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The Influence of Propyl Pyrazole Triol on the Post-Exercise Alpha Estrogen Receptor-Mediated Activation of Satellite Cells in Skeletal Muscle of Ovariectomized Rats

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THE INFLUENCE OF PROPYL PYRAZOLE TRIOL ON THE POST-EXERCISE
ALPHA ESTROGEN RECEPTOR-MEDIATED ACTIVATION OF SATELLITE
CELLS IN SKELETAL MUSCLE OF OVARIECTOMIZED RATS

by

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THESIS

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Abstract

Estrogen has been shown to augment satellite cell activation, proliferation and total number and that this may occur through an estrogen receptor (ER) mediated mechanism. The purpose of this study was to investigate the role of ERs in the post-exercise estrogen-induced augmentation of satellite cells in skeletal muscle of ovariectomized rats. Furthermore, the specific role of the ER α was examined through the use of an ER α agonist, propyl pyrazole triol (PPT). Ovariectomized rats were used (n=64) and separated into 4 groups: sham, estrogen supplemented, agonist supplemented and a combined estrogen and agonist supplemented group. These groups were further subdivided into control (unexercised) and exercise groups. Animals in the exercise group participated in an intermittent running protocol that involved animals running downhill on a motorized treadmill for 90 minutes. Surgical removal of white vastus and soleus muscles occurred 72 hours post-exercise. Muscle samples were immunostained for the satellite cell markers Pax7 and MyoD. Significant increases in total (Pax7-positive) and activated (MyoD-positive) satellite cells were found in all groups post-exercise. A further significant augmentation of total and activated satellite cells occurred in estrogen supplemented, agonist supplemented and the combined estrogen and agonist supplemented groups post-exercise in white vastus and soleus muscles relative to unsupplemented animals. These results demonstrate that both estrogen and the ER α agonist, PPT, can significantly augment satellite cell number and activation following exercise-induced muscle damage. This suggests that estrogen acts through an ER-mediated mechanism to

stimulate satellite cell activation and proliferation following exercise, with ER α playing a primary role.

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List of Abbreviations

A – agonist group; animals supplemented with PPT

Akt-mTOR – Akt-mammalian target of rapamycin

Con – control group (unexercised)

EA – estrogen and Agonist group; animals supplemented with estrogen and PPT

E – estrogen group; animals supplemented with estrogen only

Ex – exercise group

ER α – alpha estrogen receptor

ER β – beta estrogen receptor

ER – estrogen receptor

FGF – fibroblast growth factor

HGF – hepatocyte growth factor

HRT – hormone replacement therapy

IGF-I – insulin-like growth factor

IL-6 – interleukin-6

I/R – ischemia reperfusion

LIF – leukemia inhibitory factor

NO – nitric oxide

NOS – nitric oxide synthase

OVX - ovariectomized

PI3 kinase-Akt – phosphatidylinositol 3-kinase-Akt

PPT – propyl pyrazole triol

S – sham group

List of Definitions

Apoptosis – Programmed cell death.

Blunt Dissection – A surgical technique that exposes structures without cutting.

Calpain – A protease that is activated by calcium and causes the breakdown of proteins.

Chemotaxis – Refers to the movement of cells in a certain direction, in response to chemical stimuli.

Concentric Contraction – A type of contraction where force development occurs while the muscle is shortening.

Cytokines – Small proteins that are released by cells and influence cell-cell interaction, communication and behaviour of other cells.

Cytoskeleton – A part of the cytoplasm that does not include organelles or internal membrane systems.

Diestrus – A stage of the estrous cycle where estrogen levels are the lowest.

Eccentric Contraction- A type of contraction where force development occurs while the muscle is lengthening.

Hepatocyte growth factor – A growth factor that regulates the activation, proliferation and differentiation of satellite cells.

Fibroblast growth factor – A growth factor that regulates satellite cell proliferation and differentiation.

I band – A region of the sarcomere that contains actin myofilaments.

Insulin growth factor-I – A growth factor that regulates satellite cell proliferation and differentiation.

Interleukin-6 – A cytokine that regulates macrophage apoptosis and satellite cell proliferation.

Immunohistochemistry – The use of immunologic techniques for the chemical analysis of tissues where the presence of specific antigens in a tissue are marked by fluorescent dyes or enzymes.

Ischemia/Reperfusion – Occurs when there is a low level of oxygen present in a tissue (usually due to inadequate blood flow) followed by an increase in blood flow and oxygen levels. This can cause a production of reactive oxygen species and oxidative damage to the involved tissues.

Leukemia inhibitory factor – A cytokine that regulates satellite cell proliferation.

Ligand – A molecule that binds to a receptor to initiate a cellular response.

Macrophage – A leukocyte that infiltrates skeletal muscle 24-48 hours following muscle damage and is involved in the removal of cellular debris and stimulation of satellite cells.

Myoblast – Cells that give rise to skeletal muscle fibres.

Myocyte – A muscle cell.

MyoD – A marker of activated satellite cells.

Myofibril – A long, cylindrical fibre in skeletal muscle that is composed of actin and myosin myofilaments.

Myofilament – Make up myofibers. Thick ones contain the contractile protein myosin while thin ones contain the contractile protein actin.

Neutrophil – A leukocyte that is involved in the breakdown of cellular debris following muscle damage.

Ovariectomy – Surgical removal of one or both ovaries.

Pax7 – A transcription factor that is present in quiescent, activated and proliferating satellite cells. It can be stained through immunohistochemical techniques and thus acts as a positive marker for satellite cells.

Peroxidation of Lipid Bilayer – Breakdown of the lipid bilayer from an oxidant.

PPT – Propyl pyrazole triol; an estrogen agonist that acts on the estrogen receptor alpha.

Protease – A type of enzyme which breaks down proteins into smaller subunits.

Proteolysis – The cleavage (breakdown) of proteins by proteases.

Reactive Oxygen Species – Include oxidants, such as superoxide, which can cause protein degradation, peroxidation of lipid membranes and damage to DNA.

Sarcolemma – The plasma membrane of a skeletal muscle fibre.

Sarcomere – The functional, repeated subunit of striated muscle.

Sarcoplasmic reticulum – A membrane enclosed organelle in skeletal muscle that sequesters calcium. During excitation-contraction coupling, the sarcoplasmic reticulum releases calcium, allowing cross-bridge formation to occur.

Satellite Cell – Cells found in muscle that act to repair existing myofibers and/or make new myofibers.

Superoxide – A strong oxidant that can cause damage to cells.

Z disc – A region of the sarcomere where actin myofilaments are inserted.

Z disc streaming – Tearing of the Z disc in one or multiple sarcomeres.

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Chapter One

Introduction

A fundamental role of skeletal muscle is the production of gross and fine movements. Skeletal muscle is made up of fibres that contain specialized contractile proteins that allow for muscle force generation and contraction to occur. Damage sustained by muscle fibres can cause pain, swelling and ultimately impair skeletal muscle function (MacIntyre et al., 1995; Proske & Allen, 2005). Muscle repair mechanisms therefore play a vital role in the proper and optimal functioning of skeletal muscle. Key components of the indices of muscle damage, inflammation and repair are further discussed below.

1.1 Skeletal Muscle Damage

Skeletal muscle damage involves the disruption of muscle fibres and can result from a variety of factors including exercise, muscular trauma, overuse injuries and ischemia reperfusion (I/R) injuries (Armstrong et al., 1983; Gute et al., 1998; Silverman, 2007). In order to elicit muscle damage, many studies employ exercise regimes that involve eccentric contractions. Eccentric contractions occur when the muscle produces force as it lengthens. Numerous studies have demonstrated that eccentric contractions produce significantly more damage to muscle tissue than concentric contractions (Clarkson & Sayers, 1999; Gibala et al., 1995). There are various mechanical factors that play a role in this fact. Eccentric muscle contractions require the recruitment of fewer motor units and muscle fibres than concentric contractions and therefore increase the strain per fibre, causing more damage. Additionally, a smaller cross-sectional area of the

muscle is loaded, increasing the subsequent amount of damage (Enoka, 1996). Moreover, recruitment of structures not typically involved in excitation-contraction coupling, such as tendons, can occur during eccentric contractions (Brown et al., 1997). Morgan (1990) proposed a theory to explain this phenomenon. He stated that during eccentric contractions some sarcomeres become overextended and “pop,” resulting in a decreased or total lack of overlap of the actin and myosin myofilaments in that particular sarcomere. In order to maintain the load, the activation of tendons then becomes necessary, which can increase the degree damage within the muscle.

Histological evidence of muscle damage to the sarcolemma, sarcoplasmic reticulum, myofibrils and the cytoskeleton following eccentric exercise have been demonstrated in numerous studies (Armstrong et al., 1983; Friden et al., 1984; Trappe et al., 2002; Yu et al., 2003). The cytoskeleton of skeletal muscle fibres is composed of a variety of proteins including titin, desmin, α -actinin, nebulin and dystrophin. These proteins act to stabilize actin, myosin and the sarcomere as a whole (Morgan & Allan, 1999). Exercise-induced damage, particularly from eccentric contractions, can damage these cytoskeletal proteins causing Z disc streaming to occur. Z disc streaming is a common feature of exercise-induced muscle damage where the Z discs of some sarcomeres are disrupted or torn (Figure 1). This type of damage has been shown in human muscle biopsies following eccentric contractions (Newham et al., 1983; Nurenberg et al., 1992; Gibala et al., 1995; Gibala et al., 2000).

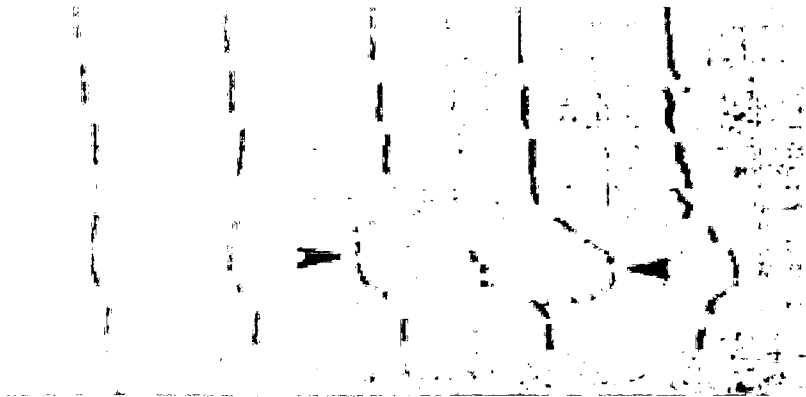


Figure 1: An electron micrograph from a human leg muscle biopsy sample following an eccentric exercise protocol. The arrows indicate Z-disc streaming in two sarcomeres (Nureberg et al., 1992).

Secondary damage (damage occurring following the initial injury) to the muscle can occur post-exercise via the stimulation of non-lysosomal proteases including calpain (Brooks et al., 1983; Van der Westuyzen et al., 1981; Zenman et al., 1985). Calpain has three isoforms: calpain-1, calpain-2 and calpain-3. While calpain-3 is involved in skeletal muscle maintenance, acting to aid in the removal of cellular debris within the sarcomere (Beckmann & Spencer, 2008), calpain-1 and calpain-2 act in a proteolytic fashion contributing to the damage of the sarcoplasmic reticulum, connective tissue, contractile tissue and mitochondrial swelling in skeletal muscle following intense exercise or eccentric contractions (Brown et al., 1997; Duncan, 1987; Verburg et al., 2009). Furthermore, since 2-5% of the total amount of calpain in the muscle fibre is located at the I and Z bands, it is thought that calpain-1 and calpain-2 may play a role in the degradation of cytoskeletal proteins in the Z disc (Belcastro et al., 1988; Verburg et al., 2009). Belcastro et al. (1988), for example, found a significant decrease in two proteins, 58 Kda and 95 Kda, following exercise-induced muscle damage. These two proteins could correspond to desmin and α -actinin, both of which make up a large portion of the Z disc. Furthermore, studies conducted *in vitro* have demonstrated the ability of calpain-1 and calpain-2 to cleave various proteins including desmin, which can cause the release of α -actinin, contributing to Z disc streaming (Goll et al., 1991; Saïdo et al., 1994; Verburg et al., 2009). These results show a plausible link between exercise-induced muscle damage, the activation of calpain and the resulting breakdown of α -actinin and desmin; contributing to Z disc streaming and further damage to the muscle fibre.

Numerous studies support post-exercise induced muscle damage by calpain following eccentric exercise. Since calpain is activated by calcium, in skeletal muscle,

intracellular levels of calcium must increase following exercise in order for this damage to occur. Duncan (1987) demonstrated this in a study that utilized skinned amphibian pectoris cutaneous muscle where an increased intracellular calcium concentration caused cellular damage including Z disc streaming, loss of myofibril organization and mitochondrial swelling. Similarly, treatment of skeletal muscle with the calcium ionophore (A23187) increased the intracellular calcium concentration within the muscle fibre and caused ultrastructural damage to occur (Publicover et al., 1978). Warren et al. (1995) has linked eccentric exercise to an increase in free cytosolic calcium levels in the muscle cell and an associated damage to the sarcolemma. Overall, the concentration of calcium within the muscle not only plays a role in excitation-contraction coupling and cross-bridge formation but it also can activate calpain-1 and calpain-2, leading to proteolysis of muscle tissue. Eccentric exercise was used in this study as a means to elicit exercise-induced muscle damage due to the strong relationship between eccentric contractions, calpain-1 and calpain-2 activation and the subsequent skeletal muscle damage.

Another source of secondary injury to muscle, which is also involved in initiating the muscle repair process, is the inflammatory response.

1.2 The Inflammatory Response

Following eccentric exercise and its associated muscle fibre damage, an inflammatory response is initiated involving the migration of leukocytes and other chemotactic agents to the site of injury (Clarkson & Sayers, 1999). Early stages of damage are characterized by an infiltration of neutrophils, followed by an increase in macrophages ED1⁺ and ED2⁺ to the site of injury (Tidball, 1995).

Neutrophils

Neutrophils are thought to be the first cells to migrate to the site of damage in skeletal muscle (Tidball, 1995). Fielding et al. (1993) demonstrated an accumulation of neutrophils in skeletal muscle 45 minutes following eccentric exercise in humans and this neutrophil accumulation remained for 5 days. The infiltration of neutrophils to the site of injury typically peaks at 12 to 24 hours post-injury, however. While the exact function of neutrophils is not known, it is postulated that they are involved in the removal of cellular debris and in stimulating the migration of macrophages to the site of injury (MacIntyre et al., 1995; Teixeira et al., 2003; Tidball, 1995). Neutrophils may also play a role in secondary muscle damage as their infiltration to the site of damage can lead to the production of reactive oxygen species including superoxide. Reactive oxygen species cause cellular damage including peroxidation of membrane lipid bilayers and the disruption of functional proteins, all of which can contribute to secondary damage post-exercise. This is a controversial topic, however, with studies showing support for (Nguyen and Tidball, 2003; Pizza et al., 2005) and against (Armstrong et al., 1983; Lapointe et al., 2002) secondary muscle damage caused by neutrophil infiltration.

Macrophages

Infiltration of macrophages to the site of damage in skeletal muscle is necessary for muscle regeneration to occur. This accumulation of macrophages peaks at 48 hours following injury (Hawke & Garry, 2001). Removal of cellular debris and necrotic tissue via phagocytosis is the primary role of macrophages in the inflammatory response; more specifically, it is postulated that this is completed by ED1⁺ macrophages (Lapointe et al.,

2002; Lescaudron et al., 1999). Studies have shown that ED2⁺ macrophages, on the other hand, do not participate in the removal of cellular debris but may play a role in the proliferation of satellite cells post-injury (Cantini et al., 1994; McLenna, 1993; St. Pierre & Tidball, 1994). In support of this, St. Pierre et al. (1994) demonstrated an accumulation of ED1⁺ macrophages in damaged myofibres 2 days following muscle reloading in mice, whereas ED2⁺ macrophages reached a peak accumulation 4 days following muscle reloading and were not directly involved in phagocytosis. Lescaudron et al. (1999) also demonstrated that muscle regeneration does not precede the infiltration of macrophages to the site of injury *in vivo*, providing evidence that macrophages may play a role in muscle regeneration. While this study does not examine the inflammatory response, the infiltration of neutrophils and macrophages to the site of damage in skeletal muscle plays an essential role in satellite cell signalling and activation, which this study did measure.

1.3 Muscle Repair: Satellite Cells

The term satellite cell was first coined by Alexander Mauro (1961) after their discovery in 1961. Satellite cells are mononucleated and fusiform in shape and reside between the basal lamina and the sarcolemma of muscles, running parallel to the myofibres. Since terminal differentiation of skeletal myocytes occurs in vertebrates, satellite cells are present to function as stem cells within skeletal muscle, aiding in postnatal muscle repair and regeneration (Mauro, 1961; Muir et al., 1965).

Until activated by various stimuli, satellite cells remain in a quiescent, undifferentiated state with a high nuclear-to-cytoplasmic ratio and few organelles (Muir et al., 1965; Schultz, 1976). In addition, the nucleus within a satellite cell is small and

contains a greater amount of condensed chromatin compared to myonuclei (Schultz, 1976). Stimuli that can activate satellite cells include myotrauma, ischemia, exercise and disease (Schultz & McCormick, 1994). Upon activation, satellite cells undergo a reduction in chromatin and an increase in organelle content and cytoplasmic-to-nuclear ratio. An increase in mitotic activity via entry into the cell cycle occurs as well, leading to the proliferation of satellite cells (Schultz & McCormick, 1994). Proliferation of satellite cells peaks 2 to 3 days following the myotrauma but can continue for up to 5 days post-trauma. At this point in time, satellite cells withdraw from the cell cycle and differentiate. When satellite cells differentiate, fusion into existing myofibres can occur, contributing to hypertrophy. In addition, satellite cells can align and fuse together forming a new myotube which is known as hyperplasia (Figure 2). The fate of a differentiating satellite cell depends on the severity of the damage and muscle regeneration is typically complete 10 days following the myotrauma (Garry et al., 1997; Hawke & Garry, 2001; Schultz & McCormick, 1994).

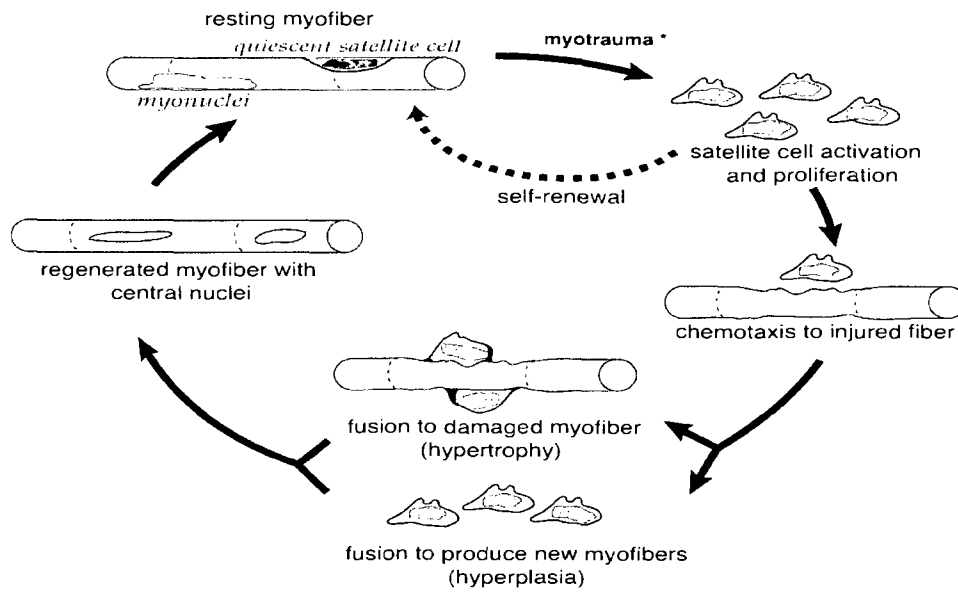


Figure 2: The satellite cell cycle (Hawke & Garry, 2001).

Satellite Cell Markers

There are various markers for satellite cell identification that are expressed or incorporated by satellite cells at different phases of activation. Some commonly used immunohistochemical markers include 5-bromo-2'-deoxyuridine (BrdU), MyoD and Pax7. BrdU is a thymine analog that identifies proliferating satellite cells (Hawke & Garry, 2001). BrdU becomes incorporated into the DNA of proliferating satellite cells acting as a nonradioactive marker that can identify replicating cells *in vivo* (Hurme & Kalimo, 1991).

Pax7 and MyoD are transcription factors that are common markers for satellite cell identification. Pax7 marks quiescent, activated and proliferating satellite cells (Hawke & Garry, 2001). MyoD, on the other hand, is not expressed in quiescent satellite cells but becomes up-regulated within 12 hours following muscle injury (Seale & Rudnicki, 2000). Activated satellite cells express MyoD and studies have shown that proliferating satellite cells express MyoD as well as other markers including Myf5 and desmin (Cornelison & Wold, 1997). Furthermore, studies utilizing MyoD(-/-) mice have demonstrated a continued proliferation of satellite cells while in the presence of differentiation-favoured conditions. As a result, it is postulated that MyoD may also play a role in satellite cell differentiation (Sabourin et al., 1999).

This study measured satellite cells 72 hours post-exercise utilizing Pax7 and MyoD as positive satellite cell markers. This time period was chosen as numerous studies have demonstrated the presence of peak levels of activated and proliferating satellite cells 72 hours post-exercise (Armstrong et al., 1983; Hawke & Garry, 2001; Hurme et al., 1991). Furthermore, Enns & Tiidus (2008) demonstrated that satellite cells labelled by

Pax7 peak at 3 days post-exercise and MyoD-labelled satellite cells peak at 2-3 days post-exercise when using the exercise protocol employed by this study.

Satellite Cell Regulation

Satellite cell activity may be regulated by numerous factors. It has been postulated that the extracellular matrix of skeletal muscle fibres store growth factors that are released through tears in the sarcolemma following eccentric exercise (Tatsumi et al., 1998). The release of these growth factors into the muscle fibre may play a role in the regulation of satellite cell activation, proliferation and differentiation. Hepatocyte growth factor (HGF), insulin-like growth factor (IGF-I), fibroblast growth factor (FGF), leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) are some such factors that may play a role in satellite cell regulation. These factors are further discussed below.

Hepatocyte Growth Factor (HGF)

HGF is a cytokine that acts on the c-met receptor located on satellite cells (Bischoff, 1997; Tatsumi et al., 1998). It is secreted by myoblasts (Anastasi et al., 1997) and by damaged tissue within the muscle (Cornelison & Wold, 1997; Tatsumi et al., 1998). Proposed roles for the action of HGF include the activation and proliferation of satellite cells (Allen et al., 1995; Tatsumi et al., 1998). Additionally, HGF acts as a chemoattractant where its release from damaged muscle tissue may allow satellite cells to migrate to the site of injury by chemotaxis (Bischoff, 1997). Gal-levi et al. (1998) also demonstrated that treatment of quiescent satellite cells with HGF caused the activation of satellite cells by triggering entry into the cell cycle, shown by an increase in DNA synthesis. Gal-levi et al. (1998) further demonstrated that HGF not only plays a role in

the activation and proliferation of satellite cells but it also inhibits differentiation by inhibiting transcriptional factors necessary for differentiation, including MyoD and myogenin.

Insulin-like Growth Factor-I (IGF-I)

IGF-I is secreted by skeletal muscle and plays a role in the promotion of satellite cell proliferation and differentiation (Chakravarthy et al., 2000; Hawke & Garry, 2001; Johnson et al., 1990; Mills et al., 2007). Chakravarthy et al. (2000), for example, demonstrated the proliferative capability of IGF-I where its injection into atrophied skeletal muscle of rats was followed by a significant increase in the proliferation of satellite cells compared to controls, leading to a significant increase in muscle mass. Furthermore, numerous studies have also shown that IGF-I plays a role in increasing skeletal muscle mass, strength and hypertrophy (Barton et al., 2007; Barton-Davis et al., 1998; Musaro et al., 1999). Since up-regulation of IGF-I occurs following eccentric exercise in humans (Hameed et al., 2007) and rats (Adams et al., 1998), IGF-I may be a key factor in skeletal muscle regeneration through the regulation of satellite cell proliferation and differentiation.

Fibroblast growth factor (FGF)

FGF is a cytokine that, like IGF-I, plays a role in satellite cell proliferation. A study by Floss et al. (1997) examined the role of FGF-6, an isoform of FGF, which is solely found in skeletal muscle. Using FGF-6 deficient mice, FGF-6(-/-), it was shown that following a crush injury, these mice had an impaired ability for muscle regeneration compared to mice with the FGF-6 gene. The authors postulated that FGF-6 may play a significant role in satellite cell activation or proliferation (Floss et al., 1997). The role that

FGF plays in the regulation of satellite cells was further examined by Scanta et al. (1999) who demonstrated that increasing the FGF receptor availability lead to an increase in satellite cell proliferation and a decrease in differentiation. Furthermore, decreasing FGF receptor availability lead to a decrease in satellite cell proliferation and an increase in differentiation of myocytes. This data shows that FGF plays a role in satellite cell proliferation and differentiation and that this may be regulated in part by receptor number and/or availability.

Leukemia inhibitory factor (LIF) & interleukin-6 (IL-6)

LIF and IL-6 belong to the same family of cytokines and are produced by a variety of cells including macrophages and myoblasts (Hawke & Garry, 2001; Hibi et al., 1996). LIF and IL-6 play a role in satellite cell regulation by stimulating cell proliferation (Austin & Burgess, 1991; Hawke & Garry, 2001). Kurek et al. (1997) utilized LIF(-/-) mice to demonstrate the regenerative role of LIF *in vivo* as the LIF knockout mice had a significantly decreased capacity for muscle regeneration following a muscle crushing injury. Cantini et al. (1995) examined IL-6 and demonstrated that it is released by activated satellite cells and macrophages, promoting satellite cell proliferation. IL-6 also acts directly on macrophages and neutrophils to induce cellular apoptosis and is therefore a regulator of both satellite cell proliferation and the inflammatory process. During satellite cell proliferation there are an increased number of satellite cells present and a consequent increase in the amount of IL-6 released. This is a regulatory mechanism that may act to ensure that the breakdown of macrophages and neutrophils occur at the site of injury while new myocytes and myotubes are being formed (Cantini et al., 1995).

Overall, there are many growth factors that play a role in satellite cell regulation. Satellite cells may also be regulated by a variety of other factors including nitric oxide, transforming growth factors, platelet-derived growth factor and hormones such as insulin and testosterone (Hawke & Garry, 2001). In order to further understand the skeletal muscle repair process, it is important to elucidate the mechanism of satellite cell activity and the circumstances in which the factors described above will act to regulate satellite cell activity. This study looked specifically at a potential mechanism of estrogen influence on post-exercise satellite cell activation and proliferation.

1.4 The Role of Estrogen

Estrogens are steroid sex hormones that include estradiol, estrone and estriol. Estradiol is produced by the ovaries and is the primary estrogen in the body (Wierman, 2007). In the past it was typically assumed that sex hormones target sex organs alone, however, recent studies have indicated that sex hormones, such as estrogen, have a number of target organs including skeletal muscle (Enns et al., 2008; Wang et al., 1999). Estrogen has two distinct receptors, estrogen receptor alpha ($ER\alpha$) and estrogen receptor beta ($ER\beta$). Both receptors are expressed in skeletal muscle, though they are encoded by separate genes (Hall et al., 2001; Wiik et al., 2003). In addition, $ER\alpha$ and $ER\beta$ have a similar binding affinity for endogenous and synthetic estrogens and can be activated when bound with a ligand (Hall et al., 2001; Kalbe et al., 2007).

Estrogen plays a role in skeletal muscle through its influence on muscle damage, inflammation and repair. Following various exercise protocols, sex differences in the degree of sustained muscle damage have been found. Komulainen et al. (1999), for

example, demonstrated an increase in cytoskeletal damage in male rats following an eccentric exercise protocol, compared to female rats. Estrogen has also been shown to attenuate damage caused by I/R injury and exercise-induced damage. In studies utilizing a rat forebrain ischemia model, administration of 17β -estradiol was shown to reduced I/R injury (Santizo et al., 2000; Wang et al., 1999).

The presence of estrogen has also been shown to influence inflammation by attenuating neutrophil and macrophage infiltration in skeletal muscle following injury. Stupka & Tiidus (2001) presented the first study examining the effects of estrogen on leukocyte infiltration following an I/R-induced injury. Estrogen was shown to exert a protective effect on skeletal muscle and attenuated the infiltration of neutrophils in female rats following I/R injury. The relationship between estrogen and neutrophil infiltration following exercise-induced damage was further examined by Tiidus et al. (2001) in ovariectomized (OVX) rats. Neutrophil infiltration in the estrogen-supplemented rats was significantly reduced post-exercise compared to the exercised placebo group. In addition, myeloperoxidase activity, a marker for neutrophil infiltration, was significantly reduced in the exercised estrogen group 1 hour post-exercise. These studies demonstrate the attenuation of neutrophil accumulation in damaged skeletal muscle in female, OVX rats with estrogen replacement. The same attenuation of neutrophil infiltration was demonstrated in estrogen supplemented male rats, suggesting that the presence of physiological levels of estrogen alone is sufficient to cause this reduction in neutrophil infiltration (Tiidus & Bombardier, 1999).

The relationship between estrogen and satellite cell activation in damaged skeletal muscle has also been examined. A study by Tiidus et al. (2005) demonstrated that

eccentrically exercised, estrogen-supplemented male rats had a significantly greater number of satellite cells present in the soleus and white vastus muscles at 72 hours post-exercise, compared to exercised male rats without estrogen-supplementation. The satellite cells in this study were identified by the marker Pax7 and it was therefore not known what stage of activation the identified satellite cells were in. Further investigation was conducted by Enns & Tiidus (2008), where OVX female rats underwent an eccentric exercise running protocol to induce skeletal muscle damage. The number of satellite cells present 72 hours post-exercise was then examined with the markers Pax7, MyoD and BrdU. This immunohistochemical analysis showed a significantly greater increase in total, activated and proliferating satellite cells post-exercise in the estrogen-supplemented exercised animals, compared to the exercised group without estrogen-supplementation. The results of this study suggest that estrogen may play a role in the activation of satellite cells following exercise-induced damage (Enns & Tiidus, 2008).

While the exact mechanism of estrogen on the skeletal muscle repair process is not fully understood, possible mechanisms have been proposed. It is thought that these mechanisms may involve an increased activation of signalling proteins that are necessary for protein synthesis. The Akt-mammalian target of rapamycin (Akt-mTOR) pathway, for example, regulates protein synthesis in the muscle and estrogen has been shown to stimulate this pathway in cardiac muscle (Sitnick et al., 2006). Stimulation of the Akt-mTOR pathway by estrogen causes mTOR to phosphorylate and activate a signalling protein, p70^{S6k}. This leads to the phosphorylation of S6, a ribosomal protein, causing increased translation of mRNA that encodes proteins necessary for protein synthesis in muscle. In support of this pathway as a mechanism for the influence of estrogen on

skeletal muscle growth, Sitnick et al. (2006), observed a decrease in the activation of Akt and p70^{sk6} in OVX rats during muscle reloading, inhibiting muscle growth.

Another pathway that may affect the skeletal muscle repair process is the phosphatidylinositol 3-kinase-Akt (PI3 kinase-Akt) pathway. Results from Patten et al. (2004) demonstrated an increased activation of PI3 kinase-Akt signalling by estrogen in cardiac muscle via interaction with the ER α . Interaction between ER α and p85, a regulatory subunit of PI3-kinase-Akt, causes an increase in the activation of this pathway which stimulates protein synthesis and muscle growth (Patten et al., 2004; Pedram et al., 2002; Sitnick et al., 2006). Since ER α 's are present in skeletal muscle, activation of the PI3-kinase-Akt pathway, via estrogen-ER α binding and the subsequent interaction with p85, may be a possible mechanism to explain how estrogen augments satellite cell activation and proliferation following muscle damage.

The participation of an estrogen receptor (ER) mediated pathway as a potential mechanism for satellite cell activation and proliferation in skeletal muscle is supported by Enns et al. (2008) where an ER antagonist, ICI 182,780, was used to completely block ERs. This blocking of ERs in estrogen-supplemented rats eliminated the estrogen-induced augmentation of satellite cells in damaged muscle fibres post-exercise. In addition, Lemoine et al. (2002) demonstrated an association between exercise and ER α s where a significant increase in ER α mRNA transcripts in skeletal muscle was found following endurance training. These studies support the involvement of ERs in skeletal muscle during exercise and muscle repair.

In order to corroborate the results from Enns et al. (2008), which suggest that estrogen acts through an ER-mediated mechanism to augment satellite cell activation and

proliferation, this study utilized the ER α agonist propyl pyrazole triol (PPT). PPT is an ER α agonist that has a 50% greater binding affinity for the ER α compared to estrogen itself (Kraichely et al., 2000; Stauffer et al., 2000). Through ER α binding, PPT has been shown to mimic the actions of estrogen by preventing body weight increases and bone mineral density losses in ovariectomized rats (Harris et al., 2002). In addition, PPT stimulates uterine growth with the same effectiveness as estrogen (Harris et al., 2002; Katzenellenbogen et al., 1995). Use of the ER α agonist, PPT, coupled with the results from Enns et al. (2008) will give further confirmation for the role that ERs play in the skeletal muscle repair process while also allowing the specific role that ER α plays in satellite cell activation and proliferation to be determined.

1.5 Research Applications

This research is potentially relevant to post-menopausal women. Since post-menopausal women have reduced levels of estrogen, these women may experience an increased susceptibility to muscle damage and delays in muscle regeneration and recovery. In fact, research has shown that post-menopausal women suffer higher rates of sarcopenia and tend to develop it at an earlier age compared to men (Sipila et al., 2001). Sarcopenia is a loss of muscle mass that typically affects those aged 65 and older and can severely affect one's muscle strength and functional ability (Thompson, 2007).

Impaired satellite cell functioning has been implicated as a possible contributor to sarcopenia (Zammit et al., 2006). Given the role that estrogen plays in satellite cell augmentation it is possible that this impairment in satellite cells, and consequent decrease in muscle mass, may arise due to the reduction of estrogen levels in post-menopausal

women. Studies have shown that while physical activity is commonly used to counteract sarcopenia, estrogen supplementation in addition to increased physical activity, decreases the development of sarcopenia to a greater extent (Dionne et al., 2000). Moreover, it has been demonstrated that estrogen alone can improve muscle recovery and repair from disuse atrophy in animal models (McClung et al., 2006). By examining the relationship between estrogen and the skeletal muscle repair process, an increased body of knowledge regarding possible preventative or treatment measures for skeletal muscle loss caused by a reduction of estrogen may become available. This may ultimately lead to increases in mobility, muscular strength and overall quality of life for post-menopausal women.

Chapter Two

The Influence of Propyl Pyrazole Triol on the Alpha Estrogen Receptor-Mediated Activation of Satellite Cells in Rat Skeletal Muscle

Statement of Problem

The purpose of this study was to investigate the role of the alpha estrogen receptor ($ER\alpha$) on the activation and proliferation of satellite cells in skeletal muscle following eccentric exercise through the administration of an $ER\alpha$ agonist (PPT) to ovariectomized female rats supplemented with or without estrogen.

Hypotheses

1. Based on previous studies it was hypothesized that supplementation with estrogen would augment satellite cell activation and proliferation post-exercise.
2. Administration of the $ER\alpha$ agonist, PPT, would mirror the effects of estrogen by augmenting satellite cell activation and proliferation post-exercise.
3. A combined administration of estrogen and the agonist would also increase satellite cell activation and proliferation post-exercise.
4. No significant differences in satellite cell activation and proliferation would exist between the estrogen supplemented, agonist supplemented and the combined estrogen and agonist supplemented animals post-exercise.

Introduction

Repeated, high-intensity eccentric muscle contractions have been shown to elicit exercise-induced muscle damage (Proske & Allen, 2005). This sets in motion a series of well documented events: an inflammatory response in skeletal muscle that is characterized by the infiltration of neutrophils and macrophages to the site of injury (Tidball et al., 1995); and the activation and proliferation of satellite cells. Satellite cells are located between the basal lamina and the sarcolemma of skeletal muscle and are involved in the muscle repair process (Mauro, 1961; Muir et al., 1965). Satellite cells remain in a mitotically quiescent state until activated by certain stimuli, including myotrauma caused by eccentric exercise. Upon activation, satellite cells proliferate and migrate to the site of injury and differentiate to repair existing myofibres and/or form new myofibres (Hawke & Garry, 2001; Schultz & McCormick, 1994).

Many studies have demonstrated sex differences on indices of muscle damage, inflammation and repair that are largely due to the female sex-hormone estrogen. Estrogen has been shown to decrease cytoskeletal damage caused by I/R injury and exercise-induced damage in rats (Komulainen et al., 1999; Santizo et al., 2000; Wang et al., 1999). The presence of estrogen has also been shown to attenuate neutrophil and macrophage infiltration in rat skeletal muscle following injury (Stupka & Tiidus, 2001; Tiidus & Bombardier, 1999; Tiidus et al., 2001).

Estrogens' influence on satellite cells was first examined by Tiidus et al. (2005) where estrogen supplemented male rats showed a significant increase in the total number of satellite cells present post-exercise compared to a sham group with no estrogen. Enns & Tiidus (2008) conducted a follow-up study to determine which satellite cell stage is

influenced by estrogen using the satellite cell markers Pax7 (total), MyoD (activated) and BrdU (proliferating). A significant increase in the presence of all markers was observed, demonstrating that estrogen plays a role in the activation and proliferation of satellite cells, as well as increasing the total number present in myofibers post-exercise.

While the exact mechanism that estrogen exerts its effect on the skeletal muscle repair process is not fully understood, possible mechanisms have been proposed. It is thought that these mechanisms involve an increased activation of signalling proteins that are necessary for protein synthesis. Estrogen, for example, has been shown to influence two such pathways: the phosphatidylinositol 3-kinase-Akt (PI3 kinase-Akt) pathway and the Akt-mammalian target of rapamycin (Akt-mTOR) pathway (Patten et al., 2004; Pedram et al., 2002; Sitnick et al., 2006). Results from Patten et al. (2004) demonstrated an increased activation of PI3 kinase-Akt signalling by estrogen in cardiac muscle via interaction with the ER α . Since ER α 's are present in skeletal muscle (Hall et al., 2001; Kalbe et al., 2006), activation of the PI3-kinase-Akt pathway, via estrogen-ER α binding, may be a possible mechanism to explain how estrogen augments satellite cell activation and proliferation following muscle damage.

In an attempt to elucidate the mechanism through which estrogen exerts its effect, Enns et al. (2008) utilized an estrogen antagonist, ICI 182,780, to study the estrogen receptor-mediated pathway in satellite cell activation and proliferation in skeletal muscle. This estrogen antagonist competitively binds to ERs with an extremely high affinity, preventing estrogen binding. OVX female rats were used with results showing a significant increase in total, activated and proliferating satellite cells post-exercise in rats supplemented with estrogen compared to those without estrogen. Use of the estrogen

antagonist, however, eliminated the observed estrogen-induced augmentation of total, activated and proliferating satellite cells post-exercise. This data, therefore, lends support to the theory that estrogen mediates satellite cell activation and proliferation in skeletal muscle through an estrogen receptor-mediated pathway.

The purpose of this study was to further examine the estrogen receptor-mediated pathway in satellite cell augmentation resulting from exercise and estrogen supplementation. In order to address this issue the ER α agonist, PPT, was used to bind to and stimulate the skeletal muscle ER α s, where the total number of satellite cells (Pax7-positive) and activated satellite cells (MyoD-positive) were examined in unexercised and exercised, OVX rats. Use of PPT allowed for the specific role of the ER α in the skeletal muscle repair process to be established. Based on results from Enns et al. (2008), it was hypothesized that estrogen would act in a receptor-mediated fashion to augment the activated and total number of satellite cells present in rat skeletal muscle, as demonstrated by a significant but similar post-exercise increase in satellite cells in the estrogen supplemented, agonist supplemented and the combined estrogen and agonist supplemented animals.

Methods

Animals

This experimental protocol was approved by the Animal Care Committee at Wilfrid Laurier University and operated in accordance to the Canada Council on Animal Care. A total of 64 female Sprague-Dawley rats were used (Charles River Laboratories, LaSalle, QC) and surgical removal of their ovaries occurred at 9 weeks of age. The animals arrived to our laboratory approximately one week post-surgery and were housed two animals per cage, in a temperature-controlled environment with a 12:12 hour light/dark cycle. Animals were allowed access to food (Teklad 22/5 Rodent Diet, Harlan-Teklad, Madison, WI) and water *ad libitum*.

Experimental Design

Upon arrival to our laboratory, animals were given one week to acclimatize to the environment. During the acclimatization period, the animals were randomly divided into 4 groups: Sham (S), Estrogen supplemented (E), Agonist supplemented (A), and Estrogen and Agonist supplemented (EA). The animals were further subdivided into Unexercised Control (Con) and Exercise (Ex) groups within the 4 groups (Figure 3). Animal weights were recorded from the day of arrival until tissue collection.

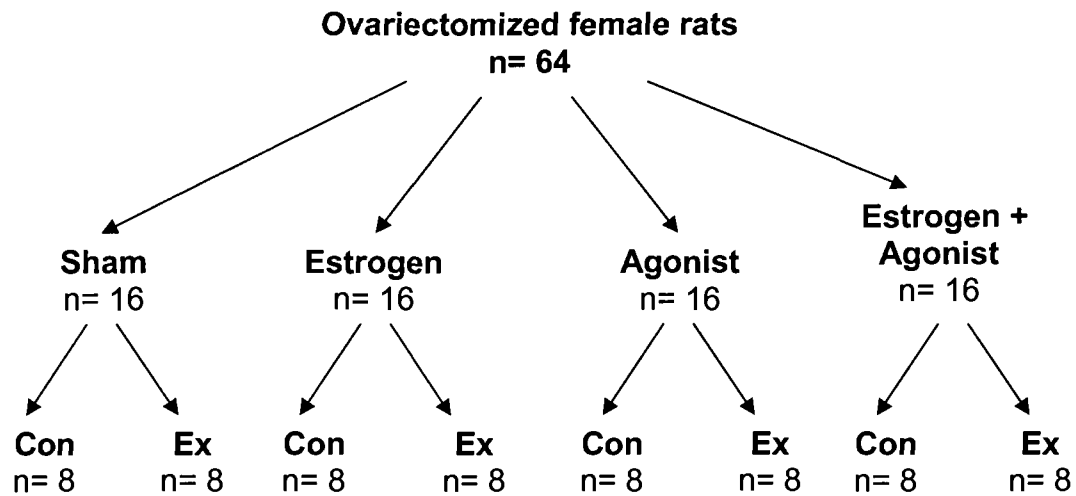


Figure 3: Schematic outline of the experimental groups. Con = control (unexercised) condition; Ex = exercised condition.

Following the acclimatization period, the animals underwent pellet implantation or a sham surgery. These procedures all occurred under aseptic conditions where the rats were anaesthetized with gaseous isoflurane. A small incision, approximately 1 cm in length, was made in the skin folds of the neck and blunt dissection was used to separate the skin from the underlying connective tissue. Implantation of an estrogen pellet (0.25 mg 17 β -estradiol, 21-day time release pellet, Innovative Research of America, Sarasota, FL) underneath the skin occurred in groups E and EA. Groups S and A underwent a sham surgery which is identical to the procedure described for groups E and EA but did not include implantation of an estrogen pellet. Following the pellet implantation or sham procedure the incision was closed with Vetabond (3 M St Paul, MN).

The ER α agonist PPT (Tocris, Ellisville, MO) was administered four days post-surgery and three days prior to the exercise protocol. Each rat in the A and EA group received 0.5mg of PPT in a dimethyl sulfoxide (DMSO) vehicle per day, via a 0.1mL subcutaneous injection for 6 days. The rats in group S and E received a 0.1mL subcutaneous injection of DMSO daily for 6 days where the last injection occurred one day prior to tissue collection. This protocol was developed to ensure an appropriate, prolonged exposure of PPT to the skeletal muscle ERs. It was based on a study by Harris et al. (2002) where the same PPT protocol activated uterine ERs as demonstrated through a significant increase in uterine weight.

The exercise protocol occurred seven days following the pellet implantation and sham surgery. This was to ensure optimal exposure of skeletal muscle ERs in groups E and EA to the estrogen hormone. All animals in the Ex groups participated in a non-fatiguing exercise protocol where the animals ran downhill on a motorized rodent

treadmill with an electric shock grid (Columbus Instruments, Columbus, OH). The animals ran at approximately 17 m/min at a -13.5° grade. An intermittent protocol was used where the animals ran for 5 minutes, followed by 2 minutes of rest, for a total of 90 minutes of running. This protocol elicits muscle damage to various muscles including the soleus and white vastus muscles (Komulainen et al., 1999). These muscles were selected due to their differing fibre types; the soleus is highly composed of type I fibres, while white vastus is predominantly composed of type IIb fibres. One and two days prior to the exercise protocol the Ex rats underwent a familiarization period on the treadmill for 5 minutes. This increased the comfort level of the rats on the treadmill and allowed the 90 minute running session to be completed with greater ease and accuracy on the run day.

Tissue Collection

Previous research has established that positively labelled Pax7 and MyoD satellite cells peak 3 days following exercise (Enns & Tiidus, 2008). The Ex animals were, therefore, sacrificed 72 hours following completion of the running protocol; Con animals were sacrificed at the same time as the Ex animals. Animals were anaesthetised with sodium pentobarbital (55 mg/kg i.p.). A toe web pinch was conducted to ensure loss of the withdrawal reflex prior to commencement of tissue collection. Blood samples were obtained from the femoral artery and were allowed to clot at room temperature. The samples were then centrifuged at 3000g for 10 min.; the serum was then removed and stored at -80°C until further analysis.

The soleus and the white vastus muscles were then surgically removed. During removal, the muscle samples were rinsed in physiological saline to remove excess blood,

blotted dry and visible connective tissue was removed. Tissue samples for immunohistochemical analysis were mounted, coated in optimal cutting temperature (OCT) medium and frozen in isopentane that was chilled to the temperature of liquid nitrogen. All tissue samples were then immediately placed into liquid nitrogen until transferred to a -80°C environment. Samples remained at this temperature until analysis. See Figure 4 for a schematic outline of the experimental protocol.

Serum Analysis

Estrogen levels were analyzed via blood serum analysis using the Coat-a-Count radioimmunoassay kit (Inter Medico, Markham, ON).

β-glucuronidase activity

Using the Barrett method, the activity of β-glucuronidase was assessed (Barrett, 1972). β-glucuronidase is a lysosomal hydrolase that is a marker of muscle damage, where increased activity indicates increased muscle damage (Salminen & Kihlstrom, 1985). Performed in triplicate, muscle samples were assayed at 420 nm and activities are expressed as the amount of substrate (5 mM *p*-nitrophenyl-β-D-glucuronide, Sigma-Aldrich) hydrolyzed per protein amount and incubation time. Protein concentration was measured using Lowry's method (Lowry et al., 1951).

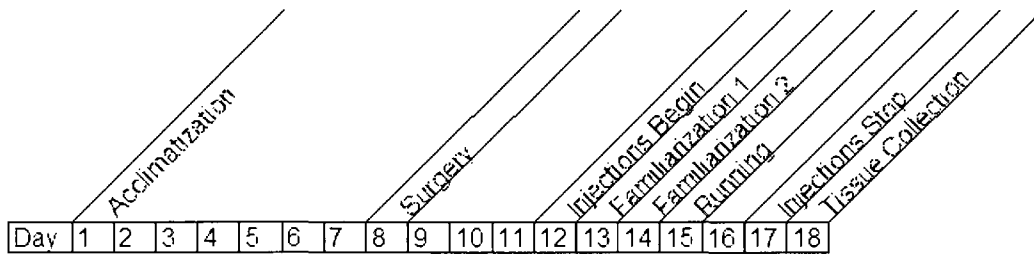


Figure 4: Schematic outline of the experimental protocol timeline.

Immunohistochemical Analysis

Using a cryostat (Leica CM3050S, Germany), muscle samples were transversely cut into 10 μm sections and placed onto Vactabond coated glass slides (Vector Laboratories, Burlington, ON). All slides were stored at -20°C until analysis. Immunohistochemical staining for markers of satellite cells, Pax7 ((Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and MyoD (Dako Canada, Mississauga, ON) were completed.

Quantification of satellite cells from the immunostains were conducted by placing the slides under a light microscope (Leica DMLS) that projects onto a computer screen at a magnification of 400x (10x ocular and 40x objective lens). For each muscle sample, 2 sections of 200 myofibers were counted for positive satellite cells, for a total of 400 counted myofibers, as previously described (Enns et al., 2008; Enns & Tiidus, 2008). These measurements are considered to give an accurate representation of the relative number of satellite cells present since myofibers staining positively for more than one satellite cell was rarely observed (unpublished observations). A positive satellite cell was identified as a dark-stained ovoid body, located between the sarcolemma and basal lamina. Figure 5 depicts representative positive cells for Pax7 and MyoD that were used during quantification.

Statistical Analysis

Data is reported as mean \pm SEM. Differences between groups were measured using a 2 (exercise) by 4 (treatment) factorial analysis of variance (ANOVA). All post-hoc tests were completed using Fisher's LSD.

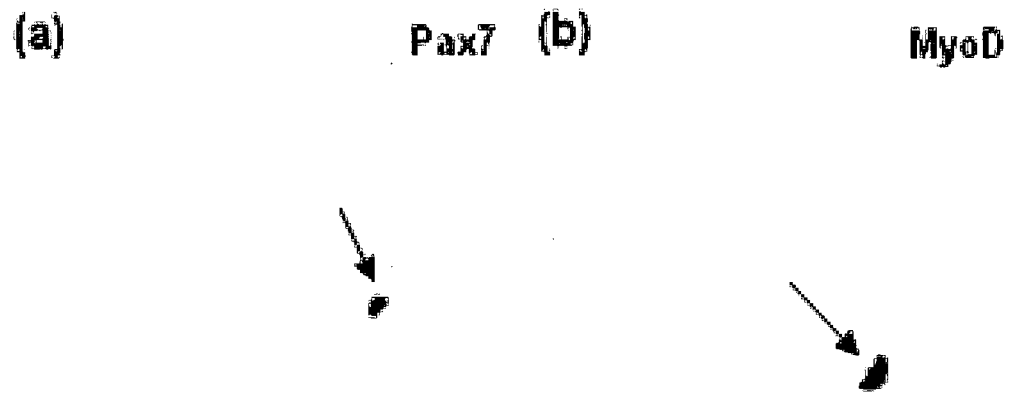


Figure 5: Representative rat skeletal muscle fibres immunostained for satellite cells; (A) Pax7 and (B) MyoD (400x magnification).

Results

Serum Estrogen Levels

Exercise treatment did not significantly influence serum estrogen levels compared to treatment-matched control animals. As a result, serum estrogen levels were pooled into four groups: Sham (S), Estrogen supplemented (E), Agonist supplemented (A) and Estrogen and Agonist supplemented (EA). As depicted in Table 1, the E and EA supplemented animals had significantly higher serum estrogen levels compared to the S and A groups ($P < 0.01$).

Body Weights

Since exercise did not influence animal body weight, animals were pooled according to treatment group. The S group had significantly greater body weights than all other treatment groups ($P < 0.01$). In addition, the EA group weighed significantly less than the E and A groups ($P < 0.05$). Estrogen and/or PPT supplementation therefore significantly reduced body weight compared to S treated rats (Table 2). These effects on body weights are typically seen in studies involving estrogen (Enns et al., 2008; Enns & Tiidus, 2008; Tiidus et al., 2005).

Uterine Weights

The activation of uterine ERs when bound with estrogen or an estrogen agonist has been shown to stimulate uterine growth (Harris et al., 2002; Katzenellenbogen et al., 1995). As such, wet uterine weights were used to verify the effective delivery of PPT and estrogen to the uterine ERs. As shown in Table 2, supplementation with estrogen and/or

PPT significantly increased uterine weight compared to the S group ($P < 0.01$). Additionally, uterine weight in the E group was significantly greater than the A group ($P < 0.05$); while the EA group had a significantly higher uterine weight compared to all treatment groups ($P < 0.05$). When the uterine weights were normalized to overall body weight of the rats, these same trends were observed (Table 2).

β -Glucuronidase Activity

Measurement of β -Glucuronidase activity is a marker of muscle damage where increased activity indicates increased muscle damage (Salminen & Kihlstrom, 1985). As expected, the Ex S group had a significant increase in β -Glucuronidase activity in the soleus muscle 72 hours post-exercise compared to the Con S group (Table 3). An attenuation of muscle damage was seen in the Ex A group and the Ex EA group demonstrated by a significantly lower β -Glucuronidase activity compared to the treatment-matched S group ($P < 0.01$). Similar, non-significant trends for β -Glucuronidase activity were seen in the white vastus muscle as shown in Table 3.

Satellite Cells

Quiescent, activated and proliferating satellite cells were measured using the marker Pax7 (Hawke & Garry, 2001). In the white vastus muscle, a main effect for exercise was observed where a significant increase in satellite cells was found in the Ex groups compared to treatment-matched Con groups ($P < 0.01$). In addition, the E, A and EA groups had a significantly greater number of satellite cells post-exercise compared with the treatment-matched S group ($P < 0.01$), as shown in Figure 6.

Figure 7 demonstrates the same effects in the soleus muscle where a significant increase in satellite cells was found in the Ex groups compared to the treatment-matched Con group ($P < 0.01$). A significant increase in satellite cells was also found in the E, A and EA groups post-exercise compared to the treatment-matched S group ($P < 0.05$).

MyoD was used to positively mark activated and proliferating satellite cells (Cornelison & Wold, 1997). As depicted in Figure 8, a main effect for exercise was observed in the white vastus muscle where a significant increase in satellite cells was found in the Ex groups compared to the treatment-matched Con groups ($P < 0.01$). The E, A and EA groups also had a significantly greater number of satellite cells present post-exercise compared with the treatment-matched S group ($P < 0.01$).

Figure 9 demonstrates the same effects in the soleus muscle where a significant increase in satellite cells was observed in the Ex groups compared with the treatment-matched Con groups ($P < 0.01$). The E, A and EA groups also had a significant increase in satellite cells post-exercise compared to the treatment-matched S group ($P < 0.01$). Furthermore, an additional significant increase in satellite cell numbers was found in the EA group post-exercise compared to the treatment-matched E and A groups ($P < 0.01$).

Table 1: Serum estradiol levels for sham, estrogen, PPT and E + PPT groups. Values are pooled Mean \pm SEM as no significant differences were observed between treatment-matched control and exercise values. * P < 0.01, compared with treatment-matched Sham group. ‡ P < 0.01, compared with treatment-matched PPT group.

	Sham	Estrogen	PPT	E + PPT
Serum Estradiol (pg ml ⁻¹)	11 \pm 0.9	91 \pm 15*‡	34 \pm 0.9	90 \pm 10*‡

Table 2: Animal body weights and uterine weights for sham, estrogen, PPT and E + PPT treatment groups. Values are pooled Mean \pm SEM as no significant differences were observed between treatment-matched control and exercise values. * P < 0.01, compared with treatment-matched Sham group. † P < 0.05, compared with treatment-matched Estrogen group. ‡ P < 0.01, compared with treatment-matched PPT group.

	Sham	Estrogen	PPT	E + PPT
Body weight, g	309 \pm 4	272 \pm 3*	278 \pm 2*	261 \pm 3*†‡
Uterine Weight, g	0.110 \pm 0.009	0.409 \pm 0.161*	0.332 \pm 0.018*†	0.460 \pm 0.022*†‡
Uterine weight, g 100g ⁻¹ body weight	0.037 \pm 0.004	0.152 \pm 0.010*	0.122 \pm 0.006*†	0.180 \pm 0.010*†‡

Table 3: Changes in soleus and white vastus muscle β -Glucuronidase activity with downhill running. Values are Mean \pm SEM. * P < 0.01, compared with treatment-matched Sham group. † P < 0.05, compared with treatment-matched Estrogen group. ‡ P < 0.05, compared with treatment-matched control group.

	Sham	Estrogen	PPT	E + PPT
Soleus				
Control	4.70 \pm 0.34	4.56 \pm 0.21	5.21 \pm 0.18	3.99 \pm 0.43
Exercise	7.18 \pm 0.92‡	6.04 \pm 1.00	5.55 \pm 0.36*	4.42 \pm 0.34*†
White Vastus				
Control	3.35 \pm 0.37	2.73 \pm 0.42	3.39 \pm 0.46	2.70 \pm 0.38
Exercise	3.45 \pm 0.33	4.16 \pm 0.46	3.14 \pm 0.33	3.42 \pm 0.38

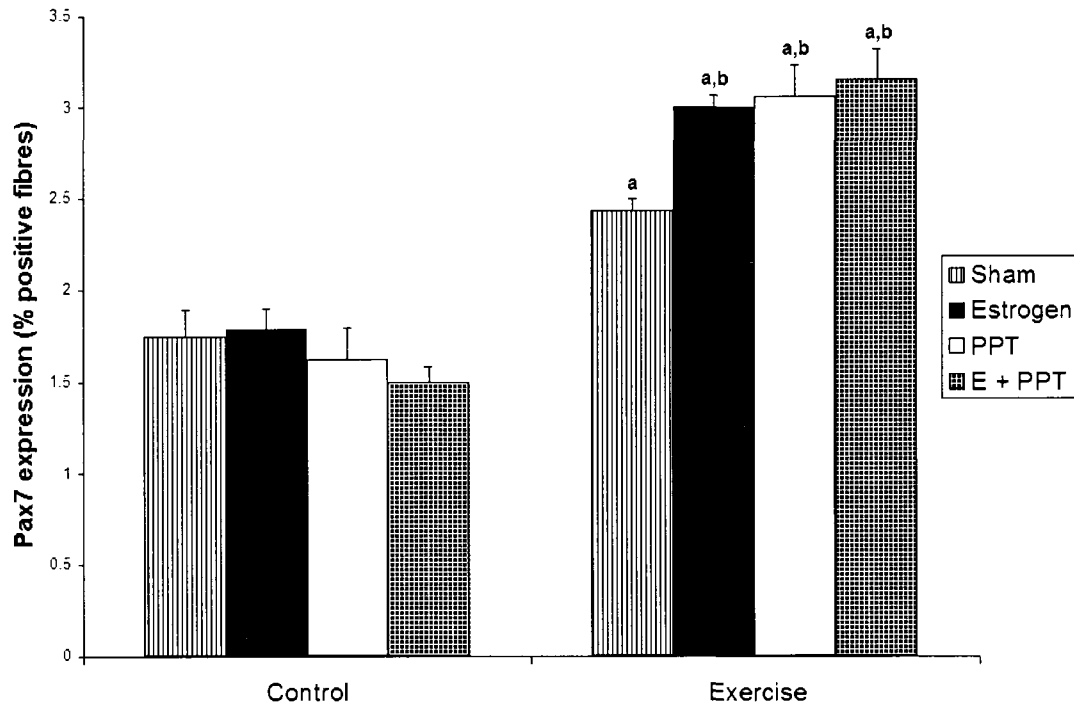


Figure 6: Effects of supplementation with estrogen, PPT and a combination of estrogen and PPT on the number of positive muscle fibres for the satellite cell marker, Pax7, in rat white vastus muscle 72 hours following downhill running. Values are Means \pm SEM. ^a P < 0.01 compared to Control (unexercised). ^b P < 0.01 compared to Sham treated.

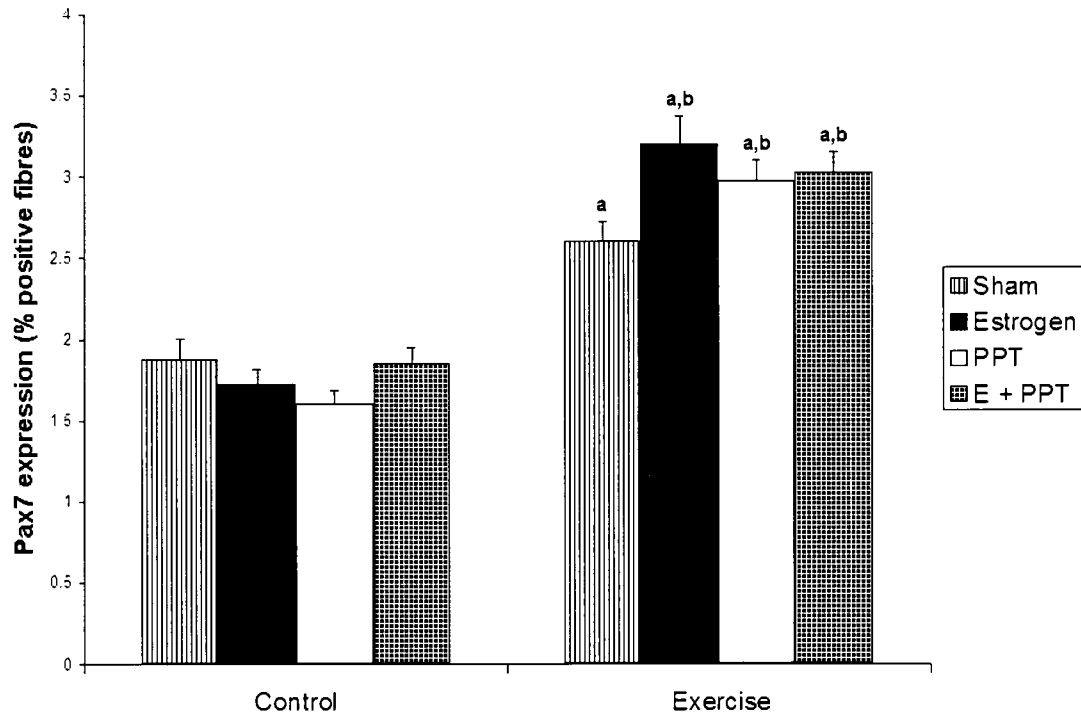


Figure 7: Effects of supplementation with estrogen, PPT and a combination of estrogen and PPT on the number of positive muscle fibres for the satellite cell marker, Pax7, in rat soleus muscle 72 hours following downhill running. Values are Means \pm SEM. ^a $P < 0.01$ compared to Control (unexercised). ^b $P < 0.05$ compared to Sham treated.

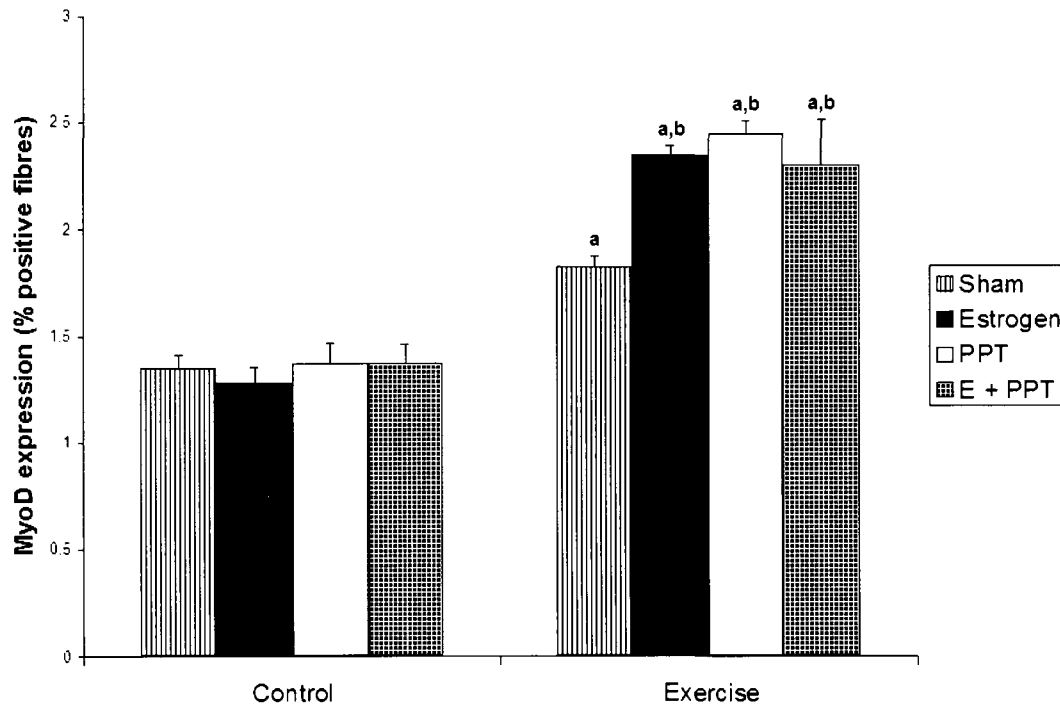


Figure 8: Effects of supplementation with estrogen, PPT and a combination of estrogen and PPT on the number of positive muscle fibres for the satellite cell marker, MyoD, in rat white vastus muscle 72 hours following downhill running. Values are Means \pm SEM. ^a $P < 0.01$ compared to Control (unexercised). ^b $P < 0.01$ compared to Sham treated.

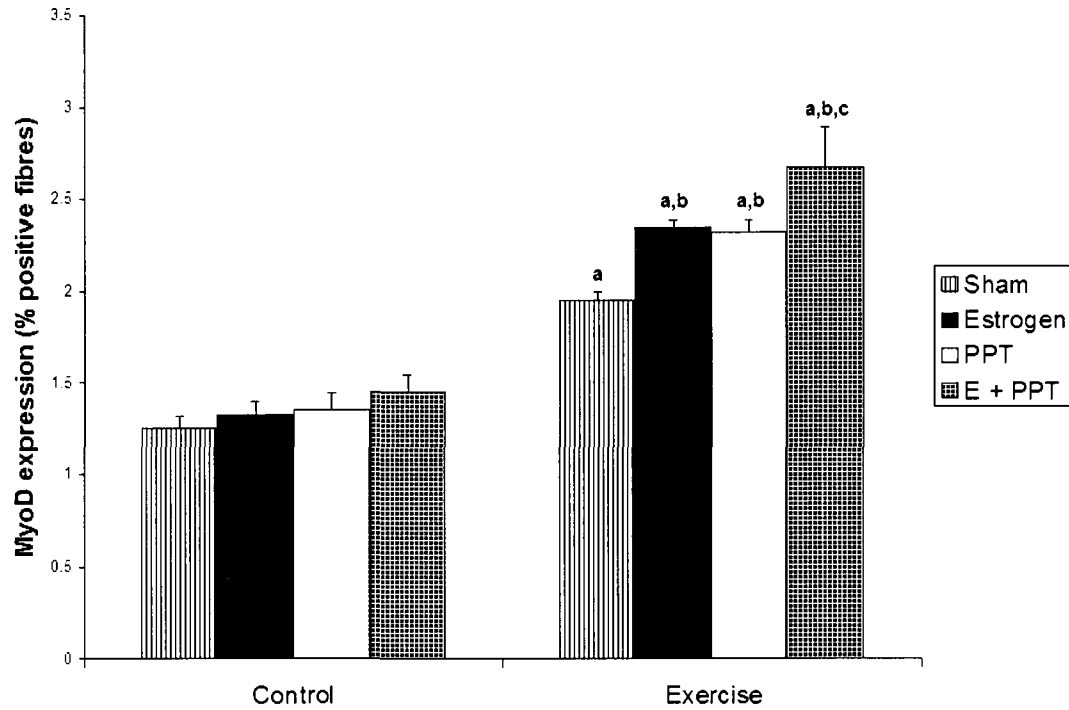


Figure 9: Effects of supplementation with estrogen, PPT and a combination of estrogen and PPT on the numbers of positive muscle fibres for the satellite cell marker, MyoD, in rat soleus muscle 72 hours following downhill running. Values are Means \pm SEM. ^a $P < 0.01$ compared to Control (unexercised). ^b $P < 0.01$ compared to Sham treated. ^c $P < 0.05$ compared to Estrogen treated and PPT treated.

Discussion

Previous research has shown that estrogen augments satellite cell proliferation in skeletal muscle post-exercise (Enns et al., 2008; Enns & Tiidus, 2008; Tiidus et al., 2005). While estrogens' mechanism of action in the skeletal muscle repair process is not fully understood, an ER-mediated pathway has been postulated. An ER α agonist, PPT, was used to examine the role of ER α in satellite cell activation.

The findings of this study show that estrogen-related augmentation of satellite cell activation and proliferation following exercise acts through an ER-mediated mechanism and that the ER α plays a major role in this regulation. Animals supplemented with estrogen, PPT and a combination of estrogen and PPT all demonstrated a significant increase in total (Pax7-positive) and activated (MyoD-positive) satellite cells post-exercise, compared to OVX sham animals. In addition, sole stimulation of ER α by PPT resulted in the same degree of satellite cell augmentation compared to animals supplemented with estrogen, indicating a key role for ER α in the skeletal muscle repair process.

These results corroborate findings by Enns et al. (2008) where use of an ER antagonist, ICI 182,780, eliminated the estrogen-induced increases in total, activated and proliferating satellite cells post-exercise. In this study, animals supplemented with a combination of estrogen and ICI 182,780 had significantly fewer satellite cells present post-exercise compared to estrogen supplemented animals as well as the sham animals. These results support estrogens' mechanism of action through estrogen receptors.

The significant increases in uterine weights observed in the PPT and estrogen plus PPT groups can verify that PPT, as used in this study, did bind ERs. Although this study

cannot fully elucidate the specific role of ER β in post-exercise augmentation of satellite cell numbers, the lack of significant difference between estrogen and PTT alone or in combination on post-exercise satellite cell numbers may suggest that the primary effects of estrogen are manifested through ER α . Different actions for ER α and ER β have been identified in other cellular processes including GLUT-4 expression; ER α acting as a positive regulator and ER β acting in an inhibitory fashion (Barros et al., 2006). The presence of the ER α has also been shown to play a role in reducing body weight in mice whereas ER β had no such effect (Brown et al., 2009). While both ER α and ER β are present in skeletal muscle and have a similar binding affinity for estrogen (Hall et al., 2001), it is possible that each receptor regulates different cellular events.

Animals supplemented with estrogen alone and in combination with PPT had an 8-fold increase in serum estrogen levels compared to animals without estrogen. Female, ovary-intact rats experience cycling estrogen levels over a three to four day estrous cycle. The physiological range of circulating estrogen in rats may be varied depending on the estrous cycle stage. Serum estrogen values as low as 10 pg ml⁻¹ in ovary intact rats have been reported (Persky et al., 2000), while values as high as 285 pg ml⁻¹ have also been found (McNulty et al., 2000). Animals in this study experienced a continued, unvaried exposure of estrogen within the normal physiological range (90-91 pg ml⁻¹).

It is noteworthy that animals supplemented with both estrogen and PPT manifested a further significant increase in activated satellite cells in the soleus muscle, relative to the smaller augmentation induced by estrogen supplemented and PPT supplemented groups alone. This finding was not seen in the white vastus muscle or in either muscle for the total number of satellite cells (Pax7-positive).

The reason for this increased augmentation of satellite cells is not known. The combination of estrogen and PPT supplementation provides a greater amount of available substrate to activate ERs. This, coupled with the observation that type I muscle fibres, including soleus, have a greater number of ER α s compared to type II muscle fibres (Lemoine et al., 2002b), could result in the activation of a greater number of ER α s and a subsequent further augmentation of satellite cells in the soleus muscle. However, as no fibre type difference was observed in Pax7-positive satellite cells this remains speculative. One can speculate that the combined increase in circulating estrogen and PPT levels may stimulate ERs outside of skeletal muscle that influence satellite cell activation specifically. However, it is not known if ERs are present on satellite cells themselves.

The downstream signalling pathways involved in satellite cell augmentation following ER α activation are not entirely understood. Various pathways that are involved in muscle growth and repair may play a role. The Akt-mTOR pathway, for example, regulates protein synthesis in muscle (Sitnick et al., 2006). Estrogen has been shown to stimulate this pathway in cardiac muscle, however, it is unclear as to whether or not estrogen stimulates this pathway through an ER-mediated process (Patten et al., 2004). Activation of ER α by estrogen has been shown to stimulate the PI3 kinase-Akt pathway, leading to an increase in protein synthesis and muscle growth (Patten et al., 2004; Pedram et al., 2002; Sitnick et al., 2006). Estrogen is also involved in the activation of two immediate early genes *c-fos* and *egr-1* in proliferating myoblasts through an ER-mediated mechanism (Kahlert et al., 1997). Stimulation of these pathways by estrogen appears to regulate muscle growth and repair to some extent; further investigation for the role of

these pathways in satellite cell activation and proliferation is needed to further elucidate these and other possibilities.

Various growth factors may also be involved in the estrogen-stimulated augmentation of satellite cell activation and proliferation. Numerous studies have reported increases in IGF-I mRNA expression in the uteri of rats following estrogen supplementation (Huynh & Pollak, 1993; Kamanga et al., 2008a). Furthermore, this estrogen-induced augmentation of IGF-I may be an ER-mediated process as use of the ER antagonist, ICI 182 780, eliminated the estrogen-induced increase in IGF-I gene expression (Huynh & Pollak, 1993; Kassem et al., 1998; Stygar et al., 2003). Since IGF-I is a known regulator of satellite cell proliferation and differentiation (Chakravarthy et al., 2000; Hawke & Garry, 2001; Johnson et al., 1990; Mills et al., 2007), it is possible that estrogen, through receptor-mediated mechanisms, may act to increase IGF-I expression in skeletal muscle leading to the augmentation of satellite cell proliferation and differentiation.

HGF is another known regulator of satellite cell activation and proliferation which may also be influenced by estrogen (Allen et al., 1995; Tatsumi et al., 1998). Estrogen is reported to increase nitric oxide synthase (NOS) and nitric oxide (NO) levels in animal (Node et al., 1997) and human models (Caulin-Glaser et al., 1997). Following skeletal muscle damage, NO has been shown to regulate the release of HGF (Tatsumi, 2002); moreover, inhibition of NOS, and subsequent decrease in NO levels, leads to a reduction in activated satellite cells (Anderson et al., 2000). Therefore, the presence of estrogen may act to indirectly augment satellite cell activation through NO-mediated stimulation of HGF. Further research into the role of estrogen and skeletal muscle ER involvement

with NO regulation of satellite cell activation and proliferation also needs to be conducted to follow up on these possibilities.

The results of this study may have potential implications for post-menopausal women. Post-menopausal women suffer higher rates of sarcopenia and tend to develop it at an earlier age compared to men (Sipila et al., 2001). Given that estrogen seems to stimulate satellite cell activity, decreased estrogen levels in post-menopausal women may be a contributing factor to the development of sarcopenia and also potentially to diminished rates of muscle repair capacity in older females.

In conclusion, estrogen mediates satellite cell activation and proliferation in skeletal muscle through an ER-mediated mechanism, with ER α playing a key role. Future research focusing on the downstream signalling pathways involved in estrogen-induced satellite cell activation will aid in the understanding of the factors and pathways involved in the skeletal muscle repair process. In addition, the differing roles of ER α and ER β in skeletal muscle damage, inflammation and repair are worthy of study as their differing roles may present pharmacological implications for estrogen and ER-specific agonists and antagonists.

Chapter Three

Discussion & Conclusions

Estrogen has been shown to play a key role in skeletal muscle by influencing skeletal muscle damage, inflammation and repair. Estrogen seems to have a protective role in skeletal muscle as it attenuates post-exercise calpain activity, decreases membrane damage and reduces the degree of damage sustained by the muscle (Enns et al., 2008; Enns & Tiidus, 2008; Tiidus et al., 2001). Estrogen also attenuates the inflammatory response by decreasing neutrophil and macrophage infiltration into the muscle post-exercise (Enns et al., 2008; Tiidus et al., 2001). Furthermore, estrogen plays a role in skeletal muscle repair, though its mechanisms of action are not yet fully understood.

The objective of this study was to examine the role of ER α in the estrogen-induced augmentation of satellite cells in skeletal muscle. While previous studies have shown an estrogen-induced augmentation of satellite cells in rat skeletal muscle post-exercise compared to OVX animals, estrogens' mechanism of action for this process was not fully understood (Enns et al., 2008; Enns & Tiidus, 2008; Tiidus et al., 2005). Given that both ER α and ER β are present in skeletal muscle (Wiik et al., 2003) and are upregulated with exercise (Lemoine et al., 2002; Wiik et al., 2005) it had been postulated that estrogen may influence satellite cells through an ER-mediated mechanism. This was examined through the use of PPT.

PPT is an ER α agonist that binds to the ER α with a 50% greater binding affinity than estrogen (Kraichely et al., 2000; Stauffer et al., 2000). PPT has been shown to effectively mimic estrogen actions in numerous studies as its administration decreases food intake, meal size and body weight (Roesch, 2005; Santallo et al., 2007), while

increasing uterine weight in OVX rats (Harris et al., 2002; Katzenellenbogen et al., 1995). In this study, the collected uterine weights confirmed that the administered doses of estrogen and PPT were sufficient to stimulate ERs. Use of PPT allowed for the specific action of the ER α in the skeletal muscle repair process to be elucidated.

The main finding of this study was that estrogen acts through an ER-mediated mechanism to augment satellite cell activation in skeletal muscle, with ER α playing a primary role. PPT stimulated a significant post-exercise increase in total (Pax7-positive) and activated (MyoD-positive) satellite cells to the same extent as estrogen. Furthermore, supplementation with PPT and a combination of estrogen and PPT may play a protective role in skeletal muscle by decreasing the degree of muscle damage; this is depicted by a decrease in β -Glucuronidase activity compared to sham treated animals post-exercise in the soleus muscle. Similar findings have been reported by Enns et al. (2008) and Enns & Tiidus (2008).

Future research focusing on the downstream signalling pathways that follow ER α activation by estrogen is needed. Activation of ER α by estrogen seems to be the initial step in a cascade of events that leads to satellite cell augmentation; though the pathways and factors involved following ER α activation remain elusive. A pathway that may potentially play a role is the PI3 kinase-Akt pathway. Activation of ER α by estrogen stimulates p85, a regulatory subunit of the PI3 kinase-Akt pathway, leading to its activation (Patten et al., 2004). Since the PI3 kinase-Akt pathway stimulates protein synthesis and muscle growth, one can speculate that its ER α -mediated activation by estrogen may also elicit a cascade of events that stimulate satellite cell activation and proliferation (Patten et al., 2004; Pedram et al., 2002; Sitnick et al., 2006).

Estrogen also increases IGF-I expression through an ER α -mediated mechanism. IGF-I is a known regulator of satellite cell proliferation and differentiation and plays a key role in skeletal muscle repair and hypertrophy (Chakravarthy et al., 2000; Hawke & Garry, 2001; Johnson et al., 1990; Mills et al., 2007). Interestingly, IGF-I, like estrogen, has also been shown to activate the PI3 kinase-Akt pathway in myoblasts (Latres et al., 2005; Rommel et al., 2001). Therefore estrogen may augment satellite cell activation and proliferation through the direct ER α -mediated stimulation of IGF-I and the PI3 kinase-pathway; in addition, IGF-I may further contribute to the skeletal muscle repair process by activating the PI3 kinase-Akt pathway as well. Overall, activation of the ER α by estrogen may, therefore, elicit various event cascades that act to augment satellite cell activation and proliferation.

IGF-I may also indirectly augment satellite cells through its influence on ERs. Estrogen does not appear to significantly influence ER transcriptional activity in non-reproductive organs as peak ER transcriptional activity reportedly occurs in the diestrus stage of the estrous cycle (Ciana et al., 2003). IGF-I, on the other hand, has been shown to increase ER transcriptional activity in mice in the absence of estrogen (Cenni & Picard, 1999). Given that ER α and ER β are expressed in human skeletal muscle in individuals with varying levels of estrogen including children, male and female adults and post-menopausal women (Wiik et al., 2009), one may speculate that ER expression in skeletal muscle may be regulated, in part, by IGF-I. IGF-I, therefore, may not only play a role in the skeletal muscle repair process by stimulating satellite cell activation and proliferation and the PI3 kinase-Akt pathway, it may also influence ER transcriptional

activity. The role IGF-I plays in the skeletal muscle repair process specifically in relation to estrogen therefore warrants further investigation.

There is some controversy over whether or not estrogen does, in fact, enhance post-exercise muscle recovery and repair. While animal models consistently show that estrogen enhances post-exercise muscle recovery and repair, human studies report equivocal evidence. Some human studies, for example, have reported that no gender differences exist in the degree of structural damage and muscle force and strength deficits incurred by the muscle fibre following eccentric exercise (Sayers & Clarkson, 2001; Stupka et al., 2001). In addition, gender differences have not been found in some studies looking at muscle strength recovery post-exercise (Sayers & Clarkson, 2001; Thompson et al., 1997). Nevertheless, there are human studies that do show decreased levels of creatine kinase in women compared to men post-exercise, indicating a decreased degree of muscle damage in women (Sewright et al., 2008; Stupka et al., 2000). These studies, coupled with animal studies that demonstrate an estrogen-induced decrease in post-exercise muscle damage, inflammation and an augmentation of muscle repair (Enns et al., 2008; Enns & Tiidus, 2008; Tiidus et al., 2001; Tiidus et al., 2005), support the role of estrogen in mediating skeletal muscle damage and repair.

The influence of estrogen on skeletal muscle damage, inflammation and repair may, therefore, have potential implications for post-menopausal women. Sarcopenia typically affects individuals aged 65 and older and involves a loss of muscle mass that can severely reduce muscular strength and functional ability (Thompson, 2007). Since an increased incidence of sarcopenia in post-menopausal women compared to men of the same age has been reported, it has been postulated that the development of sarcopenia

may be a consequence of decreased circulating estrogen levels in post-menopausal women (Sipila et al., 2001).

Given that estrogen plays a role in the skeletal muscle repair process through the augmentation of satellite cell activation and proliferation, impairment in satellite cell function may contribute to sarcopenia. In fact, an age-related decline in satellite cell activation (Conboy et al., 2003) and proliferation (Mouly et al., 2005) has been reported. In addition, Shefer et al. (2006) has reported a significant decline in the total number of satellite cells present in human myofibers with age. One may therefore speculate that the impairment of muscle repair in post-menopausal women may be due the decreased levels of circulating estrogen, causing impairment in satellite cell activation and proliferation.

In order to counteract sarcopenia, research has shown that women who participate in strength training regimes can increase muscle cross-sectional area, force and strength (Brown et al., 1997, Sipila et al., 1996). Hormone replacement therapy (HRT) has also been reported to increase muscular strength in the back extensor and flexor muscles (Heikkinen et al., 1997) and in lower limb muscle groups in post-menopausal women (Greeves et al. 1999; Sipila et al., 2001). Moreover, Dionne et al. (2000) found a further reduction in sarcopenia in women participating in both strength training and HRT compared to those participating in exercise regimes alone. HRT, therefore, seems to play a key role in increasing skeletal muscle strength and mass in post-menopausal women.

Given this positive benefit of HRT in skeletal muscle, along with its other associated benefits which include the prevention of osteoporosis, HRT also has negative effects. Use of HRT has been link to an increased risk of developing venous thromboembolic events, gallbladder disease, and breast cancer (Mosca et al., 2001). As

result, its use is not recommended for women who may already have an increased susceptibility for these diseases.

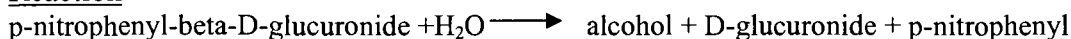
The development of pharmacological estrogen mimicking therapies that target specific pathways and ERs could have dramatic implications for post-menopausal women. Use of an ER α agonist, for example, could target skeletal muscle (amongst other organs) to augment satellite cell activation and proliferation to increase muscle repair in post-menopausal women. This may be refined to possibly avoid negative side effects from HRT that are caused by non-receptor mediated estrogen pathways or in pathways involving activation of the ER β . Significant further research needs to be conducted in this area, however.

Overall the results of this study, coupled with findings from Enns et al. (2008), demonstrate that estrogen augments satellite cell activation and proliferation through an ER-mediated mechanism. Furthermore, use of an ER α agonist revealed a key role for the ER α in the skeletal muscle repair process. Various downstream signalling pathways and factors may be involved in the estrogen-induced augmentation of satellite cell activation and proliferation. Further investigation into these ER α -mediated signalling cascades are worthy of study as they may have pharmacological and intervention implications for post-menopausal women.

Appendix A

β-Glucuronidase Enzyme Assay

Reaction



Reagent Procedure

1. Sodium acetate (anhydrous) ($\text{C}_2\text{H}_3\text{O}_2\text{Na}$) (0.1N) (mw 82.03)
0.82 g in 100 ml of H_2O pH 4.2
2. Glycine Buffer (0.1M) pH 10.8
 - A) 0.375g glycine ($\text{C}_2\text{H}_5\text{NO}_2$, mw 75.07) + 0.2922g NaCl (mw 58.44) in 50 ml H_2O
 - B) 0.4 g of NaOH (mw 40) in 100 ml of H_2OMix 52.2 ml of A and 47.8 ml of B and pH to 10.8
3. Substrate: p-nitrophenyl-beta-D-glucuronide (5mM) (sigma N-1627) (mw 315.2)
7.88 mg in 5 ml H_2O

Procedure

1. Homogenize tissue (10-20 mg; optimal 15 mg) in glass pestle using a 33.33:1 (3%) dilution using distilled water.
-use 16.66667 times the mass of tissue, do this 2 times
2. Add 50 μl of homogenate and water to appropriately labeled test tube
3. Add 450 μl of Acetate buffer to tubes using repeater pipette
4. Preincubate tubes in 37°C bath for 5 minutes
5. Add 250 μl of substrate using repeater pipette
6. Incubate for 16-18 hours
7. Add 1.5 ml of cold glycine buffer using repeater pipette
8. Cool in ice water for 10 minutes
9. Centrifuge at 3500 rpm for 10 minutes (temp 4°C)
10. Read on spectrophotometer at 420 nm.

Standard Preparation

Standard: p-nitrophenol (mw 139.1)

Standard, mM	To make:
0.125	Take 563 μl of 10 mM p-NP stock and add 437 μl dH_2O
0.0625	Take 500 μl of 0.125 mM solution and add 500 μl dH_2O
0.03125	Take 500 μl of 0.0625 mM solution and add 500 μl dH_2O
0.015625	Take 500 μl of 0.03125 mM solution and add 500 μl dH_2O
0 (distilled water)	

Appendix B

Lowry Protein Assay

PROCEDURE:

1. Use 12*75 mm culture tubes and do samples, standards, and blanks in triplicate.
2. Add 50 μ l of standards to appropriately labelled tubes.
3. Add 50 μ l of water to tubes labelled REAGENT BLANK.
4. Prepare a 20:1 dilution of homogenizing media (10 μ l media + 190 μ l water). Add 50 μ l of dilute media to tubes labelled SAMPLE BLANK.
5. Prepare a 20:1 dilution of EACH tissue sample (10 μ l sample + 190 μ l water). Add 50 μ l of dilute sample to tubes labelled SAMPLE.
6. Add 0.5 ml of alkaline copper reagent to all tubes.
7. Mix well and let stand for 10 minutes at 25°C (room temperature).
8. Add 2.0 ml of phenol reagent to each tube. Mix each tube individually IMMEDIATELY after adding phenol reagent.
9. Incubate for 5 minutes at 55°C.
10. Cool in tap water for 1 minute.
11. Read on spectrophotometer at 650 nm.

REAGENTS:

1. ALKALINE COPPER REAGENT:

0.05 g CuSO₄.5H₂O
10.00 g Na₂CO₃
0.10 g POTASSIUM SODIUM TARTRATE
2.00 g NaOH

TO 100 ml WATER ADD THE CuSO₄ AND DISSOLVE COMPLETELY. ADD THE REMAINING REAGENTS IN ORDER. STORE AT 20°C FOR 2 WEEKS.

2. FOLIN-CIOCALTEU PHENOL REAGENT: (Sigma-Aldrich, St. Louis, MO)

TO 80 ml WATER ADD 5.0 ml of 2 N PHENOL REAGENT. MAKE FRESH DAILY.

3. BOVINE SERUM ALBUMIN STANDARD, 1.0 mg/ml: (BSA, Sigma-Aldrich, St. Louis, MO)

ADD 10 mg of BSA TO 10 ml WATER. MIX BY INVERSION. SERIAL DILUTE TO OBTAIN SOLUTIONS AS FOLLOWS:

1.0 mg/ml
0.5 mg/ml
0.25 mg/ml
0.125 mg/ml
0.0 mg/ml

CALCULATION:

(A₆₅₀ of sample – A₆₅₀ of sample blank)

(A₆₅₀ of standard – A₆₅₀ of reagent blank)

Multiply by

Concentration of Standard (mg/ml) * 20 (dilution factor) = Protein concentration, mg/ml

Appendix C

Pax7 Immunohistochemistry

Use frozen sections of rat muscle cut 7-10 microns thick.

1. Let slides air dry for 5 min or until warmed up to room temperature.
2. Fix sections with 100% cold acetone for 10 min.
3. Let slides air dry for 5-10 min.
4. Permeablize cells in 0.5% Triton X-100 solution (in PBS) for 5 min.
5. Wash in PBS for 3 x 5 min.
6. Apply 0.6% H₂O₂ (in absolute methanol) for 10 min.
7. Wash in PBS for 3 x 5 min.
8. Block with 5% normal goat serum in PBS (containing 5% nonfat milk powder) for 30 min. Tap off excess.
9. Incubate in primary antibody: Pax 7 (1/20 diluted in blocking solution) for 1 h.
10. Wash in PBS for 3 x 5 min.
11. Apply bottle 3: GAM-Biotin (from Dako LSAB-2 kit) for 10 min.
12. Wash in PBS for 3 x 5 min.
13. Apply Bottle 4: Streptavidin-HRP (from Dako LSAB-2 kit) for 10 min.
14. Wash in PBS for 3 x 5 min.
15. Apply Vector NovaRed stain for 5 min (or until desired intensity is achieved).
16. Rinse in dH₂O liberally from wash bottle, then wash in bath for 2 x 5 min.
17. Counterstain using Vector Hematoxylin QS (add hematoxylin for 10-15 s and rinse off with running 37°C tap water for 30 s).
18. Place a drop of permanent mounting medium on cover slip and place slide upside down onto cover slip so that mounting medium covers section completely.
19. Invert slide and apply gentle pressure on cover slip to remove any air bubbles.

Appendix D

MyoD Immunohistochemistry

Use frozen sections of rat muscle cut 7-10 μm thick.

1. Let slides air dry for 5 min or until warmed up to room temperature.
2. Fix sections with 100% cold acetone for 10 min.
3. Let slides air dry for 5-10 min.
4. Permeabilize cells in 0.5% Triton X-100 solution (in PBS) for 5 min.
5. Wash in PBS for 3 x 5 min.
6. Apply 0.6% H_2O_2 (in absolute methanol) for 10 min.
7. Wash in PBS for 3 x 5 min.
8. Block with 5% normal goat serum in PBS (containing 5% nonfat milk powder) for 30 min. Tap off excess.
9. Apply primary antibody: MyoD (DakoCytomation, 1/50 diluted in blocking solution) overnight at 4°C (in humidity chamber).
10. Wash in PBS for 3 x 5 min.
11. Apply bottle 3: GAM-Biotin (from Dako LSAB-2 kit) for 10 min.
12. Wash in PBS for 3 x 5 min.
13. Apply Bottle 4: Streptavidin-HRP (from DakoLSAB-2 kit) for 10 min.
14. Wash in PBS for 3 x 5 min.
15. Apply DAB stain (Vector Laboratories) for 5 min (or until desired intensity is achieved).
16. Rinse in dH_2O liberally from wash bottle.
17. Counterstain using Vector Hematoxylin QS (add hematoxylin for 15 s and rinse off with running tap water for 30 s).
18. Place a drop of permanent mounting medium on cover slip and place slide upside down onto cover slip so that mounting medium covers section completely.
19. Invert slide and apply gentle pressure on cover slip to remove any air bubbles.

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