, SHIP. On the other hand, PTEN (phosphatase and tensin homolog), is a protein phosphatase that dephosphorylates PIP3 at the D3 position and acts as a negative regulator of PI3K/AKT signaling during myogenesis (Wan and Helman, 2003). PI(3,4)P2 and PI(3,4,5)P3 are capable of binding to the pleckstrin homology (PH) domain of AKT and other kinases like PDK1 (Matsui et al., 2002).

The binding of the pleckstrin homology (PH) domain of AKT to PI(3,4)P2 and PI(3,4,5)P3 on the plasma membrane releases the autoinhibitory role of this domain allowing phosphoinositide-dependent kinase-1 (PDK1) to phosphorylate AKT on Thr308. Thr308 phosphorylation partially activates AKT while full activation requires phosphorylation at Ser473 which remains as yet unsolved, although the kinase responsible is referred to as PDK2 (Stokoe et al., 1997). Other possible activation mechanisms at Ser473 involves autophosphorylation by AKT itself (Toker et al., 2000) and PDK1 phosphorylating both the Thr308 and Ser473 (Balendran et al., 1999). Furthermore, PI3K activity could be irreversibly inhibited by the fungal metabolite Wortmannin and the synthetic compound LY294002 (Figure 1.3).

One of the major downstream targets of AKT is GSK-3β, a serine/threonine protein kinase which is constitutively active in unstimulated muscle cells, phosphorylates many proteins including glycogen synthase, eIF2B, c-Myc, and cyclin D, keeping them inactive or promoting their degradation. Phosphorylation of GSK-3β suppresses its activity resulting in the activation of pathways that are normally repressed by GSK-3β (Fang et al., 2005; Vyas et al., 2002).

Data in Chapters 2 and 3 clearly point to the anti-inflammatory and pro-differentiative role of biomechanical signals. Here we hypothesized that biomechanical signals could potentially activate the IGFR1-PI3K-AKT pathway by activating and phosphorylating AKT and ultimately its downstream target, GSK3β. The activity of the
IGFR1-PI3K-AKT pathway is not known in the presence of an inflammatory environment either. So, we examined the effects of an inflammatory cytokine such as TNF-α, and/or CTS on the AKT pathway. Additionally, we also analyzed the mechanotransduction of extracellular signals into intracellular signaling events by examining the Insulin growth factor receptor, IGFR1, Insulin receptor, IR1, G Protein Coupled Receptor, GPCR, and the Integrin receptor substrate, FAK (Focal Adhesion Kinase).

4.2 MATERIALS AND METHODS

Cell culture. Same as described in Chapter 2. The PI3K inhibitor LY-294002 were purchased from Cell Signaling, CA and the specific inhibitor for the receptor tyrosine kinase, AG1024 and GPCR inhibitor, pertussis toxin was purchased from Calbiochem. The inhibitors were added to the media 1h before the start the experiments and performed as described Chapter 2.

Application of Cyclic Tensile Strain (CTS). Same as described in Chapter 2.

RNA purification and real-time polymerase chain reaction. Same as described in Chapter 2.

Western blot analysis. Same as described in Chapter 2. The antibodies used were AKT isoform sampler kit, phospho AKT sampler kit (Cell Signaling, MA), mouse monoclonal anti-β-Actin IgG (Sigma, MO) and mouse anti-phosphotyrosine antibody (Santa Cruz biotechnology, CA).

Immunoprecipitation. For FAK tyrosine phosphorylation studies, cells were switched from DMEM–20% FBS to DMEM–2% HS for 2h before the start of the experiment. After the completion of the experiment, cells were rinsed twice with ice-cold PBS and lysed in ice-cold RIPA buffer (Santa Cruz Biotech, CA). Lysates normalized for total protein, were pre-cleared with a rabbit anti-FAK (sc-557, Santa Cruz biotechnology, CA)
at 1:100 dilution for 4°C overnight. Immunocomplexes were captured with protein A/G - agarose beads for 2 h at 4°C and were subsequently washed with ice-cold RIPA buffer. Samples were boiled for 5 min and analyzed by SDS-PAGE on 8 or 10% gradient gels. After electrotransfer and membrane blocking, as previously described in Chapter 2, membranes were treated with a mouse anti-phosphotyrosine antibody (sc-7020; Santa Cruz Biotechnology, CA) and rabbit anti-FAK (sc-557, Santa Cruz Biotechnology, CA) at a 1:1000 dilution in blocking buffer overnight at 4°C. Membranes were subsequently processed and detected using an ODYSSEY imager (LI-COR Inc, NE), and quantitatively analyzed using ODYSSEY application software, version 2.1 as described in Chapter 2. Results were expressed as the mean ± SD from 3 independent experiments.

**Immunofluorescence.** Same as described in Chapter 3.

**Statistical Analysis.** Results were expressed as the mean ± standard deviation from three independent experiments performed in triplicates. Statistical analysis was performed using two-way ANOVA and post-hoc Tukey using the SPSS statistical package (v.13.0). A p < 0.05 was deemed significant and represented as, * indicating significant differences between control and TNF-α groups, § significant differences between TNF-α and CTS / TNF-α groups, and ¶ significant differences between control and CTS groups.

4.3 RESULTS

**CTS activate AKT phosphorylation at Ser473.** AKT is activated by dual regulatory mechanisms that require both its membrane relocalization from the cytosol to plasma membrane and C-terminal phosphorylation at Thr308/Ser473. Thr308 phosphorylation partially activates AKT while full activation requires phosphorylation at Ser473 which remains as yet unsolved. We first analyzed the effects of biomechanical forces on both serine and threonine sites in the presence or absence of TNF-α. Cells were multiaxially stretched to 3% for 15, 60, 120 min and protein extracts were tested for PKB phosphorylation at Thr308 and Ser473 by western blot analysis. As shown in Figure 4.2, mechanical stretch induced a rapid phosphorylation of AKT at the critical Ser473, while
the basal phosphorylation at Thr308 was not increased by the application of CTS. The phosphorylation activity peaked at 15min in the C2C12 myoblasts that were tested. The localization of phosphorylated AKT was analyzed by immunostaining after TNF-α and/or CTS application at 15, 60 and 120 min. A preferential nuclear staining in CTS groups indicates that activated AKT has a tendency for nuclear translocation (Figure 4.3).

**CTS-mediated activated AKT phosphorylates GSK-3β.** GSK-3β, a serine/threonine protein kinase which is constitutively active in unstimulated muscle cells, phosphorylates many proteins including glycogen synthase, eIF2B, c-Myc, and cyclin D, keeping them inactive or promoting their degradation. Phosphorylation of GSK-3β suppresses its activity, resulting in the activation of pathways that are normally repressed by GSK-3β (Fang et al., 2005; Vyas et al., 2002). We next examined if CTS mediated activated AKT can phosphorylate its downstream target GSK3β. Cells were stretched to 3% elongation for 15, 60, and 120 min and protein extracts were tested for GSK3β phosphorylation at Ser 9 by western blot analysis. As shown in Figure 4.4, mechanical stretch induced phosphorylation of GSK3β at Ser9. The phosphorylation activity peaked at 120 min indicating a delay as compared to AKT, which was maximal at 15 min in the C2C12 myoblasts. Immunostaining for pGSK3β shows increased staining for CTS treated groups as opposed to others (Figure 4.5).

**PI3K inhibitor, LY294002, prevents CTS mediated AKT phosphorylation at Ser473.** In general, activation of AKT by growth factors (e.g., IGF-1) is mediated by PI3K. PI(3,4)P2 and PI(3,4,5)P3 are generated mainly by D3 phosphorylation of PI(4)P2 and PI(4,5)P3, respectively, with activated class Ia or Ib PI3-Kinase. PI(3,4)P2 and PI(3,4,5)P3 are capable of binding to the pleckstrin homology (PH) domain of AKT and other kinases like PDK1 (Matsui et al., 2005). The role of PI3K was examined to elucidate the signal transduction pathways underlying the activation of PKB/AKT phosphorylation by mechanical stretch. In our study the function of PI3K was blocked by a highly selective inhibitor (LY-294002). It was found that a pretreatment for 1h with
Inhibitors of IGFR1, IR and GPCR partially abrogate the effects of CTS mediated AKT phosphorylation. Insulin-like growth factors (IGF-I and IGF-II) are potent stimulators of muscle differentiation through the induction of myogenin and MEF2. Studies on signaling through the IGF receptor have revealed that insulin receptor substrate-1 (IRS-1) is tyrosine phosphorylated at multiple sites and acts as a docking site for phosphatidylinositol 3-kinase (PI3K). At 50µm concentration AG1024, a specific inhibitor of Insulin-like growth factor receptor and Insulin Receptor kinases, partially inhibited the CTS mediated AKT phosphorylation (Figure 4.7). While the G-Protein coupled receptor inhibitor, Pertussis toxin, at concentrations of 5 and 20ng/ml, marginally inhibited CTS-mediated AKT phosphorylation (Figure 4.8).

CTS does not upregulate Focal Adhesion Kinase, FAK tyrosine phosphorylation. Integrins are strong candidates as mechanotransducers in mechanical strain-induced ERK activation (Schmidt et al., 1998). Indeed, integrin-mediated cell attachment activates many intracellular signaling pathways such as tyrosine phosphorylation cascades, calcium influx, and the mitogen-activated protein kinase (MAPK) pathway (Clark, 1995). Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase that has been reported to play an important role in integrin-mediated signal transduction pathways (Alahari et al., 2002). Studies have shown that FAK is phosphorylated at multiples sites, including Tyr397, creating a high affinity binding site for the Src homology 2 domain of Src family kinases, leading to a cascade of phosphorylation events (Calalb et al., 1995). Here, we examined if CTS induces FAK tyrosine phosphorylation which could be a potential mediator of AKT activation. Our results do not show any increased FAK

50µM LY-294002 completely blocked the stretch-induced phosphorylation of AKT (Figure 4.6). The stretch-induced phosphorylation of AKT detected 60 min after stretch stimulation was reversed by increasing concentrations of LY294002 (Figure 4.6, lanes 4-7). The CTS-mediated phosphorylation of GSK3β, a target of activated AKT, was also blocked by 50µM LY294002. These findings clearly demonstrate PI3K-dependent phosphorylation of AKT in response to mechanical stretching of C2C12 cells.
tyrosine phosphorylation after 15 or 30 min of CTS (Figure 4.9). The possibility of Integrin linked kinase, ILK mediated AKT activation is yet to be explored.

**CTS upregulates AKT2 mRNA expression and protein synthesis during SMD.** Of the three isoforms, AKT2 appears to be most intricately involved with muscle cell differentiation, as evidenced by: (i) microinjection studies of specific antibodies implicated AKT2, but not AKT1 with muscle differentiation; (ii) AKT1 is expressed constitutively whereas AKT2 expression is specifically induced during SMD; (iii) both the mRNA and protein levels of AKT1 are not changed whereas AKT2 levels are elevated during muscle differentiation; and (iv) Myod1 transcriptionally regulates AKT2 by binding to the AKT2 promotor region, and AKT2 in turn triggers Myod1-MEF2 transactivation capacity, resulting in myogenin expression (Altomare et al., 1998; Calera and Pilch, 1998; Vandromme et al., 2001). Collectively, these observations strongly suggest that AKT2, but not AKT1, plays a specific role in myogenesis under physiological conditions.

Therefore, we first examined the expression patterns of AKT2 mRNA expression over a period of 4 days of differentiation. The induction of AKT2 showed a marked increase with a 22-fold expression at 72 h of differentiation as compared to the undifferentiated myoblasts (Figure 4.10). Next we examined the effects of CTS on the expression of AKT2 mRNA expression and AKT2 protein synthesis in differentiating C2C12 cells. C2C12 myoblasts were either untreated (control) or exposed to rhTNF-α, CTS, or CTS+rhTNF-α. Within 24 h of initiating differentiation, C2C12 cells exhibited an increased AKT2 mRNA expression (Figure 4.11), and this expression was about 1.5-fold increased in response to CTS. TNF-α exposure suppressed more than 46% of the AKT2 mRNA expression induced by DM. Further analysis after 48 h of CTS or CTS and TNF-α treatment revealed a similar pattern with marked differences. The AKT2 protein followed a similar picture with increased synthesis with CTS treated groups while the synthesis is suppressed by TNF-α (Figure 4.12).
4.4 DISCUSSION

The primary focus of this study is to identify the effects of mechanical forces on skeletal muscle differentiation during inflammatory conditions and dissect the actions of these forces on the IGFR1-PI3K-AKT pathway. The results demonstrate that biomechanical signals stimulate rapid, sustained, activation of IGFR1-PI3K-AKT pathway in C2C12 cells to drive myogenic differentiation and prevent the atrophy pathways initiated by FOXO transcription factors. It is well accepted that muscle cells are responsive to biomechanical signals such as those experienced during exercise and can upregulate skeletal muscle hypertrophy (Aikawa et al., 2001; Cheema and Brown, 2005). Mechanical signals have been shown to phosphorylate and activate AKT and its downstream target mTOR, resulting in increased proteins synthesis in myotubes (Hornberger et al., 2004). Similarly, mechanical stretch activates epidermal growth factor receptor and angiotensin II type 1 receptor, resulting in AKT phosphorylation (Kippenberger et al., 2004). Although much progress has been made in defining the mechanisms by which AKT governs myogenesis at the transcriptional level, the effects of inflammation on this signal transduction pathway are poorly defined. We here have additionally examined the effects of TNF-α alone or in addition to CTS on IGFR1-PI3K-AKT pathway and the early events leading to AKT activation.

Earlier studies have shown that ectopic expression of activated AKT1 can promote extensive SMD in different myoblast cell lines even in the absence of IGF-I, and can reverse the inhibitory effects of PI3K inhibitors like Wortmannin and LY294002 on myogenic differentiation (Rommel et al., 1999; Jiang et al., 1998). AKT has 3 different distinct domains (Figure 4.1). The plecstrin homology domain (PH) is involved with binding to the PIP3, the regulatory domain with sites for phosphorylation and its activation and the catalytic domain, the kinase active region. AKT is activated by dual regulatory mechanisms that require both its membrane relocalization from the cytosol to plasma membrane and c-terminal phosphorylation at Thr308/Ser473 (Hemmings, 1997).

By means of an in vitro model system, utilizing C2C12 myoblast-like cells, cyclic equibiaxial stretching, and a proinflammatory cytokine, TNF-α, we have studied the effects of mechanical forces on the AKT activation pathway. We have shown earlier that
TNF-α inhibits skeletal muscle differentiation via activation of the NF-κB signal transduction pathway and its subsequent induction of Nos2a and NO production leading to MYOD1 protein loss (Di marco et al., 2005; Chandran et al., 2007). Moreover CTS of magnitudes 3% have been shown to be anti-inflammatory, suppressing the actions of TNF-α and augmenting myogenesis during skeletal muscle differentiation. Our results show that CTS activates AKT preferentially at the Ser473 within 15 minutes of treatment.

One of the major downstream targets of AKT is GSK-3β. We examined the effects of CTS-mediated AKT phosphorylation on GSK-3β to confirm the kinase activity following CTS. GSK3β was phosphorylated by CTS as opposed to the untreated groups. A delay in activity, as compared to AKT phosphorylation, possibly could be a result of the intermediate steps that are involved before GSK3β phosphorylation.

Studies on signaling through the IGF receptor have revealed that insulin receptor substrate-1 (IRS-1) is tyrosine phosphorylated at multiple sites and acts as a docking site for phosphatidylinositol 3-kinase (PI3K). PI(3,4)P2 and PI(3,4,5)P3 are generated mainly by D3 phosphorylation of PI(4)P2 and PI(4,5)P3, respectively, with activated class Ia or Ib PI3-Kinase. PI(3,4)P2 could be also generated by 5-dephosphorylation of PI(3,4,5)P3 by Src homology 2 (SH-2)-containing 5-phosphatase, SHIP. On the other hand, PTEN (phosphatase and tensin homolog), is a protein phosphatase that dephosphorylates PIP3 at the D3 position and acts as a negative regulator of PI3K/Akt signaling during myogenesis (Wan and Helman, 2003). PI(3,4)P2 and PI(3,4,5)P3 are capable of binding to the pleckstrin homology (PH) domain of AKT and other kinases like PDK1 (Matsui et al., 2005). The binding of the pleckstrin homology (PH) domain of AKT to PI(3,4)P2 and PI(3,4,5)P3 on the plasma membrane releases the autoinhibitory role of this domain, allowing phosphoinositide-dependent kinase-1 (PDK1) to phosphorylate AKT on Thr308. Thr308 phosphorylation partially activates AKT while full activation requires phosphorylation at Ser473 which remains as yet unsolved. Our results show, after blocking the PI3K activity by LY294002, that CTS-mediated phosphorylation of AKT is blocked at the critical Ser473.

Insulin-like growth factors (IGF-I and IGF-II) are potent stimulators of muscle differentiation through the induction of myogenin and MEF2. Studies on signaling
through the IGF receptor have revealed that insulin receptor substrate-1 (IRS-1) is tyrosine phosphorylated at multiple sites and acts as a docking site for phosphatidylinositol 3-kinase (PI3K). Next we explored how mechanotransduction occurs at the receptor level by inhibiting IGFR1 / IR kinases, as well as GPCR using AG1024 and Pertussis toxin, respectively. The results demonstrate that IGFR1, IR and GPCR may play a partial role in CTS-mediated AKT activation. While the possibility of focal adhesion kinase, FAK-mediated phosphorylation was not observed, other substrates such as integrin-linked kinase, ILK, which has been implicated in the Ser473 phosphorylation of AKT, is likely and yet to be explored.

Although, stretch induced activation of IGFR1-PI3K-AKT pathway has been shown in whole muscle models and in vitro cell culture models (Hornberger et al., 2004; Kippenberger et al., 2004), our findings are the first to demonstrate that CTS is a potent myogenic signal that dramatically upregulates myogenesis despite the presence of a strong pro-inflammatory environment. Moreover we have analyzed the effects of CTS-mediated active AKT on GSK3β and FOXO atrophy pathway. We have dissected out the mechanotransduction of biomechanical signals and it likely activates IGFR1, IR1 and GPCR, among other possible mechanisms. Thus, biomechanical signals play an essential role in muscle regeneration and muscle atrophy by activating AKT and subsequently its downstream targets.
<table>
<thead>
<tr>
<th>Primer/Probe</th>
<th>Sequence</th>
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<tr>
<td>Rps18</td>
<td>Sense 5’-ATC ATC GAG AGC GAC CTG GAA CG-3’</td>
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<tr>
<td></td>
<td>Anti-sense 5’-GCC AGT GGT CTT GGT GTG CTG AC-3’</td>
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<tr>
<td>MuRF</td>
<td>Sense 5’-GGCATCGCCCCAAAAGAACTT-3’</td>
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<tr>
<td></td>
<td>Anti-sense 5’-AATGTTGCCCACCCAGCACA-3’</td>
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<tr>
<td>MAFbx</td>
<td>Sense 5’-TTGGAGAAGACAGCTATTTTGGA-3’</td>
</tr>
<tr>
<td></td>
<td>Anti-sense 5’-TCCTGAAGACACCCTTTGGGA-3’</td>
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**Table 4.1**

SYBR green Primer sequences for Real-time PCR (IV)
Figure 4.1. Schematic of catalytic subunits of AKT and its key phosphorylation sites.

All the AKT/PKB isoforms consist of a conserved domain structure: an amino terminal pleckstrin homology (PH) domain, followed by a kinase domain related to protein kinases A and C (containing Thr308 in AKT), and a C-terminal regulatory domain (containing Ser473 in AKT). PH domains have high-affinity recognition of phosphoinositide head groups. Activation of PI3K leads to D3 phosphorylation and generation of PI(3,4)P2 and PI(3,4,5)P3 which recruit AKT by binding to the PH domain and exposing the Thr308 site in the kinase domain. Subsequently, phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates Thr308. Although phosphorylation of Thr308 partially activates AKT, phosphorylation of Ser473 in a C-terminal regulatory domain is required in order to induce full activation in AKT which remains as yet unsolved, although the kinase responsible is referred to as PDK2. Other possible activation mechanisms at Ser 473 involves autophosphorylation by AKT itself and lastly PDK1 phosphorylating both Thr 308 and Ser 473.
Figure 4.2 Activation of AKT by phosphorylation at Ser473 site by CTS. The time course of PKB/AKT phosphorylation after the application of CTS and/or TNF-α in C2C12 cells. The cells were incubated in flexible collagen coated plates and grown to 80% confluence followed by a change to DMEM containing 2% HS just before the start of experiments. CTS of 3% magnitude and 0.05 Hz frequency was applied in the presence or absence of TNF-a for 15, 60, 120 min. Proteins were obtained and western blotting was performed as described under “Materials and Methods,” with phosphospecific antibodies directed toward Thr308 and Ser473, respectively. Equal loading was monitored by using antibodies directed against total PKB/AKT. The blot shows representative results (n = 3) and the graphs represent mean ± SD for 3 separate experiments. p-phospho.
Figure 4.3 Immunostaining for AKT phosphorylation at Ser473 after the application of CTS and/or TNF-α. C2C12 myoblasts grown on Bioflex plates in the presence or absence of TNF-α were subjected to CTS for various time intervals and analyzed for the presence of pSer473AKT in cells by immunofluorescence at 15, 60, and 120 min. pSer473AKT stained red while actin stained green. Increased amounts of staining could be appreciated in CTS and CTS+TNF-α groups at 120 min. Data is a representation of one of three separate experiments.
Figure 4.4 CTS-mediated activated AKT phosphorylates GSK-3β. The time course of GSK-3β phosphorylation after the application of CTS and/or TNF-α in C2C12 cells. The cells were incubated in flexible collagen coated plates and grown to 80% confluence followed by a change to DMEM containing 2% HS just before the start of experiments. CTS of 3% magnitude and 0.05 Hz frequency was applied in the presence or absence of TNF-α for 15, 60, 120 min. Proteins were obtained and western blotting was performed as described under “Materials and Methods,” with phosphospecific antibodies directed toward GSK3β. Equal loading was monitored by using antibodies directed against β–actin. The blot shows representative results (n = 3) and the graphs represent mean ± SD for 3 separate experiments. p-phospho.
Figure 4.5 Immunostaining for pGSK3β after the application of CTS and/or TNF-α for 2h. C2C12 myoblasts, grown on Bioflex plates in the presence or absence of TNF-α, were subjected to CTS for various time intervals and analyzed for the presence of pGSK3β in cells by immunofluorescence at 120 min. pGSK3β stained red while actin stained green. Increased amounts of staining could be appreciated in CTS and CTS + TNF-α groups. Data is a representation of one of three separate experiments. DM - Differentiation Medium.
PI3K inhibitor, LY294002 prevents CTS-mediated AKT phosphorylation at Ser473 but not Thr308. C2C12 cells were grown in GM and switched to 2% HS and pretreated with PI3K inhibitor LY294002 (10 or 50 μM) and stretched for 1h, after which total protein lysate was obtained and subjected to Western blot analysis for Ser473 and Thr308 phosphorylation of AKT and Ser9 phosphorylation of GSK3β. CTS mediated phosphorylation at Ser473 was prevented by pretreatment with LY294002, while Thr 308 was not affected by CTS or pretreatment with the inhibitor. The blot shows representative results (n=3). GM - Growth Medium, DM - Differentiation Medium, p-phospho.
Figure 4.7 CTS-mediated AKT phosphorylation is partially abrogated by a specific tyrosine kinase inhibitor AG1024. C2C12 cells were grown in GM and switched to 2% HS and pretreated with IGFR1 / IR kinase inhibitor AG1024 (1 or 10mM) and stretched for 1h, after which total protein lysate was obtained and subjected to Western blot analysis for Ser473 phosphorylation of AKT. CTS mediated phosphorylation of Ser 373 was partially abrogated by pretreatment with AG1024. The blot shows representative results (n=3). p-phospho.
Figure 4.8 CTS-mediated AKT phosphorylation is partially abrogated by G protein coupled receptor inhibitor, pertussis toxin. C2C12 cells were grown in GM and switched to 2% HS and pretreated for 12 h with G-Protein coupled receptor inhibitor, pertussis toxin (5 or 10 ng/ml), and stretched for 1h, after which total protein lysate was obtained and subjected to Western blot analysis for Ser473 phosphorylation of AKT. CTS-mediated phosphorylation of Ser373 was partially abrogated by pretreatment with pertussis toxin at both 5 and 10ng/ml. The blot shows representative results (n=3). p-phospho.
Figure 4.9 CTS does not activate focal adhesion kinase, FAK tyrosine phosphorylation. C2C12 cells were grown in GM and switched from DMEM–20% FBS to DMEM–2% HS and stretched for 15 min or 30 min, after which total protein lysate was obtained and immunoprecipitated with a rabbit anti-FAK antibody (sc-557, Santa Cruz Biotechnology, CA) at 1:100 dilution at 4°C overnight. Immunocomplexes were captured with protein A/G-agarose and analyzed by SDS-PAGE. Results here are a representation from 3 independent experiments with similar results.
Figure 4.10 **AKT2 mRNA temporal profiling.** mRNA expression profiles for myogenic transcription factors *AKT2*, after 24, 48, 72 and 96h differentiation shows a rapid rise in expression. Undiff- Undifferentiated myoblasts, diff 24h – Differentiation medium 24h, diff 48h – Differentiation medium 48h, diff 72h – Differentiation medium 72h, diff 96h – Differentiation medium 96h. Data exhibit mean and SD of 3 separate experiments.
Figure 4.11 Effects of CTS on AKT2 mRNA expression. C2C12 myoblasts grown on Bioflex plates in the presence or absence of TNF-α were subjected to CTS for various time intervals and analyzed for (A) AKT2 mRNA expression by RT-PCR. Data exhibit mean and SD of 3 separate experiments, * indicates $p<0.05$ between control and TNF-α treated cells, § indicates $p<0.05$ between TNF-α and CTS / TNF-α treated cells, and ¶ indicates $p<0.05$ between control and CTS treated cells.
Figure 4.12 Effects of CTS on AKT2 protein synthesis. C2C12 myoblasts grown on Bioflex plates in the presence or absence of TNF-α were subjected to CTS for various time intervals and analyzed for AKT2 protein levels by Western blots. Data is a representation from one of three separate experiments. RFU, relative fluorescence unit.
Figure 4.13 Schematic of actions of CTS on the IGFR1-PI3K-AKT pathway. Mechanical signals possibly activate the IGFR1-PI3K-AKT pathway by (1) increasing IGF secretion and causing autocrine activation of IGFR1, (2) phosphorylating and activating the receptors such as IGFR1, IR1 and GPCR among others and (3) possibly releasing AKT from the actin cytoskeleton.
Chapter 5
Discussion

Skeletal muscle differentiation is a well established process in which specific molecular and cellular events occur in a precisely defined spatial and temporal manner. The effect of biomechanical forces on skeletal muscle differentiation processes is controversial with some studies reporting these forces as being pro-differentiative (Rauch and Loughna, 2004; Sakiyama et al., 2005), while others as anti-differentiative (Kumar et al., 2004). Despite these contradictory claims, the effects of biomechanical signals on skeletal myogenesis during chronic inflammatory conditions remain largely unexplored. The primary research goal of this dissertation was to identify the effects of mechanical forces on skeletal muscle differentiation in the presence and/or absence of inflammation and dissect the actions of these forces on the regenerative IGFR1-PI3K-AKT pathway.

Summary of Results.
I. CTS upregulates myogenesis in the presence or absence of inflammation.

TNF-α inhibits skeletal muscle differentiation via activation of the NF-κB signal transduction pathway and its subsequent induction of Nos2a and NO production, leading to MYOD1 protein loss (Di marco et al., 2005; Guttridge et al., 2000). The results of our study demonstrate that C2C12 myoblast-like cells respond to biomechanical signals in a magnitude-dependent manner, inhibiting rhTNF-α-induced Nos2a expression to various degrees between the magnitudes of 3% and 18% of strain. However, the maximal inhibition of rhTNF-α actions was observed at 3% elongation.

At low physiological levels, CTS causes increased expression, synthesis, and nuclear translocation of the “early commitment factor”, MYOD1, which is one of the
initial events in the skeletal muscle differentiation process. Furthermore, CTS upregulated the expression and synthesis of other myogenic factors such as myogenin, Mef2a, and Cdkn1a, even in the presence of TNF-α. The function of bHLH and MEF2 transcription factors, in the context of myogenic differentiation, is to upregulate induction of skeletal muscle structural proteins. CTS markedly upregulates the transcriptional activation of Myh1, Myh2, and Myh4 isoforms, and the synthesis of MYHC and TPM1 proteins. The results demonstrate that biomechanical signals stimulate rapid and sustained activation of bHLH and MEF2 family transcription factors in C2C12 cells. Collectively, these observations underscore the importance of biomechanical signals in muscle regeneration, suggesting that the extracellular mechanical forces are converted into intracellular biochemical events that can initiate and sustain myogenic differentiation.

II. CTS activates the IGFR1-PI3K-AKT pathway and mechanotransduction occurs at the receptor level.

Mechanical signals have been shown to phosphorylate and activate AKT and its downstream target, mTOR, resulting in increased proteins synthesis in myotubes (Hornberger et al., 2004). Although much progress has been made in defining the mechanisms by which AKT governs myogenesis at the transcriptional level, the effects of inflammation on this signal transduction pathway are poorly defined. Here we have examined the effects of TNF-α alone or in addition to CTS on the IGFR1-PI3K-AKT pathway and the early events leading to AKT activation.

By means of an in vitro model system, utilizing C2C12 myoblast-like cells, cyclic equibiaxial stretching, and a proinflammatory cytokine, TNF-α, we have studied the effects of mechanical forces on AKT activation pathway. The results of our study demonstrate that biomechanical signals stimulate rapid, sustained, activation of the IGFR1-PI3K-AKT pathway in C2C12 cells to drive myogenic differentiation. We also found that one of the AKT targets, GSK3β, was phosphorylated by CTS, as opposed to the unstretched groups. A delay in activity, as compared to AKT phosphorylation, possibly could be a result of the intermediate steps that are involved before GSK3β
phosphorylation. Further, our results show that, after blocking the PI3K activity by LY294002, the CTS-mediated phosphorylation of AKT Ser 473 is blocked. Finally, by using different inhibitors, we identified that mechanotransduction occurs at the multiple receptor level, including but not limited to IGFR1, IR and GPCR.

**Conclusion.**

While numerous studies have examined the mechanoresponsiveness of muscle tissues *in vivo*, only a few studies have elucidated the mechanisms mediating stretch-induced upregulation of myogenesis *in vitro*. Elucidation of such intracellular mechanisms is critical because it provides pieces of missing information to the response of muscle tissues during exercise and other mechanical activities. Biomechanical signal mediated myogenesis is likely to be beneficial if the augmented myogenesis could be reproduced *in vivo*. The current findings show that biomechanical signals could activate myogenesis in the presence or absence of inflammation by possibly abrogating the proinflammatory NF-κB pathway, and activating myogenesis by upregulating the IGFR1-PI3K-AKT pathway. While acknowledging that other potential mechanisms by which mechanotransduction occurs in muscle tissue, these findings are one step closer to understanding the intracellular mechanisms relevant to muscle responses.


Dodou E, Xu SM and Black BL. (2003) mef2c is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo. Mech Dev. 120: 1021-1032.


