EFFECTS OF E2 AND ECCENTRIC EXERCISE ON SKELETAL MUSCLE

ANALYSES OF THE EFFECTS OF 17β-ESTRADIOL ON SKELETAL MUSCLE AND GLOBAL GENE EXPRESSION FOLLOWING ACUTE ECCENTRIC EXERCISE

By

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TITLE: Analyses of the effects of 17β-estradiol on skeletal muscle and global gene expression following acute eccentric exercise.

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ABSTRACT

Introduction: 17β-estradiol (E2) has proposed anti-oxidant and membrane stabilizing properties that may attenuate exercise-induced damage, inflammation and alter gene expression. The purpose of this thesis was to determine if acute E2 supplementation would affect the oxidative stress, membrane damage, inflammation and global mRNA expression induced by eccentric exercise. **Methods:** 18 healthy young males were randomly assigned to 8 days of placebo (CON) or E2 (EXP) supplementation. Blood and muscle samples were collected at baseline (BL), following supplementation (PS), +3 hours (3H) and +48 hours (48H) after 150 single-leg eccentric contractions. Blood samples were analyzed for hormone concentration, creatine kinase (CK) activity and total antioxidant capacity (TAC). Inflammation was quantified by neutrophil and macrophage infiltration. Genes selected *a priori* for oxidative stress defense, membrane homeostasis and growth were analyzed with real-time RT-PCR. High density oligonucleotide based microarrays were screened for novel differences in mRNA expression. **Results:** A primary finding was that increased serum E2 did not affect anti-oxidant capacity, creatine kinase efflux or mRNA content of genes related to oxidative stress defence and membrane homeostasis. E2 did attenuate neutrophil infiltration into muscle but did not affect macrophage density. Microarray analysis revealed that exercise induced differential expression of 611 genes at 3H and confirmed that E2 did not affect mRNA content. Genes were manually clustered into biological categories and from this dataset the signaling pathways for RhoA

and NFAT were identified as transcriptionally active. Both pathways regulate hypertrophic signaling through the AP-1 transcription factor complex.

Conclusions and significance: A major contribution of this thesis is that E2 may affect exercise induced inflammation through mechanisms that that do not affect oxidative stress or membrane stability. Additionally, the transcriptional activation of STARS/RhoA/AP1 and NFAT/AP1 indicates that both are important for early repair and remodelling signaling after a single bout of unaccustomed eccentric exercise.

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To Christine: for all the love, laughter and strength to make it all worthwhile

ABBREVIATIONS

ACTA2	-	actin, alpha 2, smooth muscle, aorta
ACTN1	-	actinin, alpha 1
ADP	-	adenosine diphosphate
ADRB2	-	adrenergic, beta-2-, receptor, surface
ANOVA	-	analysis of variance
AP-1	-	activator protein 1
AST	-	aspartate aminotransferase
ATF3	-	activating transcription factor 3
ATP	-	adenosine triphosphate
AU	-	arbitrary units
β2M	-	β2-microglobulin
BL	-	baseline
BHQ-1	-	black hole quencher - 1
CA-II	-	carbonic anhydrase isoenzyme II
CaMK	-	Ca^{2+}-calmodulin dependant kinase
CAPZA1	-	capping protein muscle Z-line, alpha 1
cDNA	-	complementary DNA
CK	-	creatine kinase
Cn	-	calcineurin
CON	-	control group
CORO1C	-	coronin, actin binding protein, 1C
C_T	-	critical threshold
DEXA	-	dual energy X-ray absorptiometry
DIAPH1	-	diaphanous homolog 1 (Drosophila)
DMPK	-	dystrophia myotonica-protein kinase
DNA	-	deoxyribonucleic acid
DOMS	-	delayed onset muscle soreness
DUSP	-	dual specificity phosphatase
E1	-	estrone
E2	-	17β-estradiol
E3	-	estriol
EASIA	-	enzyme amplified-sensitivity immunosorbent assays
EC	-	excitation – contraction
eNOS	-	endothelial nitric oxide synthase
EM	-	electron microscopy
ER	-	estrogen receptor
ERE	-	estrogen response element
ERK1/2	-	extracellular regulated kinase 1/2
EXP	-	experimental group
FAM	-	6-carboxyfluorescein
FDR	-	false discovery rate
FLNB	-	filamin B, beta

FOXO1	-	forkhead box O1
GAP	-	GTPase-activating protein
GDP	-	guanosine diphosphate
GEF	-	guanidine exchange factor
GEO	-	Gene Expression Omnibus
GO	-	gene ontology
GRP78	-	glucose related protein 78
GSK-3β	-	glycogen synthase kinase 3 beta
GSTA2	-	glutathione s-transferase alpha 2
GTP	-	guanosine triphosphate
HB-EGF	-	heparin-binding EGF-like growth factor
HMOX1	-	hemeoxygenase 1
HRP	-	horseradish peroxidase
HSP	-	heat shock protein
IGF-I	-	insulin-like growth factor 1
IL	-	interleukin
iNOS	-	inducible nitric oxide synthase
IPA	-	Ingenuity Pathway Analysis
JNK	-	c-Jun N-terminal kinase
LDH	-	lactate dehydrogenase
MAP3K	-	mitogen-activated protein kinase kinase kinase
MAPK	-	mitogen-activated protein kinase
MAPKAPK2	-	mitogen-activated protein kinase-activated protein kinase 2
MHC	-	myosin heavy chain
MPO	-	myeloperoxidase
mRNA	-	messenger ribonucleic acid
MRF	-	myogenic regulatory factor
mTOR	-	mammalian target of rapamycin
NFAT	-	nuclear factor of activated T cells
NFκB	-	nuclear factor κ B
NO	-	nitric oxide
Nrf-2	-	nuclear factor E2-related factor 2
NQO1	-	NAD(P)H dehydrogenase – quinone 1
OD	-	optical density
OVX	-	ovariectomized
PCr	-	phosphocreatine
PKA	-	protein kinase A
PKC	-	protein kinase C
PLA_2	-	phospholipases A2
PP2CA	-	protein phosphatase 2, catalytic subunit, alpha isoform
PS	-	post supplementation
RCAN1	-	regulator of calcineurin signaling 1
RhoA	-	ras homologue gene family, member A
RND3	-	Rho family GTPase 3

ROS	-	reactive oxygen species
RT-PCR	-	reverse transcription polymerase chain reaction
SD	-	standard deviation
SEM	-	standard error of the mean
SERCA	-	sarcoplasmic reticulum Ca^{2+} ATPase
SHBG	-	sex-hormone-binding globulin
SOD	-	superoxide dismutase
SR	-	sarcoplasmic reticulum
SREBP 2	-	sterol regulatory element binding protein 2
SRF	-	serum response factor
STARS	-	striated muscle activator of Rho signaling
TAC	-	total antioxidant capacity
TGF-β	-	transforming growth factor-β
TNFα	-	tumour necrosis factor-α
VO_2	-	volume of oxygen
XIRP1	-	Xin actin-binding repeat containing 1

STATEMENT OF CONTRIBUTION

Manuscript 1: The principal investigator of this study was MAT. All data was collected and analyzed at McMaster University in the laboratory of MAT, aside from serum creatine kinase activity which was measured by the core laboratory at Hamilton Health Sciences Centre. Study conception and design were done by LGM and MAT, and the ethic proposal was written by LGM. The participants were recruited by LGM and EAP and the study was coordinated by LGM. The data was collected by LGM, EAP, ACA SKB and MAT. RNA extraction was performed by LGM and HAR. Real-time RT-PCR procedures were performed by LGM. Immunochemistry was performed by XM. TAC was performed by IS. Data interpretation was performed by LGM. The manuscript was written and prepared by LGM.

Manuscript 2: The principal investigator of this study was MAT. All data was collected and analyzed at McMaster University in the laboratory of MAT, with the exception of the microarray analysis done at the Buck Institute for Age research. Study conception and design were done by LGM and MAT, and the ethic proposal was written by LGM. The participants were recruited by LGM and EAP and the study was coordinated by LGM. The data was collected by LGM, EAP, ACA SKB and MAT. RNA extraction was performed by LGM and HAR. Real-time RT-PCR procedures were performed by LGM. Western blotting was

performed by LGM and GT. Bioinformatics analysis was performed by SM and AEH. Data interpretation was performed by LGM. The manuscript was written and prepared by LGM.

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CHAPTER 1: GENERAL LITERATURE REVIEW

1.0.1 Introduction

Accounting for ~55% of the total body mass, skeletal muscle is a highly adaptable tissue with the ability to respond to changes in physical activity [1]. Its structure and function can be modified in response to the stimuli of altered activity, load or nutrition to allow for the most economical design for the conditions at that time [2]. During periods of childhood growth and increased physical load the dominant adaptive response is an increase in muscle mass (hypertrophy), while negative stimuli such as aging, disease and inactivity result in a loss of muscle mass (atrophy) [3,4]. The rate and extent of change induced by these challenges are regulated by genetic controls, controls likely responsible for the inherent differences in musculature and athletic performance found between individuals [4].

Depending on the duration and intensity of the activity and the predominant source of energy used, exercise can be broadly categorized as either endurance or resistance. The phenotypic changes within muscle that result from either type are specific to the exercise performed. Endurance exercise is continuous physical activity over long periods of time performed at submaximal intensity. Energy is primarily supplied from mitochondrial respiration; therefore the systems that mobilize, transport and oxidize glucose and lipids are the ones stressed to meet the metabolic demand. To accommodate this muscle cells enhance their ability to aerobically generate ATP by increasing the density of

mitochondria, the activity of oxidative enzymes and the transport capacity of the mitochondria, collectively known as mitochondrial biogenesis [5,6]. Resistance exercise is performed over shorter periods of time at near maximal intensity, generating large amounts of force and relying on sources of energy such as stored phosphocreatine (PCr) and anaerobic metabolism of glucose. The high forces and metabolic cost associated with resistance exercise stress the anaerobic energy systems and contractile apparatus of the muscle cell, thereby stimulating hypertrophy [7,8,9].

1.0.2 Skeletal muscle function

Skeletal muscle is composed of long cylindrical multinucleated cells called myofibres. Within each myofibre, the contractile proteins actin and myosin are organized into functional units called sarcomeres. Within the cell actin exists in two forms, as the single monomer G-actin (globular) or polymerized in the two-stranded helical structure F-actin (filamentous) that is bound to the Z-line. Actin treadmilling, the process of cycling between these two forms, is tightly controlled by actin-binding proteins that influence capping at the Z-line (capping protein muscle Z-line, alpha 1 (CAPZA1)), cross-linking between filaments (α-actinin) and severing (actin-depolymerizing factor) [10]. Myosin proteins are larger thick filaments bound to the midline (M-line) of the sarcomere with light and heavy chains that are responsible for the contractile force generated by muscle. According to the sliding filament theory of contraction, thick myosin

filaments actively ratchet along thin actin filaments, shortening the length of the sarcomere and generating tension within the myofibre. Upon relaxation, myosin ceases to bind to actin and the two filaments passively slide past one another to their resting state.

The sequence of events that begins with an action potential stimulus from the motor neuron and ends with mechanical force generated by the sarcomere is excitation-contraction (EC) coupling. Once at the myocyte, electrical signals travel along the outer membrane, entering the cell through transverse (T-) tubules where charge movement is "sensed" by a voltage-sensitive dihydropyridine receptor. Direct coupling between dihydropyridine and ryanodine calcium (Ca^{2+}) receptors on the sarcoplasmic reticulum (SR) releases Ca^{2+} ions into the cytosol where they bind to the regulatory subunit of troponin (troponin C). This initiates the translocation of troponin-tropomyosin complex away from actin to uncover binding sites for myosin and subsequently allow a power stroke to generate force. Contraction ends when Ca^{2+} is sequestered by the energy dependant sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) and concentrations are returned to resting levels [11].

Muscle contractions are classified as either: i) concentric if the force generated is greater than the load and the muscle shortens, ii) isometric if force generated equals the load and there is no change in length or iii) eccentric if the

load is greater than the force generated and the muscle lengthens under tension. Based on the contractile properties of muscle, the amount of tension that can be generated increases as velocity shifts from positive to negative. As a result, maximal eccentric contractions can generate up to 30% more force than concentric contractions [12]. Common examples of eccentric contractions are single joint exercises that forcibly lengthen a muscle group such as the elbow flexors or knee extensors and whole body exercises that incorporate braking or the slowing down of joint movement such as downhill running or stepping.

1.1.1 Eccentric exercise

Repeated high-intensity eccentric contractions induce a greater amount of muscle damage than other forms of exercise [13,14,15]. Although the exact reason is unknown, a generally accepted mechanism to explain this phenomenon was proposed by Proske and Morgan in 2001 [16]. During any contraction, sarcomeres of varying resting lengths are recruited to resist stretching. Though some sarcomeres are at or near the optimal length to generate force, others are longer and less able to resist the large load associated with an eccentric contraction. Those sarcomeres that lie on the descending limb of the length-tension curve continue to lengthen, further reducing the amount of force that they can

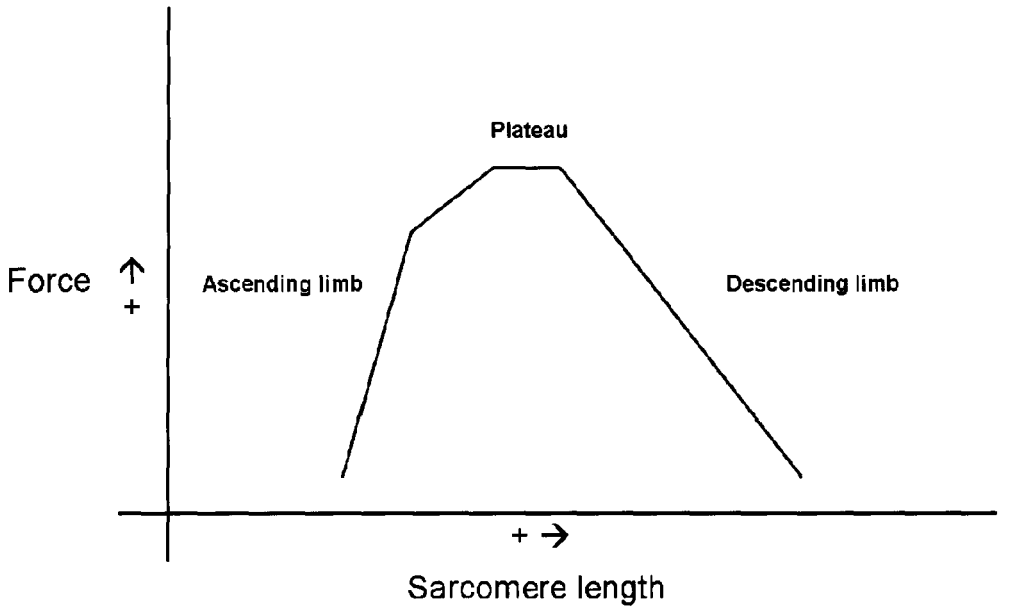

Figure 1. Schematic of the length-tension relationship of a single sarcomere.

generate until no overlap exists between the actin and myosin filaments (Fig. 1). After relaxation the disrupted actin and myosin filaments may not reinterdigitate, remaining disrupted and non-functional. With repeated eccentric contractions the number of sarcomeres that become overstretched increases which may damage sarcolemmal, SR and T-tubule membranes, leading to the uncontrolled release of Ca^{2+} and signaling for inflammation and apoptosis.

1.1.2 Characteristics of eccentric exercise induced muscle damage

Although repeated contractions of any type induce short-term fatigue, eccentric contractions are uniquely characterized by a change in length-tension relationship and delayed onset muscle soreness [15]. Changes in the length-

tension relationship are evident as a reduction in the magnitude of maximum force and an increase in the optimal muscle length for force generation as disrupted sarcomeres become dysfunctional and increase the total series compliance of the muscle [17,18]. Repeated eccentric contractions in feline medial gastrocnemius muscle reduce the peak active tension to 31.4 ± 2.8% of baseline values while increasing optimum muscle length by 7.1 ± 0.3 mm [17]. This is similar to the reductions in peak active tension of 50-55% that occur in the 48 hours after exercise observed in humans, reductions that only recover to 80% after two weeks [17,19,20,21]. In additional to the effect on maximal force, the optimum joint angle increases from 90 to 160 degrees of elbow flexion [17,19,20,21]. The magnitude of these changes is thought to be dependant on two factors: 1) the length at which the contractions are performed and 2) the amount of damage incurred [18]. Contractions performed at longer muscle lengths or those that result in greater damage to the myofibre have the greatest increase in optimum length and the largest reductions in peak isometric tension [18].

Z-line streaming is the misalignment of the normally linear Z-line as sarcomeres are stressed and ruptured. Muscle biopsies collected after unaccustomed exercise and viewed with electron microscopy (EM) show fibre disturbances that can be counted to quantify the damage present. Interestingly, the percentage of fibres exhibiting this form of structural damage following an eccentric stepping protocol increases from 32% immediately after exercise to 55%

approximately 30 hours later [22]. The phenomenon of increased damage after exercise was confirmed by a second study in which samples collected 3 days after eccentric cycling showed disruption of Z-discs in 52% of fibres, more than the 32% observed immediately after completion [23]. Thought to be the weak component of the myofibre, the initial ultrastructural damage likely occurs as the Z-line ruptures under high load [23]. However, the increased damage measured in the 24-48 hours after exercise may be due to the inflammatory processes and Ca^{2+} imbalance that stimulate the proteolytic activity of tumour necrosis factor-α (TNF-α) and calpain-3 [13,24,25].

Eccentric contractions are also characterized by a muscle tenderness or pain that peaks in the 24-48 hours after exercise; called delayed onset muscle soreness (DOMS) [16]. Although the exact cause of DOMS is unknown it has been suggested that exercise-induced inflammation may be a potential mechanism [26]. Signaled by muscle damage and degradation, inflammatory cells are recruited to the site of injury where their accumulation may induce muscle soreness as they release substances known to sensitize nerve endings III and IV for pain (nociceptors) [27,28]. The influx of protein-rich fluid (oedema) into muscle tissue as part of the inflammatory response raises osmotic pressure and temperature, which can further activate group IV nociceptors [29]. In addition, soreness may also result from movement as the increased intramuscular pressure of contraction may also activate these sensitized nerve endings [26].

Rupture of the cell membrane releases intramuscular enzymes and proteins into the lymphatic system, eventually making their way into the circulatory system where they provide an indirect marker of muscle damage. The enzymes and proteins typically measured in blood following unaccustomed exercise include lactate dehydrogenase (LDH), aspartate aminotransferase (AST), carbonic anhydrase isoenzyme II (CA-II), creatine kinase (CK), myoglobin, troponin, and myosin heavy chain (MHC) [30]. Of these CK is enzyme most commonly used as a marker of muscle damage because of the large magnitude of change typically observed and its relatively low cost of measurement [13]. However it should be noted that serum levels are affected by factors that restrict its use for the precise quantification of muscle damage [30]. First, the blood content of any intramuscular enzyme or protein is a balance between its rate of appearance and clearance. This balance can be altered by inflammation, myonecreosis, and variations in physical activity before and after damage [13]. Second, the large inter-subject variability typically observed with this marker can potentially introduce a Type II error. Differences in peak CK response as high as 100-fold have been reported following 24 maximal eccentric contractions of the elbow flexors [31]. The reason for this high variability is unknown.

1.1.3 Stages of damage and repair following eccentric exercise

The damage and subsequent repair to muscle tissue induced by repeated eccentric contractions can be separated into stages that are defined by their timing relative to the exercise bout and the primary mechanisms involved. Initially, the mechanical stress experienced by the myocyte during high intensity exercise damages the cellular ultrastructure, the SR and sarcolemma [32]. Mentioned previously, Z-line streaming is typically observed immediately after eccentric exercise [22,23]. Localized to regions of sarcomere damage, disruption of the SR membrane and SERCA activity is also evident and results in Ca^{2+} dysregulation [33,34]. This is illustrated by a 29% reduction in uptake and 39% reduction in controlled release of Ca^{2+} by SERCA in rats after 90 minutes of downhill running [34]. Leakage across the sarcolemma through stretch-activated channels is a second potential source of cytosolic Ca^{2+}. Following repeated bouts of eccentric contractions, increases in cytosolic Ca^{2+} can be inhibited by as much as 89% with the use of the stretch-activated channel blockers streptomycin and gadolinium [35]. The mechanical shearing and displacement of the sarcomeres also ruptures T-tubules, further dysregulating Na^{+} and Ca^{2+} intracellular concentrations [36]. Together these results indicate that eccentric exercise can alter normal ion concentrations within the cytosol.

The metabolic stress of exercise may also be a source of immediate damage to the myocyte. During high-intensity exercise, decreased intracellular

concentrations of PCr and a lower ATP/ADP ratio negatively influence the amount of ATP available for use by SERCA, impairing Ca^{2+} removal from the cytosol [37,38]. SERCA activity can be further inhibited by the elevated oxidative stress [39], lower pH [40] and increased temperature [40] associated with exercise. Greater O_2 consumption by the mitochondria during oxidative metabolism increases the production of ROS [41,42], which can oxidize and negatively influence sulfhydryl groups on SERCA that are important for activation [39]. H^+ ions produced by the oxidation of pyruvate also affect SERCA as they have been found to release Ca^{2+} from binding sites important for uptake [40,43]. In addition Ca^{2+} uptake by SERCA decreases with increasing temperature, possibly a result of a conformational change that inhibits activity [40]. It should be noted however that eccentric contractions do not consume as much ATP as concentric contractions to generate equivalent amounts of force, suggesting that mechanical stress may be more influential than metabolic stress in promoting the muscle damage associated with eccentric exercise [32].

In the hours following the end of exercise, lipolytic and proteolytic enzymes within the myocyte begin the autogenic breakdown of damaged structures. Elevated cytosolic Ca^{2+} concentrations from the initial stage of damage are an important signal for the activation of non-lysosomal cysteine proteases (calpains). Within skeletal muscle there are three calpains: calpain 1, calpain 2 and calpain 3 [44]. Almost exclusively expressed in skeletal muscle, calpain 3

(p94) is activated at the lowest cytosolic concentration of the three, 0.5 μM [44]. Calpain 1 (μ-calpain) and calpain 2 (m-calpain), named for the micromolar and millimolar concentrations of Ca^{2+} they were initially reported to require for activation, are ubiquitously expressed [11,45]. Because of the wide range of concentrations necessary for activation, it is thought that μ-calpain and calpain 3 are important for regulation of function and m-calpain is active in fibre degeneration, possibly because of the supraphysiological concentrations necessary for its activation [25,44]. Localized to the cell membrane and titin [25,44,46], calpains initiate degradation by cleaving large myofibrillar proteins at specific proteolytic sites such as the troponin complex, α-actinin and the Z-line proteins fodrin and desmin [25,28,45,47]. In addition, m-calpain also lyses proteins important for Ca^{2+} release from the SR, disrupting E-C coupling [48]. Following a single 30-min bout of eccentric knee extensions, neither μ-calpain nor calpain-3 were activated within the 3 h immediately after exercise [49]. However, by 24 h approximately 35% of the total calpain-3 present was activated without changes in μ-calpain activity [49]. *In vitro* work in skinned rat muscle fibres has identified that autolysis of calpain-3 is dependant on increased concentrations of Ca^{2+} and not mechanical stretch [50]. These actions suggest that the increased Z-line streaming and prolonged reduction in peak active tension typical in the 48 hours after eccentric exercise are at least partially a result of Ca^{2+} dysregulation and subsequent calpain 3 activity [44,51].

Cytoplasmic phospholipases A2 (PLA_2) are another powerful family of autogenic enzymes that contribute to cell breakdown. Following stretch-induced muscle damage, subfamilies of PLA_2 are activated in calcium-dependant and independent manners to target mitochondrial and membrane phospholipids, liberating free fatty acids and negatively affecting cellular membrane integrity [44,52,53]. The efflux of the muscle enzymes (LDH and CK) from the additional membrane damage can be completely attenuated with the use of protease inhibitors that block PLA_2 activity, without reducing myofilament damage [44,54,55]. In addition to their proteolytic properties, PLA_2s also contribute to the hypertrophic signaling activated by mechanical stretch [56]. C57 mouse skeletal myotubes cultured and exposed to mechanical stretch displayed increased signaling through the extracellular regulated kinase 1/2 (ERK1/2) pathway following exposure to a metabolite of PLA_2 in a dose dependant manner [56].

The third stage of exercise-induced muscular damage and repair is acute inflammation. Prevalent over the first 2-4 days, eccentric exercise generates a local inflammatory response in muscle characterized by the movement of plasma (oedema), plasma proteins and inflammatory cells into the site of injury [26,57,58]. The primary functions of this response are the clearance of necrotic tissue and cellular debris and the subsequent promotion of repair and regeneration [13,58,59]. Dominated by the infiltration of neutrophils and macrophages, recent findings indicate that these inflammatory cells may induce secondary tissue

damage during phagocytosis and chemotactic signalling to ensure that all damaged material is removed while providing an additional stimulus for recovery [12,28].

Neutrophils are quickly released in the blood stream after increased muscle loading, becoming the first responders to damaged tissue [57,58,59]. Neutrophil density in muscle tissue increases as early as 2-4 h after eccentric exercise and continues to remain elevated for several days [60,61,62,63,64,65]. Attracted to the site of damage by the chemoattractive agents interleukin-8 (IL-8), IL-1β and TNFα [59,66], neutrophils release cytolytic and cytotoxic molecules to aid in proteolysis, debris removal and additional chemotactic signalling [13,28,66]. Their use of a superoxide-dependant mechanism for tissue breakdown may negatively affect local healthy tissue and contribute to the total amount of muscle damage [28]. Although not a strong oxidant *in vivo*, superoxide dismutation to hydrogen peroxide in the presence of myeloperoxidase (MPO) (expressed by infiltrated neutrophils) can produce the lytic, non-radial oxidant hypochlorous acid [28]. Inhibition of the catalytic subunit required for the generation of superoxide attenuates muscle membrane lysis without affecting exercise stimulated muscle growth, indicating that neutrophils while important for phagocytosis do not contribute to muscle repair [28,67].

Signalled by damaged tissue and neutrophils, monocytes invade muscle where they mature and differentiate into macrophages as neutrophil numbers start to decline [58,59,66]. Density increases of 2 – 4-fold have been reported within 24 hours after eccentric exercise and can remain elevated for as long as 14 days, depending on the intensity of muscle damage [65,68,69,70,71,72]. The macrophages that appear at the site of injury can be divided into subtypes according to specific markers unique to their time-dependant roles in the inflammatory response [57,73,74]. In first hours after exercise, ED1+ macrophages accumulate in the muscle to remove damaged myofibrillar material together with neutrophil action [66,74]. These phagocytotic macrophages use a nitric oxide (NO)-dependant, superoxide-independent mechanism to lyse target cells and promote muscle damage [28]. Their presence is highest in areas with the greatest tissue damage and declines as the cellular debris is removed [75,76]. ED2+ macrophages that appear later do not invade necrotic myofibres, functioning instead as a stimulus for regeneration and repair [66,74]. These non-phagocytotic macrophages express cytokines important for muscle repair that include heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-β (TGF-β) and TNF- α [28,77,78]. Although the influence of the macrophage-derived factors has not been confirmed, attenuated muscle regeneration following the depletion of macrophages indicates that they are required for rebuilding and remodelling damaged tissue [79].

1.1.4 Adaptation to exercise

The adaptability of skeletal muscle can be attributed to the metabolic and morphological changes that occur after a change in intensity or type of exercise. By altering the genetic expression pattern (transcriptome), the muscle is able to shift towards a phenotype better suited to cope with subsequent bouts of exercise [6,7]. Specific to the volume, intensity and mode of exercise these adaptations are initiated by a variety of primary stimuli, regulated by the activation or inhibition of signaling pathways and culminate with the transcription of target mRNA [80]. The complete processes of adaptation to exercise are complex and new mechanisms continue to be discovered; therefore, only selected adaptations that pertain to this thesis will be considered (Fig. 2).

Primary stimuli

Calcium

As previously mentioned, muscle contraction is initiated by the release of Ca^{2+} ions from the SR and ended when Ca^{2+} is sequestered by SERCA [11]. Cytosolic concentrations of ~50 nM at rest rise more than 100-fold to 2-20 μM during contraction,

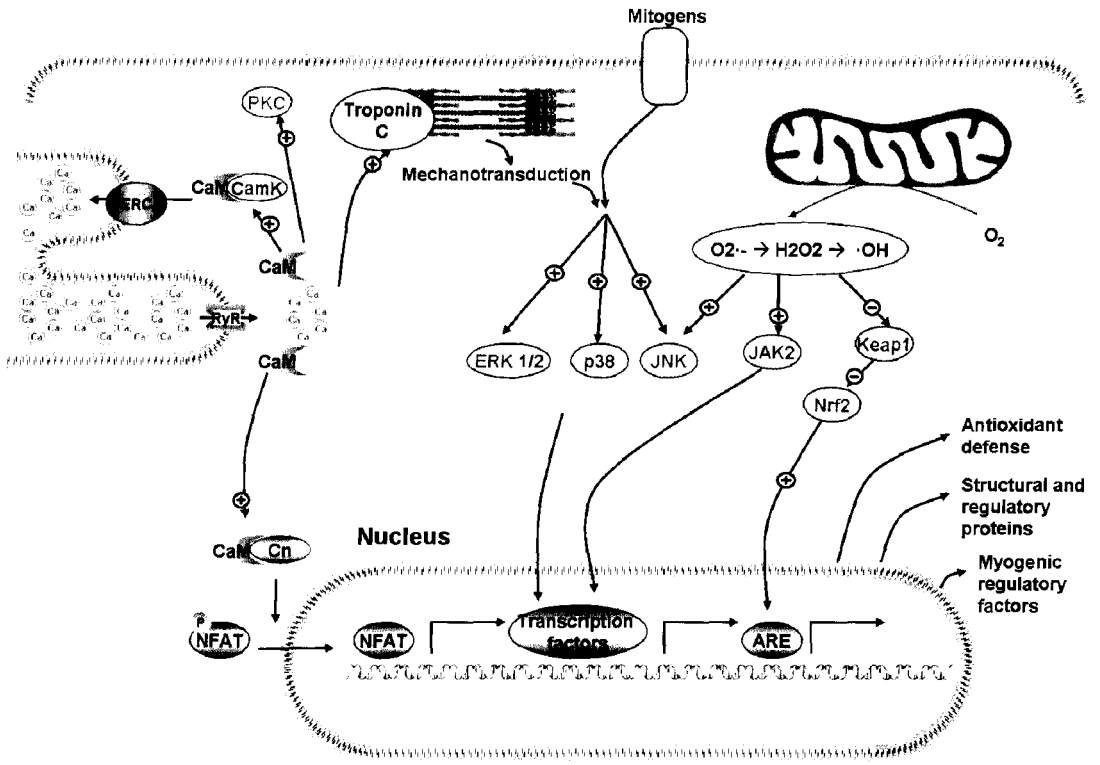

Figure 2. Adaptation pathways stimulated by resistance exercise.
SERCA – sarcoplasmic reticulum Ca^{2+} ATPase, CaM – calmodulin, CaMK – Ca^{2+}-calmodulin dependant kinases, PKC – protein kinase C, Cn – calcineurin, NFAT – nuclear factor of activated T-cells, ARE – antioxidant response element, Nrf2 - nuclear factor E2-related factor 2, RyR – ryanodine receptor

with fast twitch fibres experiencing a twofold greater change than slow twitch fibres [25,81]. In addition, the amplitude and duration of Ca^{2+} concentration change experienced by an active muscle parallels the differences in intensity and duration between resistance and endurance exercise [82]. These differences in Ca^{2+} concentration may provide a link between muscle contraction and the expression of genes to best adapt to the functional demands [82,83]. Primary pathways involved in myogenesis dependant on Ca^{2+} include the Ca^{2+}-calmodulin

dependant kinases (CaMKs), protein kinase C (PKC) and calcineurin-NFAT [4,11,82].

Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules produced as a normal byproduct of metabolism. Because of an unpaired valence shell electron, superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) readily oxidize proteins, lipids and DNA negatively affecting their function [84,85]. During exercise the amount of oxygen utilized by the mitochondrion can increase by as much as 100-fold to meet the higher demand for ATP [86]. Increased oxidative phosphorylation increases the amount of electron leakage from the electron transport chain generating $O_2^{\cdot-}$, which is converted to H_2O_2 by superoxide dismutase (SOD) and $^{\cdot}OH$ following a metal-mediated Fenton reaction [86]. ROS production may be further increased by xanthine oxidase enzymes that catalyze hypoxanthine and xanthine during purine catabolism, also generating $O_2^{\cdot-}$ [87]. In addition to metabolism, ROS are also released by neutrophils as part of the inflammatory response for tissue breakdown [88].

As a primary stimulus, the effects of ROS are dependant on their concentration within the cell. At low concentrations ROS induce adaptive transcriptional responses to defend against oxidative stress [89,90,91,92]. As primary messengers they activate the signaling pathways of JNK-AP1 (activator

protein 1) and Jak2-IRF1, inducing transcription of genes that modulate inducible nitric oxide synthase (iNOS), protect cellular function and regulate cell differentiation and antioxidant defense [93,94]. Another mechanism for the defense against oxidative stress of ROS is the nuclear factor E2-related factor 2 (Nrf2)-antioxidant response. Altered cellular redox status reduces the ubiquitylation of Nrf2 by its regulator Keap1 [95]. This activates the antioxidant response element (ARE) to induce transcription of genes encoding for the detoxification enzymes GSTA2 and NQO1[95]. However, the accumulation of damaged proteins, lipids and DNA with long term exposure can permanently disrupt cellular function, inhibit ATP production and increase ROS production inducing cell death [91].

Mechanotransduction

Resistance exercise imparts mechanical stress to contracting muscles. The conversion of mechanical signals into biological responses is mechanotransduction, an important activator of signaling cascades. Although the sensors specific to mechanotransduction are unknown, they participate in the development and maintenance of skeletal muscle function [56]. Kumar *et al.* observed that passively stretching murine diaphragm muscle in both axial and transverse directions phosphorylated the mitogen-activated protein kinase (MAPK) pathways ERK1/2 and p38, activating the downstream kinase p90 RSK and the transcriptions factors Elk-1 and AP-1 [96]. However, the upstream

activators of these responses differed between the two directions of force. Axial loading primarily activated PI3K and PKC while protein kinase A (PKA) activation only followed transverse loading [96]. This suggests that mechanical stretch not only signals skeletal muscle adaptation, but is also specific to the orientation and magnitude of the applied force.

Signaling pathways

Calcineurin

Following initiation, signaling cascades for adaptation use the biochemical action of kinases and phosphorylases to regulate the magnitude and duration of their action. Calcineurin, a Ca^{2+} dependant serine/threonine protein phosphatase, is known to regulate growth signaling in cardiac and skeletal muscle [97,98,99]. Within the cytosol Ca^{2+} binds to the regulatory protein calmodulin, which then binds to a domain on calcineurin thereby activating it [97]. One of the mechanisms of action regulated by calcineurin is the transcription factor nuclear factor of activated T cells (NFAT). Dephosphorylating its conserved serine residues, calcineurin allows the translocation of NFAT into the nucleus to promote gene transcription [100,101]. Interacting with insulin-like growth factor 1 (IGF-I), calcineurin also activates the transcription factor GATA-2 to induce hypertrophy in skeletal myocytes [102]. Calcineurin signaling may also play a role in fiber-type regulation as it activates slow fibre gene expression and mediates fast to slow fibre-type conversion [103,104,105]. Although still

unknown, these seemingly opposite adaptations may be activated by the various Ca^{2+} concentrations and exposures experienced during different exercise protocols.

MAPK

The MAPK pathways respond to extracellular signals by inducing intracellular responses. The growth factors, cytokines and environmental stress associated with exercise influence key proteins in these pathways [106,107]. Of the four that compose the MAPK family, c-Jun NH2-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase 1/2 (ERK1/2) are activated by unaccustomed and damaging resistance exercise [108,109,110]. Once phosphorylated, they activate sarcoplasmic proteins or translate to the nucleus where they phosphorylate early the pro-hypertrophic transcription factors JUN and FOS, members of the AP-1 transcription factor complex [111,112,113]. Although all three have been found to be activated following resistance exercise, localized regulation of these parallel signaling pathways is dependant on factors that include the mode and intensity of exercise and training status [114].

Gene transcription

Protein content is a balance of transcription and stability of mRNA, translation into protein by the ribosome and protein breakdown [2,80,115]. The induction of target genes by secondary messengers increases the expression of

mRNA for proteins important to adaptation [2]. Repeated exercise elevates transcriptional activity leading to elevated protein content that over time underlies the long term adaptation to exercise [80]. Potential targets of hypertrophic signaling include: transcription factors, genes encoding structural and regulatory proteins and myogenic regulatory factors.

AP-1

AP-1 is a transcriptional factor involved in the cellular processes of differentiation, proliferation and transformation that can be activated through the cascades for NFAT, the MAPK family and the small GTPase protein RhoA [101,112,113,116]. Its activity is regulated by members of the FOS and JUN gene families that combine in various heterodimers or homodimers to induce the expression of different target genes [117]. As immediate early genes, the specific response of the FOS and JUN families is thought to regulate genetic events for adaptation in a manner dependant on the exercise stimulus [117]. Marked increased expression (23 to 800-fold increases) of FOS and JUNB occurs within 2-4 h of a single bout of eccentric exercise before quickly returning to baseline levels within 24 h [118,119]. Thirty minutes of high intensity running also increased the immediate expression of FOS and JUN family members, although not to the same magnitude (7.0 to 17.8-fold) [117].

Structural and regulatory proteins

Induced by exercise-induced muscle damage, genes encoding for structural and regulatory proteins assist in the repair and remodelling process by managing stress and strengthening muscle architecture. Capping protein (actin filament) muscle Z-line alpha 1 (CAPZA1) and regulator of calcineurin signaling 1 (RCAN1) are two examples of these proteins. CAPZA1 binds the barbed ends of actin at the sarcomeric Z-lines and is crucial for myofibrillogenesis [120]. Anchoring the thin filament to the Z-disc by directly interacting with α-actinin, CAPZA1 is important for the positioning of the thin filament during the early stages of myofibrillogenesis [121]. RCAN1 is activated by calcineurin which in turn acts as an endogenous feedback regulator of calcineurin activity, inhibiting NFAT signaling [122,123,124,125,126]. The exact role of RCAN1 in skeletal muscle adaptation is unknown as it has been identified as a mediator of calcineurin signaling for hypertrophy [83] and a regulator of fibre type switching [105]. Previous work in our lab has indicated that both of these genes are significantly induced by a single bout of eccentric exercise [127].

Myogenic regulatory factors

Satellite cells provide a regenerative capacity to damaged skeletal muscle. Following their activation, proliferation and differentiation into myoblasts, they fuse with existing myofibres, assisting in growth and repair by increasing myonuclei number and myofibre size [80,128]. Normally quiescent between the basal lamina and sarcolemma of mature myofibres, specification and

differentiation of satellite cells is dependant on the expression of four myogenic regulatory factors (MRFs): MyoD, Myf5, myogenin and MRF4 [128,129]. In addition to their roles with satellite cells, MRFs also regulate the expression of the muscle specific genes for desmin [130], troponin 1 [131] and myosin light chain [132]. Within the 24 h after resistance exercise, mRNA expression levels are higher for MRF4, MyoD and myogenin in human skeletal muscle indicating that the response of MRFs to increased load occurs rapidly to regulate adaptation [133,134].

1.2.1 Estrogen

Estrogens are three naturally occurring female sex hormones primarily produced in the ovaries in females and by the testes, brain and adipose tissue in males. Two of them, estrone (E1) and 17β-estradiol (E2), are synthesized from their respective precursors androstenedione and testosterone by the enzyme aromatase [135]. The third estrogen, estriol (E3), is primarily formed in the liver from E2 [136]. Of them, E2 is the most abundant in healthy perimenopausal females (Table 1) and exhibits the most potent estrogenic properties [136]. It is transported via the circulatory system from its site of origin bound to sex-hormone-binding globulin (SHBG), although binding to albumin also occurs with lower affinity [137]. Upon reaching a target cell, E2 dissociates from its transporter to passively diffuse across the membrane where its actions are regulated by the structure of the hormone, the estrogen receptor involved and the

coactivators and corepressors present within the cell to modulate the transcriptional response [136].

Phase	17β-Estradiol (E2) pg/ml	Estrone (E1) pg/ml	Estriol (E3) pg/ml
Follicular	40-200	30-100	3-11
Preovulatory	250-500	50-200	-
Luteal	100-150	50-115	6-16
Premenstrual	40-50	15-40	-
Postmenopausal	< 20	15-80	3-11

Table 1. Serum concentrations of estrogens across the menstrual cycle in healthy women [136].

The primary action of estrogen on the growth, differentiation and function of tissues occurs after binding to one of two estrogen receptor (ER) subtypes, ERα and ERβ [136,138,139]. As ligand-activated transcription factors they reside in the cytoplasm, nucleus and mitochondria where they bind with estrogen in a homodimeric or heterodimeric form to activate transcription at specific DNA sequences called estrogen response elements (ERE) [140,141]. ERs also affect gene transcription indirectly by modifying the activity of the transcription factors AP-1 and nuclear factor κ B (NFκB) [142,143]. Although circulating concentrations of E2 are much higher in women than men, protein quantities of ERα and ERβ in skeletal muscle are similar [144,145].

Estrogens also affect cellular function in a more rapid manner than the traditional mechanism of transcriptional activation and protein synthesis. Recently

defined membrane-localized ERs contribute to the regulation of gene transcription by protein phosphorylation [146]. Residing as monomers, these membrane-bound ERs bind with estrogen to form homodimers that activate G proteins and growth factor receptors [146]. This promotes secondary signaling through endothelial Nitric Oxide Synthase (eNOS), MAPK and signaling cascades, altering protein localization and function and affecting cellular differentiation, migration and survival [146,147,148].

1.2.2 Interaction of E2 and exercise

The effect of E2 on skeletal muscle is relatively unknown, although it is reported to attenuate exercise-induced muscle damage and alter the subsequent events of inflammation and repair. Following unaccustomed exercise, exposure to exogenous and endogenous E2 in animal studies reduces the level of damage to muscle membranes [71,149,150,151,152] and structural proteins [153] while lowering inflammation-related leukocyte concentrations in blood and muscle [64,154,155,156]. E2 also increases satellite cell activation and proliferation in rats after damaging exercise improving the potential for growth and recovery, although the mechanisms for its influence are unknown at this time [154,157,158]. In contrast however, human studies do not support these effects as consistently as animal studies, often reporting similar responses between men and women [61,159,160]. Further speculation suggests that differences in age, exercise and hormones other than E2 may confound the results of human studies.

1.2.3 Estrogen and muscle damage

As previously mentioned, the appearance of muscle proteins in circulating blood is an indirect indicator of membrane damage. Early work by Amelink and colleagues identified that intact female rats have lower CK efflux than males and ovariectomized (OVX) females after exercise [149,150,151,161]. Increases in serum CK activity of almost 400% after high intensity running were completely [151,161] or partially [149] attenuated in both males and females with naturally occurring or supplemented E2. These findings are further supported by more recent studies that also show varying degrees of CK release inhibition with E2 in rodents [84,155,160].

Although women have lower serum CK activity at rest [162,163], some human studies report less activity and others report similar activity after exercise. Following cycling and eccentric exercises, studies by Shumate et al. and Sewright et al. have observed lower CK activities in women than men [164,165,166]. However, men and women have also have been reported to have equal absolute (4 fold) [167] and relative (5-fold) [168] increases in CK activity following downhill running. Comparisons between oral contraceptive users and eumenorrheic women also provide mixed results. In one study, women with higher E2 have attenuated CK activity after downhill running [166] yet show no difference following 25 eccentric arm contractions and a 50-min bench stepping exercise [169,170].

Avoiding the problems associated with using indirect measurements, muscle damage can be directly measured by assessing myofibrillar disturbances. Following an eccentric exercise protocol, the number of areas of focal and extensive Z-disc streaming increased with exercise but was not different between sexes [64]. A second study by the same group also reported similar amounts of extensive Z-disc streaming between men and women after an initial and repeated exercise bouts 5-6 weeks apart [71]. Although the number of studies is small, these results suggest that E2 does not attenuate myofibrillar disturbances after eccentric exercise in the human based research reported to date.

1.2.4 Estrogen and inflammation

Animal studies measuring neutrophil infiltration into damaged muscle after exposure to E2 often demonstrate an attenuated response. Female mice exhibit lower myofibre invasion than males 24 h following an eccentric contraction protocol when infiltration rates are highest [156]. Infiltration into soleus and plantaris muscles in male rats 24 h after treadmill running was completely attenuated in females [89]. Similar E2 based attenuations have also been reported in male and OVX rats after 8 to 21 days of supplementation and treadmill running [89,154,155,171]. Neutrophil infiltration induced by ischemia-reperfusion injury is also greater in OVX rats than intact females and OVX rats supplemented with E2 [152].

Similar to the trend observed with CK appearance, human studies also report an inconsistent neutrophil response to exercise-induced muscle damage with E2. Following eccentric exercise, Stupka et al. reported a very strong trend ($P = 0.052$) for less neutrophil infiltration in women than men (0.74 ± 0.59 cells/mm^2 vs. 2.70 ± 2.18 cells/mm^2), determining that one more subject in each group would have yielded significance [64]. However, a second study with a similar exercise protocol reported no sex difference after a first bout of exercise and a greater neutrophil infiltration in women after a second identical bout 5-6 weeks later [71]. Greater neutrophil accumulation in muscle was also reported in women vs. men 2 h after 300 maximal eccentric contractions [61].

Macrophage infiltration as a measure of inflammatory response is less common in the studies investigating E2 and exercise, but parallel the difference seen in the animal and human studies already presented. Following treadmill running, E2 supplementation for OVX rats attenuates exercise-induced macrophage infiltration in a pattern similar to neutrophil infiltration [154,171]. Although not attenuated, female mice experience a delayed macrophage infiltration after eccentric exercise reaching peak concentrations 2 days later (7 vs. 5) than males [156]. In the only study to compare men and women, the number of macrophages invading muscle after repeated bouts of eccentric exercise showed significant but similar increases after each bout [71].

1.2.5 Potential mechanisms of protection by E2

Although the effects of E2 on exercise-induced damage to skeletal muscle have been well documented, the mechanisms for protection have not been definitively identified. Aside from transcriptional modifications, E2 may also influence skeletal muscle with potential antioxidant and membrane stabilizing properties. During *in vitro* experiments in which liposomes were incubated with varying concentrations of all three estrogens, E2 had the greatest inhibition of peroxidation byproduct formation [172,173].

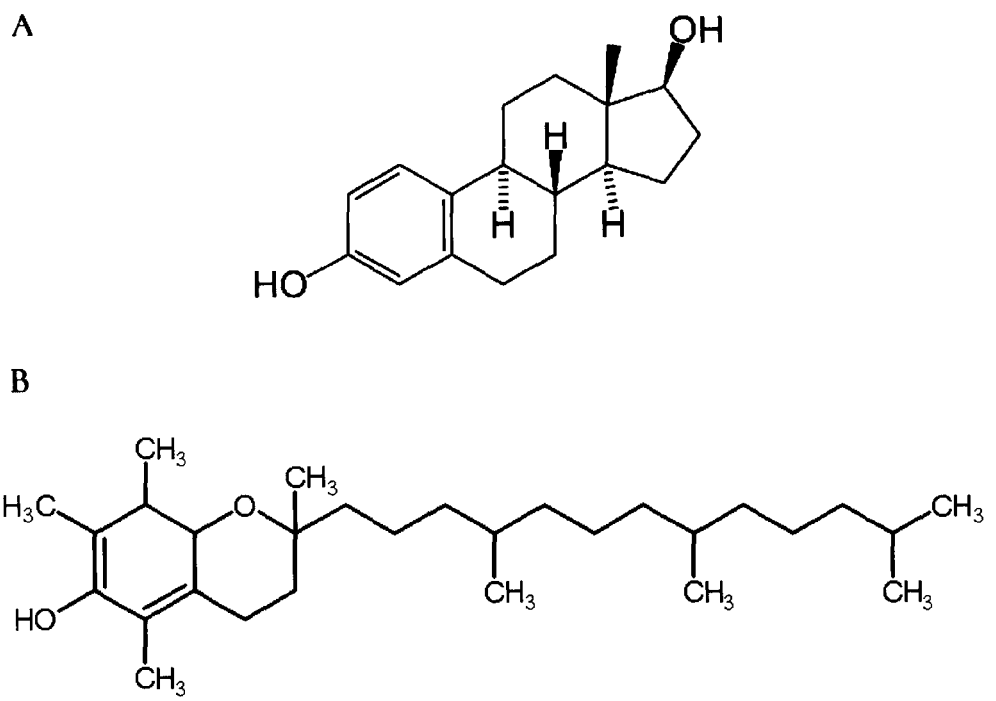

Figure 3. Chemical structures of E2 (A) and Vitamin E (B).

Additionally, E2 decreased the consumption of the antioxidants vitamin E and glutathione and increased total antioxidant capacity (TAC) in plasma following passive muscle strain in OVX rats compared to placebo [160]. A hydroxyl group in a similar location as vitamin E (Fig. 3) may allow E2 to donate hydrogen atoms, thereby terminating peroxidation chain reactions and improving antioxidant indexes [12,84,158,172,173,174,175].

As a fat soluble hormone E2 directly interacts with phospholipids in the cell membrane improving fluidity [84,176]. When incubated with E2, liposomes have lower membrane fluidity (improved stability) in a concentration dependant manner [177,178]. This may be similar to the intercalation of cholesterol into the membrane bilayer that subsequently improves mechanical strength and elasticity [177,178]. Reduced membrane damage could improve calcium homeostasis by: lowering calpain activity, forming fewer neutrophil attracting peptides and reducing neutrophil adhesion and infiltration into the muscle [155,179]. The potential antioxidant properties of E2 may also improve membrane stability by limiting the amount of damage caused by peroxidative chain reactions [180].

1.3.1 Purpose of Thesis

The research experiments performed and described in this thesis were designed to examine the effects of the sex hormone E2 on antioxidant capacity, muscle damage, inflammation and gene transcription after a single bout of

eccentric exercise in men. We hypothesized that reported antioxidant and membrane-stabilizing properties of E2 would attenuate the exercise-induced muscle damage that occurs after unaccustomed eccentric contractions. We aimed to identify these differences by measuring total antioxidant capacity, markers of membrane damage, inflammatory cell infiltration and alterations in the expression of genes involved in oxidative stress, cholesterogenesis, lipogenesis and growth. The effect of E2 administration on the skeletal muscle transcriptome profile has not been determined, alone or following a single bout of intense eccentric exercise. As such, the second aim of our investigation was to discover novel differential gene expression relevant to E2 exposure and/or the recovery from damaging eccentric exercise with the use of high density oligonucleotide based microarrays, furthering our understanding of the activation of repair and remodelling mechanisms and providing insights for future research.

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CHAPTER 2: MANUSCRIPT 1

IN REVIEW:

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17β-estradiol attenuates exercise-induced neutrophil infiltration in men.

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Running Head: E2 and eccentric exercise, muscle damage & inflammation.

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ABSTRACT

17β-estradiol (E2) attenuates exercise-induced muscle damage and inflammation in some models. Eighteen men completed 150 single-leg lengthening contractions after random assignment to 8 days of control (CON) or E2 supplementation (EXP). Muscle biopsies and blood samples were collected at baseline (BL), following 8 day supplementation, + 3 (3H) and + 48 (48H) hours after exercise. Blood samples were analyzed for sex hormone concentration, creatine kinase (CK) activity and total antioxidant capacity (TAC). The mRNA content of genes involved in membrane repair (forkhead box O1 (FOXO1), caveolin 1 and sterol regulatory element binding protein 2 (SREBP 2)) and antioxidant defense (superoxide dismutase 1(SOD1) and 2 (SOD2)) were measured by RT-PCR. Immunohistochemistry was used to quantify muscle neutrophil (myeloperoxidase) and macrophage (CD68) content. Serum E2 concentration increased 2.5 fold with supplementation ($P < 0.001$), attenuating neutrophil infiltration at 3H ($P < 0.05$) and 48H ($P < 0.001$), and the induction of SOD1 at 48H ($P = 0.02$). Macrophage density at 48H ($P < 0.05$), and SOD2 mRNA at 3H ($P = 0.01$), increased but were not affected by E2. Serum CK was higher at 48H for both groups ($P < 0.05$). FOXO1, Caveolin 1 and SREBP-2 expression were 2.8 fold ($P < 0.05$), 1.4 fold ($P < 0.05$) and 1.5 fold ($P < 0.001$) higher at 3H after exercise with no effect of E2. These findings suggest that E2 attenuates neutrophil infiltration into skeletal muscle following acute lengthening muscle contractions but the mechanism does

not appear to be lesser oxidative stress or membrane stability and may indicate lesser neutrophil/endothelial interaction.

Key words: eccentric exercise, mRNA expression, sex differences, leukocytes, oxidative stress

INTRODUCTION

Skeletal muscle is a heterogeneous and adaptable tissue, able to function across a spectrum of physiological and metabolic conditions and remodel in response to changing demands. When subjected to unaccustomed exercise, particularly contractions that forcibly lengthen muscle (also known as eccentric contractions), myofibres are stressed beyond their functional capacity and damage may occur (14, 46). This damage can be measured in the 48 hours following exercise as: structural disturbances at cellular and subcellular levels (19, 52), muscle protein appearance in blood (46, 52), tissue inflammatory cell infiltration (31, 52), and increased soreness and impaired muscle function (15, 19). Many genes that are involved in post exercise membrane homeostasis, stress management and growth are also affected, changing the myofibre transcriptome (41, 48). Because of the substantial effect on muscle tissue, eccentric contractions are commonly used for exercise-induced muscle damage and/or inflammation research.

Most animal studies report differences in measures of exercise-induced muscle damage, inflammation and repair between males and females (2, 35, 56). Manipulation of the female sex hormone 17β-estradiol (E2) indicates that it is involved and may be responsible for these differences. Following exercise, adult females and animals supplemented with E2 show attenuated damage to muscle membranes (2-4, 58, 59), structural proteins (35), and lower inflammatory cell counts in blood and muscle (21, 56, 57, 68). Not only does E2 provide a level of

cellular protection, it also enhances the potential for growth and recovery by affecting satellite cell activation and proliferation (21, 22, 66).

Human studies are less consistent providing mixed results on the effectiveness of E2 to attenuate exercise-induced muscle damage. Although serum activity levels of the muscle protein creatine kinase (CK) are generally lower in women at rest (5), after exercise they can either remain lower (51, 58) or increase to the levels not different from those found in men (23, 54). With responses that can be attenuated (57), equal (58) or greater (39) in women, a consistent sex difference in exercise-induced inflammation has also not been determined. Factors beyond the influence of E2 alone that are better controlled in animals such as sex organ removal and high dose hormone replacement may explain why there is more consistency in murine research yet there continues to be inconsistencies in human research.

E2 may provide protection from exercise-induced muscle damage by acting both as an antioxidant and a membrane stabilizer. As an antioxidant, it limits the amount of lipid peroxidation caused by free radicals (73). As a membrane stabilizer, it interacts with the cell membrane changing membrane fluidity and stability in a manner similar to cholesterol (67, 72). Estrogens have also been identified as regulators of gene expression, although it is unknown if their action is due to receptor mediation or an indirect effect of their antioxidant properties

(11). These activities occur in model membrane systems (72, 73) but have not been confirmed *in vivo*, allowing the debate to continue regarding the influence of E2 upon exercise-induced muscle damage.

The current study was designed to investigate the effects of E2 on total antioxidant capacity, muscle damage, gene expression and inflammation following a single bout of eccentric exercise. Given the proposed influences of E2, we hypothesized that supplementation would result in increased anti-oxidant enzyme capacity, reduced exercise-induced membrane damage, modified gene expression, and attenuated inflammatory cell infiltration.

MATERIALS AND METHODS

Subjects and anthropometrics. Eighteen young healthy men volunteered as participants in this study. All subjects were pre-screened to ensure that they were healthy, fit and had not regularly participated in resistance exercise in the preceding 6 months. They were given an information sheet describing all of the testing procedures before providing written consent to participate. The study conformed to the standards outlined in the *Declaration of Helsinki* and was given approval by the Research Ethics Board of McMaster University. Body composition was measured using dual energy x-ray absorptiometry (DEXA) scans (Hologic QDR 1000W, Waltham, MA). Thigh muscle cross-sectional area was

calculated using anthropomorphic measurements of mid-thigh circumference and skinfold thickness (32). The subject demographics were (mean ± SD): age, 21 ± 2 y; height, 181 ± 5 cm; weight, 76.9 ± 12.8 kg.

Supplementation protocol. Subjects were assigned in a randomized, double-blind manner to either a control (CON, N=9) or experimental (EXP, N=9) group. CON subjects consumed 400 mg glucose polymer (Polycose; Abbott Laboratories, Ross Division, St. Laurent, Quebec, Canada) for 10 days. EXP subjects consumed ~ 300 mg glucose with 1 mg E2 (Estrace; Shire BioChem, Inc., St. Laurent, Quebec, Canada) for 2 days followed by 2 mg E2 for 8 days, similar to a protocol previously used by our group (18). Glucose and E2 tablets were concealed in gelatin capsules. On the morning of the ninth day, subjects reported to the laboratory and performed the exercise protocol. Supplementation continued until the day of the final biopsy and blood collection to maintain serum E2 concentrations throughout the collection protocol. Subjects in both groups were instructed to take one pill at the same time each day and return any unused pills. All subjects reported 100% compliance.

Exercise protocol and tissue collection. Muscle damage was induced with a previously developed eccentric exercise protocol (7). Approximately 2 weeks before the exercise protocol, subjects were given a familiarization session with a Biodex isokinetic dynamometer (System 3, Biodex Medical Systems Inc.,

Ronkonkoma, NY). On the testing day, following a short warm-up (10 min of light cycling), subjects were seated in the dynamometer with their right leg strapped to a lever arm. The lever arm was programmed to extend their leg to 150° of flexion (where 180° is full extension) at a moderate speed (30°/s), then flex their leg to 90° of flexion at a faster speed (120°/s). Subjects did not have to contract maximally during the extension phase. During the flexion phase, subjects were instructed to attempt to maximally resist flexion of the knee (i.e. voluntary 'maximal' contraction) against the descending lever arm throughout the entire range of motion. The complete test consisted of 15 sets of 10 repetitions, each set separated by 1 minute of rest.

Prior to each tissue collection, subjects abstained from any other form of physical exertion (within 72 h), avoided alcohol (within 48 h), ate their habitual diet (within 48 h), and abstained from caffeine (within 12 h). Each subject consumed a 350 Kcal defined formula diet (57% carbohydrates, 15% protein and 28% fat) two hours before each muscle biopsy and did not eat again until after the final biopsy of each session was taken. These nutritional and activity controls were taken to ensure that the muscle damage would be the only variable to differentially affect the outcomes between biopsies (70).

Muscle biopsies were taken from the vastus lateralis of the control (left) leg during the familiarization session (baseline, BL) and after 8 days of

supplementation (post supplementation, PS) and the exercised (right) leg 3 hours (3H) and 48 hours (48H) after exercise, in anatomically distinct sites approximately 6 cm apart (40). The post exercise collection times were chosen because they represent two distinct phases of recovery from muscle damage (14). Blood was drawn from the antecubital vein at the same collection times. Muscle and blood samples were processed and stored for future measurements.

Blood enzyme and hormone concentrations. Serum creatine kinase (CK) activity was measured by the core laboratory at Hamilton Health Sciences Centre in batches containing all samples for each individual. Serum E2 (Fertigenix-E2-EASIA, Biosource Europe S.A, Nivelles, Belgium) and testosterone (Fertigenix-TESTO-EASIA, Biosource Europe S.A, Nivelles, Belgium) concentrations were measured by enzyme amplified-sensitivity immunosorbent assays (EASIA) according to manufacturer's specifications using BL and PS blood collections. All hormone measurements were done in duplicate.

Total Antioxidant Capacity (TAC). Plasma samples collected at BL and PS were analyzed for TAC using a commercially available TAC-peroxyl assay (Northwest Life Science Specialties, Vancouver, WA). Samples were thawed to room temperature and diluted 20 fold using the supplied sample dilution buffer to bring the measurements within the range of the standards. Assay buffer and luminol reagent were added to Trolox standards and samples according to manufacturer's

specifications. Luminescence was measured using a 20/20n Single Tube Luminometer (Turner Biosystems, Sunnyvale, CA). The induction time was measured as the x-axis intercept of the linear portion of the curve at the maximum rate of change. The difference between BL and PS induction times (δt) was calculated for each subject. Increased TAC following supplementation would lengthen induction time resulting in a positive δt, reduced TAC would shorten induction time resulting in a negative δt.

Immunohistochemistry. Frozen muscle was serially cross-sectioned to 5 μm thickness using a cryostat (Microm International, Walldorf, Germany), dried overnight and stored until analysis. Negative control sections were included in all analyses. Slides were fixed in cold acetone for 15 min. Endogenous peroxidase activity was blocked using a liquid substrate kit (00-2014, Zymed Laboratories, San Francisco, CA). The slides were blocked with 1% goat serum (D3002S, Dako Diagnostics Canada, Mississauga, ON) for 15 min. The primary antibody was diluted in goat serum, and positive slides were incubated for 30 min. The slides were then incubated with secondary goat anti-mouse antibody (95-6543-B, Zymed Laboratories) for 15 min and with peroxidase (95-6543-B, Zymed Laboratories) for an additional 15 min. A kit (00-2007, Zymed Laboratories) was used for color development. The primary antibodies used were monoclonal mouse anti-human myeloperoxidase (M0748, Dako) at a 1:300 dilution for neutrophil

detection and monoclonal mouse anti-human CD68 (M0814, Dako) at a 1:100 dilution for macrophage detection.

Neutrophils and macrophages in the total cross-sectional area were counted and expressed as number of positive cells per square millimeter of muscle. This method has been previously published by our laboratory (58).

RNA extraction. The total RNA was extracted from the frozen skeletal muscle biopsy as described previously in detail by our group (40). Briefly, ~30 mg of skeletal muscle was homogenized on ice in 2 mL of Trizol Reagent (Life Technologies; Cat. No. 15596, Gaithersburg, MD). The homogenate was incubated for 10 min at room temperature, followed by phase separation using 200 μL of chloroform and precipitation of the total RNA from the aqueous phase using 500 μL of isopropyl alcohol. The RNA pellet was then washed three times in 75% ethanol and re-suspended in 15 μL DEPC-treated water, aliquoted, and stored at -86°C. The concentration and purity of the RNA was determined using a UV spectrophotometer (Shimadzu UV-1201; Mandel Scientific, Guelph, Ontario) at the absorbance of 260/280 nm. Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{280}) of the samples was 1.7 before DNase treatment. RNA integrity was assessed in a randomly chosen subset of samples using agarose gel

electrophoresis, and the OD ratio of 28S to 18S rRNA was consistently greater than 1 for each sample.

DNase treatment. Prior to cDNA microarray and real time quantitative RT-PCR analysis, the isolated RNA samples were treated with DNA-free™ recombinant DNase I (Ambion Inc, Austin, TX) according to the manufacturer's instructions to remove any potential genomic DNA contamination.

Real-time RT-PCR analysis. Changes in gene expression relative to baseline values were measured using real-time polymerase chain reaction (RT-PCR). Cu/Zn superoxide dismutase (SOD1) and Mn SOD (SOD2) were chosen for their role in oxidative stress management. Forkhead box O1 (FOXO1), Caveolin 1 and sterol regulatory element binding protein 2 (SREBP 2) were selected for their involvement in cholesterol and lipid homeostasis for membrane repair (41). The selected housekeeping gene was β2-microglobulin. It has been shown in previous work to remain constant following eccentric exercise (40), and this was again confirmed in the current study. The primer and probe sequences for these genes can be found in Table 1.

RT-PCR was completed using a TaqMan® real-time method. The primers and a probe to each target gene were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez/?db=gene) with Primer 3

designer (http://frodo.wi.mit.edu/primer3-0.4.0/input.htm). All target gene probes were labeled with FAM at their 5′ ends and BHQ-1 at their 3′ ends. Duplex RT-PCR was performed on an iCycler real-time PCR system (Bio-Rad Laboratories, Hercules, CA) in the One-step TaqMan® RT-PCR Master Mix Reagents (Roche, Branchburg, New Jersey) according to the manufacture's instruction with target gene primers, target probe, housekeeping gene primers and housekeeping gene probe in the same reaction (43). Determination of significant gene expression change was done as previously described (43). The genes of interest were normalized to the housekeeping gene, β2-microglobulin by following the standard method. Briefly, C_T values of the housekeeping gene were subtracted from the C_T values of the gene of interest giving a δC_T. This is equivalent to the $\log_2$ difference between endogenous control and target gene (10). Values were then normalized to baseline, $\delta\delta C_T$. The $\delta\delta C_T$ of each replicate is presented as normalized data. All samples were run in triplicate, fluorescence emission was detected using FAM and Tamra filters, and C_T was automatically calculated.

Western blotting. Muscle biopsy samples were homogenized and prepared for polyacrylamide gel electrophoresis using methods previously described (61). Briefly, frozen skeletal muscle tissue samples (~30 mg) were hand homogenized in 25 μl of phosphate buffer (50 mM Kpi, 5 mM EDTA, 0.5 mM DTT, 1.15%KCl (w/v)) per milligram of tissue. A protease inhibitor cocktail (Sigma, St. Louis, Missouri) was added to the phosphate buffer immediately prior to use at a ratio of

1:1,000. Samples were centrifuged at 600*g* for 10 min at 4°C and the supernatant aliquoted for analyses. Protein concentrations of each sample were determined using the method described by Lowry et al (38).

Samples were loaded on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. Membranes were blocked with 5% BSA (wt/vol) in Tris-buffered saline with 0.1% Tween (vol/vol) (TBST) and incubated overnight at 4° in primary antibody: total ERK1/2 (Cell Signaling Technology, Danvers, MA; no. 9102, 1:1,000); ERK1/2 Thr^{202}/Tyr^{204} (Cell Signaling Technology; no. 9101, 1:1,000); total AKT (Cell Signaling Technology; no. 9272, 1:1,000); Akt Ser^{473} (Cell Signaling Technology; no. 9271, 1:1,000). After washing in TBST, membranes were incubated in HRP-linked anti-rabbit IgG secondary antibody (Amersham Biosciences, Piscataway, NJ; no. NA934V, 1:6000), washed with TBST and developed using ECL (Amersham Biosciences; model no. RPN2106). Densitometry was performed on scanned images of x-ray film (Biomax XAR; Kodak, Rochester, New York) using Image J v1.40g software (National Institutes of Health, Bethesda, Maryland).

Statistical Analysis. Student's unpaired t-tests were used to identify statistical differences in: subject characteristics, total work, and TAC. 2-way repeated measures ANOVAs (supplementation group x time) were used to identify

differences in: CK activity, E2 concentration, testosterone concentration, neutrophil infiltration, macrophage infiltration and the linear 2-$\delta\delta C_T$ data sets of gene expression measured with RT-PCR. When statistical significance was achieved, Tukey's honestly significance difference post-hoc test was used to determine the significance among the means. STATISTICA for Windows 5.0 (Statsoft, Tulsa OK) was used to perform t-tests and ANOVAs. The threshold for significance was set at $P \leq 0.05$. Data are presented as mean ± SEM unless otherwise indicated.

RESULTS

Subject and work characteristics.

Both groups were similar in age, weight, height and body fat percentage (Table 2). All subjects completed the required 150 eccentric contractions. The total work completed was not different between groups ($P = 0.47$). The average thigh cross-sectional area was not different between groups ($P = 0.47$).

E2 supplementation altered serum E2 and testosterone concentrations.

Serum levels of E2 and testosterone following 8 days of supplementation are presented in Table 3. E2 concentrations were 2.5 fold higher ($P < 0.001$), and testosterone concentrations were 26% lower ($P = 0.01$), in the EXP group than the CON group. Although the magnitude of change was smaller than previous

research (18, 28, 69), the direction of change was similar. E2 and testosterone concentrations were not different between groups at BL and remained unchanged in the CON group.

Baseline E2 concentrations are within the expected range of values for men and are similar to lower values found in women in the follicular phase (40-200 pg/ml); elevated values in the EXP group are similar to the lower concentrations found in luteal phase women (100-150 pg/ml) (27).

E2 supplementation affected exercise induced muscle inflammation.

Neutrophil infiltration increased at 3 h (4.3 fold; $P < 0.05$), and 48 h (7.1 fold; $P < 0.001$), post-exercise in the CON group (Fig. 1). The neutrophil infiltration values for the EXP group did not change from baseline.

Macrophage infiltration increased 2.6 fold ($P < 0.05$) 48 h following exercise (Fig. 2). There was no difference between groups for macrophage infiltration.

Signalling proteins were not affected by E2 or eccentric exercise.

Phosphorylation of the signaling proteins ERK1/2 (Thr ^{202}/Tyr ^{204}) and Akt (Ser ^{473}) were not significantly different from baseline at any time or between supplementation groups (Figure 3).

Total antioxidant capacity and SOD2 mRNA are not affected by E2.

TAC was not altered following E2 supplementation (Table 4).

SOD1 mRNA content was greater in the CON group than the EXP group 48 h after exercise ($P = 0.02$) (Fig. 4). SOD2 mRNA content was 2.3 fold higher 3 h after exercise, regardless of group ($P = 0.01$) (Fig. 4).

Eccentric exercise induced muscle damage.

Serum creatine kinase activity was elevated 4.6 fold ($P = 0.04$) 48 hours after exercise (Table 5). The EXP values did not differ from the CON values at any time.

FOXO1, Caveolin 1 and SREBP-2 were upregulated 3 h after exercise 2.8 fold ($P = 0.002$), 1.4 fold ($P = 0.016$), and 1.5 fold ($P = 0.007$), respectively (Fig. 5). The mRNA abundance changes induced by acute exercise were similar for both groups.

DISCUSSION

Research conducted on the potential influence for E2 to alter exercise-induced muscle damage and inflammation has produced inconsistent results, particularly in human studies. We examined the *in vivo* effects of E2 on membrane damage,

antioxidant capacity, inflammation, and gene expression following a single bout of eccentric exercise. In an attempt to minimize other influences of sex *per se* (i.e., XX, XY chromosomes, body fat differences, etc.), we recruited only men and manipulated their sex hormone concentration with supplementation in a repeated measures design such that the only manipulated parameter was serum E2 concentration. Exercise-induced neutrophil infiltration and SOD1 induction were both attenuated by E2 while CK activity, antioxidant capacity, macrophage infiltration and expression of the other genes examined were unaffected.

E2 eliminated the elevated skeletal muscle neutrophil abundance that occurred following a single bout of eccentric exercise, an observation supported by animal studies that report either partial (60%) (68), or complete attenuation (33, 65), of neutrophil accumulation with E2 in the 24 hours after exercise. When a comparison was made between men and women following a single bout of eccentric exercise, both sexes showed increases in the number of cells containing leukocyte common antigen; however, there was a very strong trend ($P = 0.052$) for less infiltration in women than men (0.74 ± 0.59 cells/mm^2 vs. 2.70 ± 2.18 cells/mm^2) (57). Neutrophils are rapidly responding leukocytes that eliminate the cellular debris resulting from damage (25, 44, 63), whose adhesion and infiltration is primarily regulated by cytokines and cell adhesion molecules (16, 47). The different neutrophil densities we measured may be a result of improved membrane stability and reduced oxidative stress, which maintains calcium homeostasis and

reduces the signals for inflammation (65). However, we found that E2 did not influence membrane damage or antioxidant capacity, suggesting that another mechanism(s) is/are linking E2 concentration and neutrophil response.

Neutrophil recruitment is also influenced by endothelial nitric oxide synthase (eNOS) activity following damage (36, 53). Independent of gene transcription, E2 increases eNOS phosphorylation via two signaling proteins in a biphasic manner (53). The initial increase is mediated by mitogen-activated protein kinase/extra-cellular regulated kinase (MAPK/ERK), acutely activating eNOS within 5 minutes (13). Twenty minutes after exercise, the second increase in eNOS phosphorylation is mediated by protein kinase Akt (29, 53). As a result, mice implanted with E2 have an 3.2-fold increase in eNOS activity and attenuated leukocyte infiltration into muscle tissue following damage (53). To address this possibility, we measured ERK1/2 and Akt activation status and found that both were unchanged regardless of time or intervention in our study. It is important to note that the timing of the first biopsy following exercise (+3H) occurred after the reported peak activities of these signaling proteins and phosphorylation states may have returned to baseline. Although not identified here, it is possible that E2 altered eNOS activity immediately after the cessation of exercise, thereby preventing neutrophil infiltration. The latter two possibilities need to be further explored in subsequent studies with earlier time-points.

Skeletal muscle macrophage infiltration increased 48 h after exercise, regardless of E2 concentration. This is in agreement with previous results by our group and others that indicate an approximate 2-3 fold increase in macrophage density in the 24-48 hours after a single bout of eccentric exercise (7, 8, 42, 58). Of those studies, one compared men and women and although men showed a greater increase in density, the trend was not significant (58). Macrophages typically infiltrate damaged cells following neutrophils to regulate the immune response to injury and release growth factors important for regeneration and repair (63). Early responding neutrophils release cytokines important for the subsequent accumulation of macrophages, suggesting that an attenuated neutrophil response would result in attenuated macrophage recruitment. However, myogenic precursor cells initiate monocyte recruitment following damage when neutrophil infiltration is eliminated (12, 44), maintaining the growth response.

Differences in exercise-induced muscle damage between sexes may be due to antioxidant properties of E2 that reduce lipid peroxidation of the cell membrane and oxidative stress (22, 34, 55). With a hydroxyl group in a similar location as Vitamin E it may donate a hydrogen atom to reactive oxygen species (ROS), thereby attenuating oxidation of other molecules (9, 60). Although the exact mechanism is unknown, E2 terminates peroxidation chain reactions, inhibits lipid oxidation in microsomes, liposomes and macrophages and enhances TAC in rats (24, 60). However, we did not observe a change in TAC with E2, in contrast with

animal studies likely because of differences in E2 concentration. For example, the 10-20 μM used to achieve significant inhibition of lipid peroxidation in culture (60) is much higher than the E2 values seen in our subjects (350.1 ± 42.9 pM), and the normal range seen in women across the menstrual cycle (~150-750 pM) (27). The attenuation of neutrophil infiltration without a change in TAC is not consistent with proposed antioxidant effects of E2 (at least at physiological concentrations), reducing oxidative stress and thereby inhibiting inflammation (34). This suggests that at physiological levels there does not seem to be a direct antioxidant effect of E2.

E2 did attenuate the late induction of SOD1 mRNA but did not affect the SOD2 increase immediately after exercise. Acute induction of SOD1 and SOD2 occurs immediately following exercise as these antioxidant defense enzymes convert ROS to less active molecules (30, 31). ROS associated with exercise are generated by two main sources: increased O_2 consumption during exercise increases ROS production (26, 45), and release of ROS from activated neutrophils for apoptotic and chemotactic signaling following exercise-induced muscle damage (63, 74). As the elevated E2 concentration did not alter SOD1 or SOD2 expression at 3H and did not affect TAC, it likely did not act as an antioxidant buffering the ROS generated during exercise. It is more likely the attenuation in SOD1 mRNA was a result of the E2 attenuated neutrophil infiltration releasing a

smaller amount of ROS for inflammatory signaling and transcriptional activation of SOD1.

Membrane stabilizing properties are a second mechanism by which E2 may protect muscle from exercise-induced damage. E2 improves membrane fluidity in cell culture (64), and lowers the efflux of the muscle proteins CK following exercise (2-4, 17, 52, 58); however, this is not a consistent result. Although CK activity increased 4.6 fold following exercise, indicating that the exercise successfully induced membrane damage, we did not find a difference with E2. Equal absolute (4 fold) (23), and relative (5-fold) (54), increases in CK activity between men and women following downhill running support both the magnitude of damage and lack of protection we observed. The inconsistencies may be due to differences in study designs. The early studies identifying a lower CK with E2 were conducted with rats after a 2 hour flat running protocol (2, 3, 6), while human based research using eccentric exercise typically demonstrate no differences with E2 (23, 50, 54, 62). As well, improvements in membrane stability in liposomes only occurred with concentrations much higher (4-40 μM) than the *in vivo* concentrations mentioned earlier (71, 72). E2 may affect cell membranes in animals or at high concentration, but our results indicate that changing E2 alone does not reduce CK release from muscle after high intensity eccentric exercise.

The genes selected for their activity during membrane repair were induced early after exercise but were not affected by E2. The activation of caveolin 1 and SREBP2 at 3H is in agreement with results previously reported by our laboratory in which they were both measured 3.2 fold higher with DNA microarray screening following a similar eccentric exercise protocol (41). All three genes are involved in the production of elements necessary for membrane biosynthesis (41): FOXO1 and SREBP2 increase the production of lipids (1, 20), and caveolin 1 is a lipid binding protein in the plasma membrane (37, 49). Together, the synchronized upregulation of these mRNA species after exercise indicates an early pre-translational level of control following exercise to provide material to maintain lipid and cholesterol homeostasis, a modification not affected by E2.

This is the first study to investigate the effects of E2 on muscle damage, inflammation and repair following eccentric exercise using hormone manipulation in men. Although E2 is proposed to have antioxidant properties and the ability to stabilize cell membranes protecting them from exercise-induced damage, we did not observe either of these effects. We did find an attenuation of neutrophil infiltration, which may have resulted from direct neutrophil/endothelial interaction though further investigation is needed to confirm or refute this possibility.

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FIGURE LEGENDS

Figure 1. Density of myeloperoxidase (MPO) positive cells identifying neutrophil infiltration in CON (open bars, N = 9) and EXP (gray bars, N = 9) groups. BL = baseline, PS = post supplementation, 3H = 3 hours post exercise, 48H = 48 hours post exercise. * $P < 0.05$ and *** $P < 0.001$ compared to treatment baseline. $^{\dagger\dagger}P < 0.01$ between treatments. Values are mean ± S.E.M.

Figure 2. Density of CD68 positive cells identifying macrophage infiltration in CON (open bars, N = 9) and EXP (gray bars, N = 9) groups. BL = baseline, PS = post supplementation, 3H = 3 hours post exercise, 48H = 48 hours post exercise. *$P < 0.05$ main effect of time when compared to baseline. Values are mean ± S.E.M.

Figure 3. Fold change of phosphorylated/total ratio of signaling pathways in CON (open bars, N=9) and EXP (gray bars, N= 9) groups from baseline after eccentric exercise. BL = baseline, 3H = 3 hours post exercise, 48H = 48 hours post exercise. Graph A – ERK1/2 (Thr ^{202}/Tyr ^{204}). Graph B – PKB/Akt (Ser^{473}). Values are mean ± S.E.M.

Figure 4. Expression fold change of oxidative stress genes in CON (open bars, N = 9) and EXP (gray bars, N = 9) groups from baseline after supplementation and exercise protocols. PS = post supplementation, 3H = 3 hours post exercise, 48H =

48 hours post exercise. Graph A - SOD1. Graph B – SOD2. *P < 0.05 main effect of time when compared to 48H. †P < 0.05 between treatments. Values are mean ± S.E.M.

Figure 5. Expression fold change of membrane homeostasis genes in CON (open bars, N=9) and EXP (gray bars, N= 9) groups from baseline after supplementation and exercise protocols. PS = post supplementation, 3H = 3 hours post exercise, 48H = 48 hours post exercise. Graph A – FOXO1. Graph B – Caveolin 1. Graph C – SREBP-2. *P < 0.05 main effect of time when compared to 48H. Values are mean ± S.E.M.

Table 1. *Primer and probe sequences for membrane homeostasis, antioxidant defense and housekeeping genes*

Gene	Left Primer	Right Primer	Probe
SOD1	ctcaggagaccattgcatca	cagcgtttcctgtctttgtact	cttcatttccacctttgcccaagtcat
SOD2	ggacaaacctcagccctaac	gccgtcagcttctccttaaac	agccatcaaactggactttggttcctt
FOXO1	agatctacgagtggatggtcaa	acacgaatgaacttgctgtgta	agcgtgccctacttcaaggataa
SREBP2	tgagccaggaagccctctat	aagaatccgtgagcggtcta	agatgaaccatctcatgctctgtggc
Caveolin 1	cagtgcatcagccgtgtcta	tcccaacagcttcaaagagtg	catctacgtccacaccgtctgtgacc
β2M	ggctatccagcgtactccaa	gatgaaacccagacacatagca	tcaggtttactcaacgtcatccagcagag

Table 2. *Subject and eccentric exercise trial characteristics*

	CON	EXP
No. of subjects	9	9
Age, yr	21.1 ± 0.8	20.9 ± 0.9
Height, cm	181.6 ± 2.0	180.9 ± 1.4
Weight, kg	73.4 ± 3.8	80.4 ± 4.6
Body fat, %	14.7 ± 1.7	20.1 ± 2.4
Quadriceps CSA, cm^2	74.4 ± 3.3	78.5 ± 4.1
Work, kJ	24.9 ± 2.9	25.1 ± 1.3

Values are means ± SEM

Table 3. *Serum hormone concentrations after 8d of supplementation with either placebo (CON) or E2 (EXP)*

	CON	EXP
Estradiol (pg/ml)	38.4 ± 2.8	95.4 ± 11.7***
Testosterone (nmol/L)	14.6 ± 2.0	10.8 ± 2.1**

Values are means ± SEM **P < 0.01 and ***P < 0.001 when compared to CON. N = 9/group.

Table 4. *Plasma total antioxidant capacity represented by change in induction time (δt) in CON and EXP groups following 8 days of supplementation*

	δt (sec)
CON	-47.6 ± 63.9
EXP	-25.3 ± 60.6

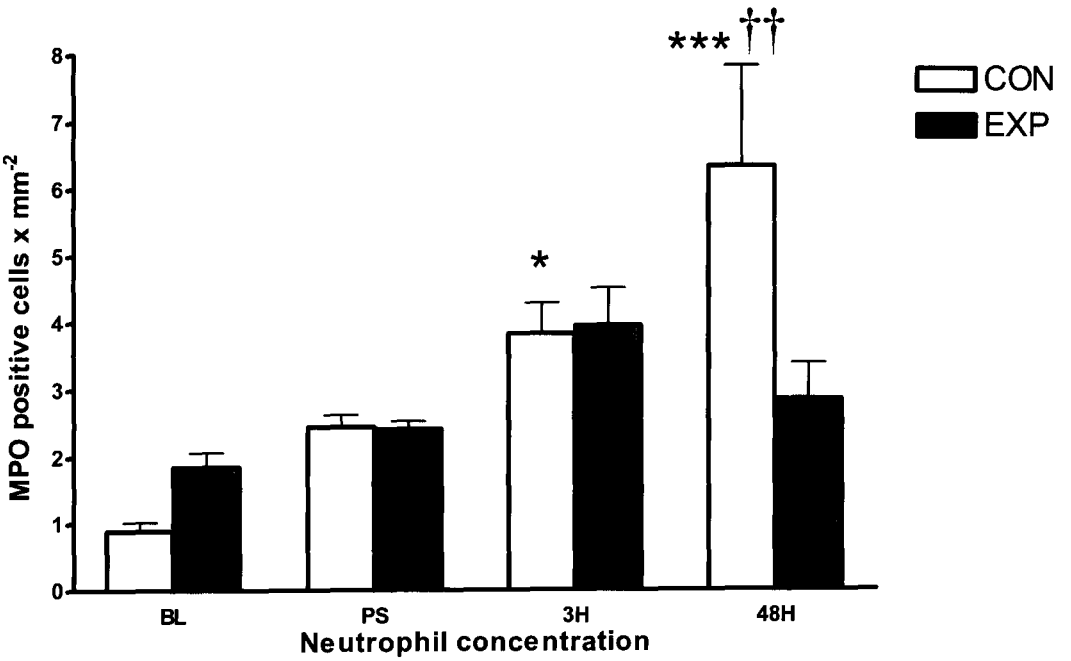
Values are means ± SEM. CON (N = 9) or EXP (N = 9).

Table 5. *Serum creatine kinase activity following 150 eccentric contractions in CON and EXP groups*

	Serum Creatine Kinase concentration (U/L)			
	Baseline	Post supplementation	3 hours	48 hours*
CON	113.6 ± 22.0	104.1 ± 19.8	235.7 ± 48.2	489.3 ± 73.0
EXP	207.1 ± 33.6	165.3 ± 29.8	268.5 ± 53.8	966.5 ± 531.0

Values are mean ± SEM. *P < 0.05 main effect for group compared to baseline. CON (N = 7), EXP (N = 8).

Figure 1.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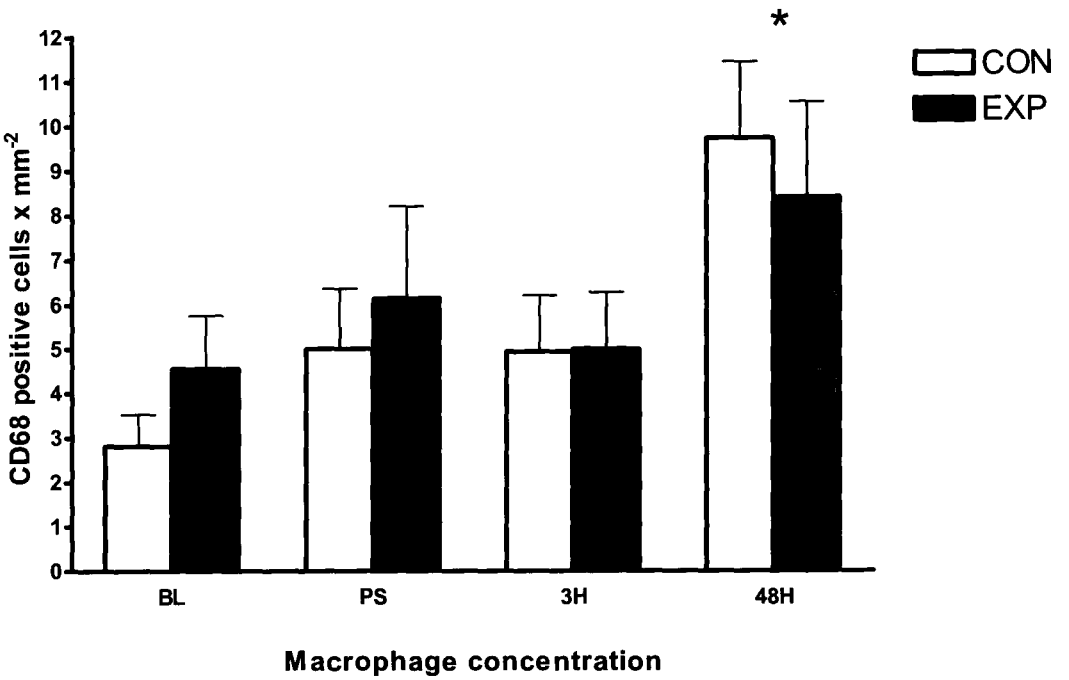
Neutrophil concentration

Figure 2.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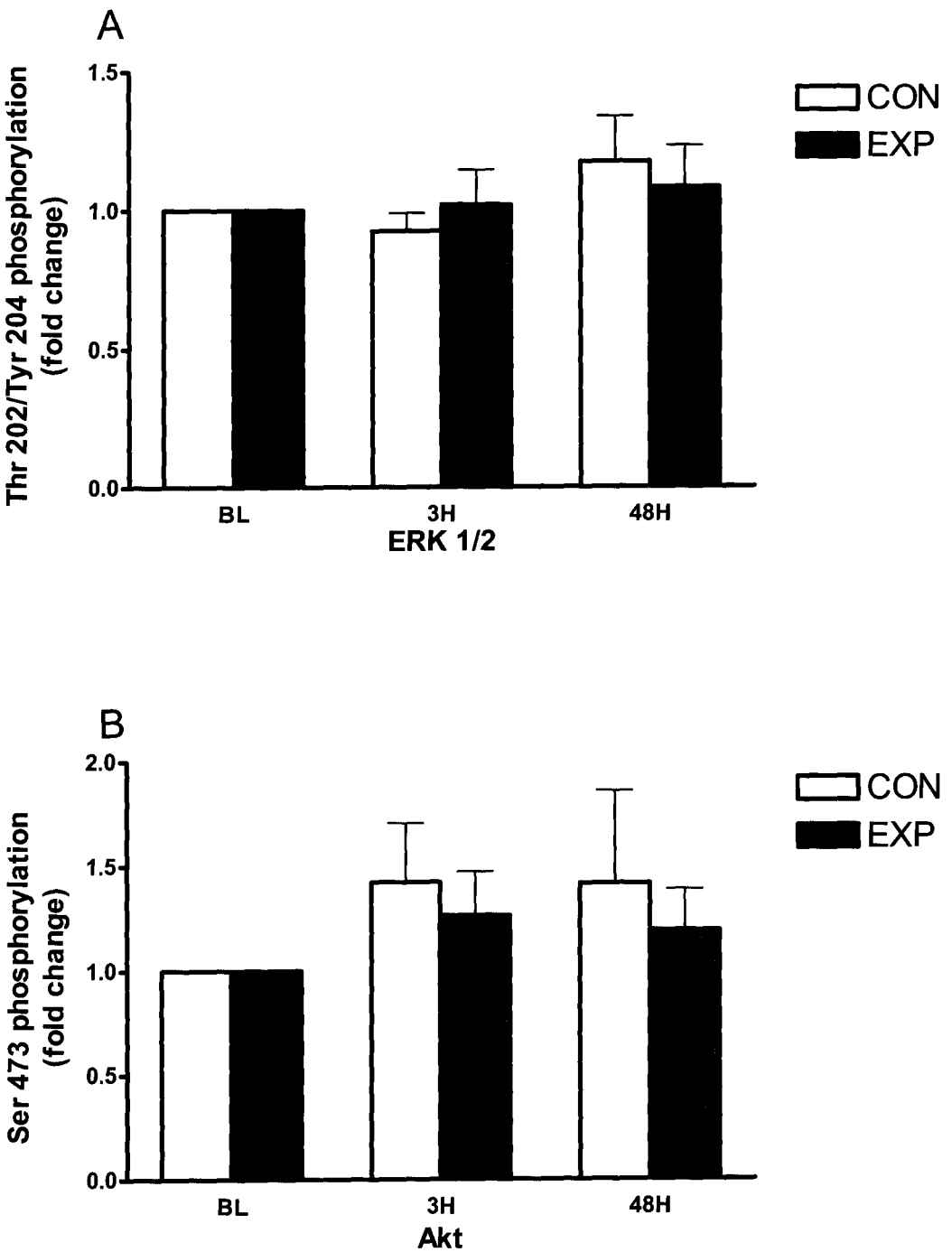
48H
Macrophage concentration

Figure 3.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Thr 202/Tyr 204 phosphorylation (fold change)
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3H
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B
Ser 473 phosphorylation (fold change)
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1.0
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BL
3H
48H
Akt
CON
EXP

Figure 4.

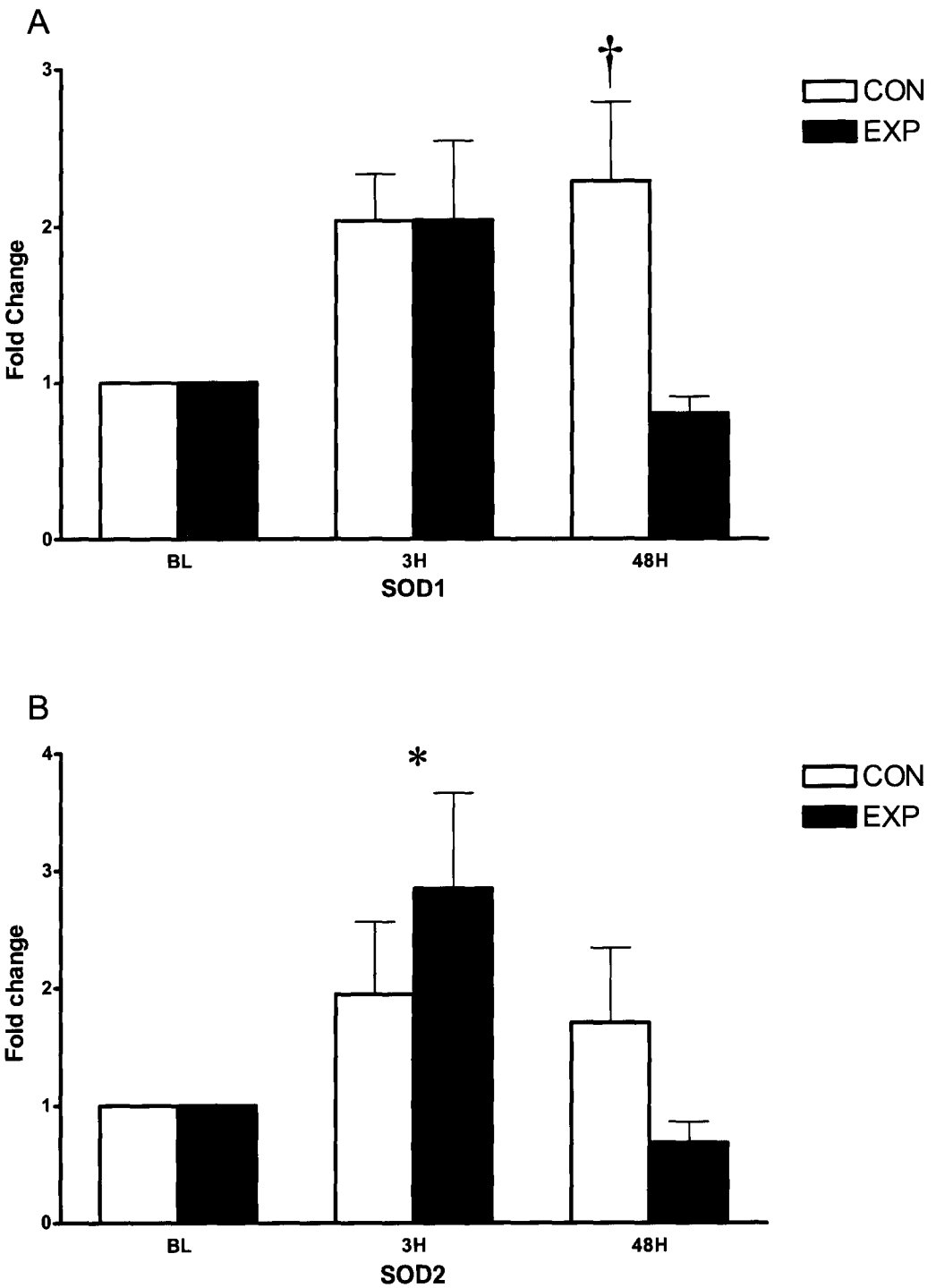
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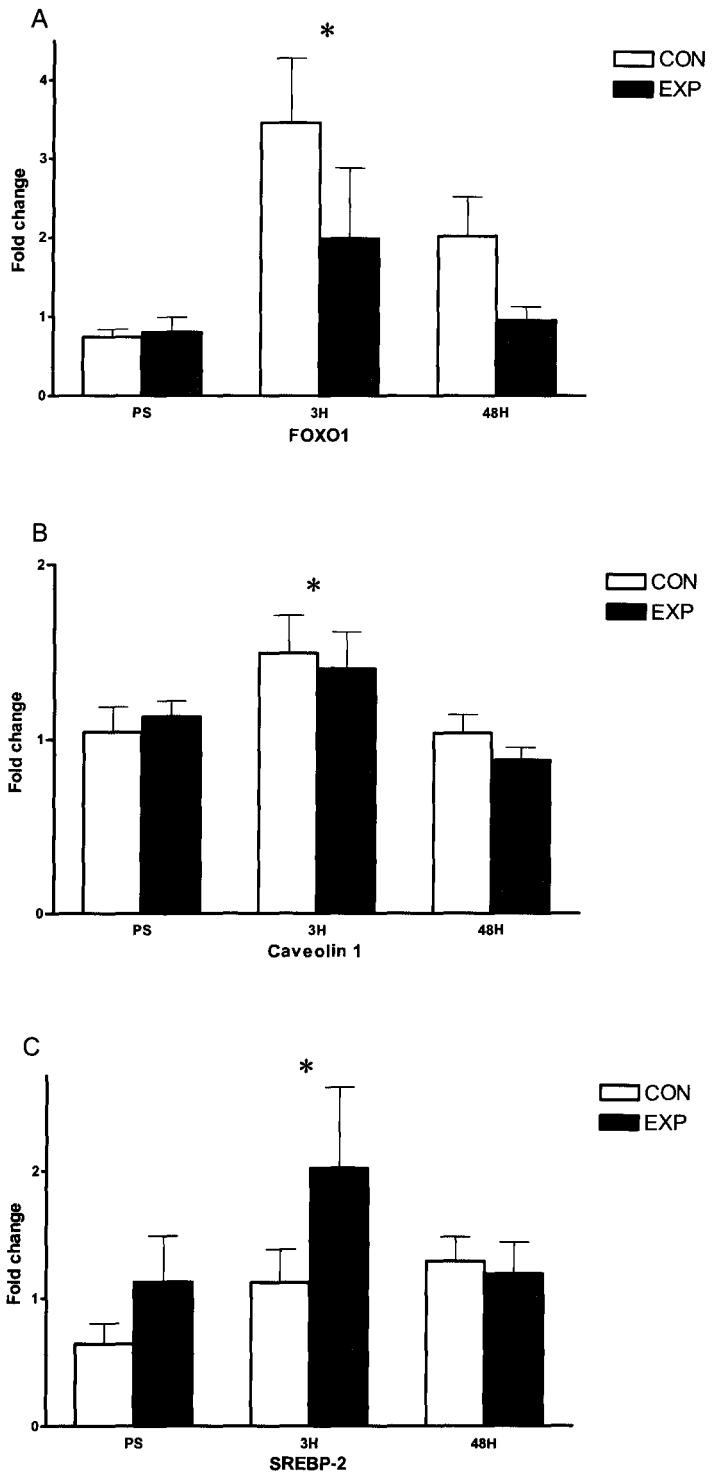
Figure 5.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SREBP-2

CHAPTER 3: MANUSCRIPT 2

IN REVIEW:

PLoS ONE

Eccentric exercise activates novel transcriptional regulation of hypertrophic signaling pathways not affected by hormone changes.

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Short title: mRNA regulation after exercise

ABSTRACT

Unaccustomed eccentric exercise damages skeletal muscle tissue, activating mechanisms of recovery and remodelling that may be influenced by female sex hormone 17β-estradiol (E2). Using high density oligonucleotide based microarrays, we screened for differences in mRNA expression caused by E2 and eccentric exercise. After random assignment to 8 days of either placebo (CON) or E2 (EXP), eighteen men performed 150 single-leg eccentric contractions. Muscle biopsies were collected at baseline (BL), following supplementation (PS), +3 hours (3H) and +48 hours (48H) after exercise. Serum E2 concentrations increased significantly with supplementation ($P < 0.001$) but did not affect microarray results. Exercise led to early transcriptional changes in striated muscle activator of Rho signaling (STARS), Rho family GTPase 3 (RND3), mitogen activated protein kinase (MAPK) regulation and the downstream transcription factor FOS. Targeted RT-PCR analysis identified concurrent induction of negative regulators of calcineurin signaling RCAN ($P < 0.001$) and HMOX1 ($P = 0.009$). Protein contents were elevated for RND3 at 3H ($P = 0.02$) and FOS at 48H ($P < 0.05$). These findings indicate that early RhoA and NFAT signaling and regulation are altered following exercise for muscle remodelling and repair, but are not affected by E2.

Key words: eccentric exercise, calcineurin, mRNA analysis, RhoA, nuclear factor of activated T-cells.

INTRODUCTION

Myofibres have the capacity to be remodeled to best meet their functional and metabolic demands. Changes in physical activity can initiate a remodelling process toward increased (hypertrophy) or decreased (atrophy) muscle mass [1,2,3]. Through protein signaling pathways, integration of chemical, mechanical and bioenergetic signals change the genetic expression patterns for cell size, function and metabolic processes [4]. Physical activities that incorporate unaccustomed eccentric contractions are typically associated with high levels of muscle damage, inflammation and delayed onset muscle soreness (DOMS) [5,6,7]. Eccentric contractions, identified by a lengthening while under tension, create an insult to myofibres which may be characterized by: damage to the sarcoplasmic reticulum [5], t-tubules [8] and structural proteins [6,9,10], the presence of muscle protein in blood [11,12], Z-line streaming [13,14], soreness and fatigue [7,8].

Murine and rodent research have often indicated an attenuation of exercise induced membrane damage [15,16,17,18,19], structural proteins [20], and inflammation [21,22,23,24] along with enhanced satellite cell activation [21,25,26] with exposure to the sex hormone 17β-estradiol (E2). This reduced muscle damage and improved recovery may result from potential antioxidant, membrane stabilizing, or gene regulation properties of E2 [27,28,29,30,31]. As

the most abundant estrogen, E2 exerts estrogenic properties that affect the differentiation, growth and function of reproductive, skeletal, neural and muscular tissues [31,32]. However, human studies do not consistently support the effectiveness of E2 to attenuate exercise-induced muscle damage, mostly reporting similar values for CK efflux, inflammation and loss of muscle function when comparing men and women [6,33].

Recent microarray analyses have uncovered novel transcriptional programs that coordinate the regeneration and repair of damaged muscle following eccentric exercise [10,34,35]. These studies have indentified clusters of genes representing important mechanisms for recovery and adaptation that include the regulation of inflammation [10,35], growth [10,35], stress response proteins [10,34] and membrane biosynthesis [34]. Similar analyses have also described sex differences in the expression levels of genes involved in metabolism and growth inhibition that may result from variations in body composition and hormone content [36,37,38]. Specifically, women express a higher abundance of mRNA for several genes involved in fat metabolism that include trifunctional protein β and lipoprotein lipase [36], and greater expression of the negative regulators of the anabolism growth factor receptor-bound 10 and activin A receptor IIB [37].

The effect of E2 administration on the transcriptome expression profile of skeletal muscle following a single bout of intense eccentric exercise has not yet been

evaluated. In this study we used microarray analysis to identify how global mRNA abundance is altered by E2 supplementation in men after 150 eccentric contractions. We hypothesized that the anti-oxidant and membrane stabilizing properties of E2 would attenuate the amount of muscle damage experienced, thereby modifying the expression of mRNA species involved in membrane homeostasis, growth and stress management. Furthermore, we hypothesized that the use of gene array analysis would allow us to detect novel genes relevant to the hypertrophic growth signaling stimulated by intense exercise.

MATERIALS AND METHODS

Ethics statement. All participants were given an information sheet describing all of the testing procedures before providing written consent to participate. The study conformed to the standards outlined in the *Declaration of Helsinki* and was given approval by the Research Ethics Board of McMaster University (05-438).

Subjects and anthropometrics. Eighteen young healthy men volunteered as participants in this study. All subjects were pre-screened to ensure that they were healthy, fit and had not regularly participated in resistance exercise in the preceding 6 months. Body composition was measured using dual energy x-ray absorptiometry (DEXA) scans (GE Lunar Prodigy, Fairfield, CT). Thigh muscle cross-sectional area was calculated using anthropomorphic measurements of mid-

thigh circumference and skinfold thickness [39] to control for potential differences in total work completed. The subject demographics were (mean ± SD): age, 21 ± 2 y; height, 181 ± 5 cm; weight, 76.9 ± 12.8 kg.

Supplementation protocol. Subjects were assigned in a randomized, double-blind manner to either a control (CON, N=9) or experimental (EXP, N=9) group. CON subjects consumed 400 mg glucose polymer (Polycose; Abbott Laboratories, Ross Division, St. Laurent, Quebec, Canada) for 10 days. EXP subjects consumed ~300 mg glucose with 1 mg E2 (Estrace; Shire BioChem, Inc., St. Laurent, Quebec, Canada) for 2 days followed by 2 mg E2 for 8 days. This protocol has been previously used by our group to increase serum E2 concentrations to the range observed during the luteal phase of the menstrual cycle [40]. Glucose and E2 tablets were concealed in gelatin capsules. On the morning of the ninth day, subjects reported to the laboratory and performed the exercise protocol. Supplementation continued until the day of the final biopsy and blood collection to maintain serum E2 concentrations throughout the collection protocol. Subjects in both groups were instructed to take one pill at the same time each day and return any unused pills. All subjects reported 100% compliance.

Exercise protocol and tissue collection. Muscle damage was induced with a previously developed eccentric exercise protocol [41]. Approximately 2 weeks before the exercise protocol, subjects were given a familiarization session with a

Biodex isokinetic dynamometer (System 3, Biodex Medical Systems Inc., Ronkonkoma, NY). On the testing day, following a short warm-up (10 min of light cycling), subjects were seated in the dynamometer with their right leg strapped to a lever arm. The lever arm was programmed to extend their leg to 150° of flexion (where 180° is full extension) at a moderate speed (30°/s), then flex their leg to 90° of flexion at a faster speed (120°/s). Subjects did not have to contract maximally during the extension phase. During the flexion phase, subjects were instructed to attempt to maximally resist flexion of the knee (i.e. voluntary 'maximal' contraction) against the descending lever arm throughout the entire range of motion. The complete test consisted of 15 sets of 10 repetitions, each set separated by 1 minute of rest.

Prior to each tissue collection, subjects abstained from any other form of physical exertion (within 72 h), avoided alcohol (within 48 h), ate their habitual diet (within 48 h), and abstained from caffeine (within 12 h). Each subject consumed a 350 Kcal defined formula diet (57% carbohydrates, 15% protein and 28% fat) two hours before each muscle biopsy and did not eat again until after the final biopsy of each session was taken. These nutritional and activity controls were taken to ensure that the muscle damage would be the only variable to differentially affect the outcomes between biopsies [42].

Muscle biopsies were taken from the vastus lateralis of the control (left) leg during the familiarization session (baseline, BL) and after 8 days of supplementation (post supplementation, PS) and the exercised (right) leg 3 hours (3H) and 48 hours (48H) after exercise, in anatomically distinct sites approximately 6 cm apart [43]. The post exercise collection times were chosen because they represent two distinct phases of recovery from muscle damage [6]. The muscle biopsies were quickly dissected of fat and connective tissue, sectioned into RNase-free cryovials (~30 mg/piece), flash frozen with liquid nitrogen and stored at -86°C until analysis. Blood samples were drawn from the antecubital vein into heparinized tubes at the same collection times, placed on ice, centrifuged at 1750 g at 4°C for 10 min and stored at -20°C for analyses.

Blood hormone concentrations. Serum E2 (Fertigenix-E2-EASIA, Biosource Europe S.A, Nivelles, Belgium) and testosterone (Fertigenix-TESTO-EASIA, Biosource Europe S.A, Nivelles, Belgium) concentrations were measured by enzyme amplified-sensitivity immunosorbent assays (EASIA) according to manufacturer's specifications using BL and PS blood collections. All hormone measurements were done in duplicate.

RNA extraction. The total RNA was extracted from the frozen skeletal muscle biopsy as described previously in detail by our group [43]. Briefly, ~30 mg of skeletal muscle was homogenized on ice in 2 mL of Trizol Reagent (Life

Technologies, Cat. No. 15596, Gaithersburg, MD). The homogenate was incubated for 10 min at room temperature, followed by phase separation using 200 μL of chloroform and precipitation of the total RNA from the aqueous phase using 500 μL of isopropyl alcohol. The RNA pellet was then washed three times in 75% ethanol and re-suspended in 15 μL DEPC-treated water, aliquoted, and stored at -86°C. The concentration and purity of the RNA was determined using a UV spectrophotometer (Shimadzu UV-1201; Mandel Scientific, Guelph, Ontario) at the absorbance of 260/280 nm. Measurements were done in duplicate and had an average coefficient of variation (CV) of <10%. The average purity (OD_{260}/OD_{280}) of the samples was 1.7 before DNase treatment. RNA integrity was assessed in a randomly chosen subset of samples using agarose gel electrophoresis, and the OD ratio of 28S to 18S rRNA was consistently greater than 1 for each sample.

DNase treatment. Prior to microarray chipping and real time quantitative RT-PCR analysis, the isolated RNA samples were treated with DNA-free™ recombinant DNase I (Ambion Inc, Austin, TX) according to the manufacturer's instructions to remove any potential genomic DNA contamination.

Microarray analysis. The resulting total RNA samples were further assessed for integrity prior to chipping using a Nanodrop Spectrophotometer and the Agilent Bioanalyzer Nano Chip System. Samples which passed this initial quality control

assurance step were then amplified one round, using an Illumina TotalPrep Kit (Ambion) to generate cDNA then cRNA according to the manufacturer's instructions. This was again assessed for quality by using the Nanodrop and Bioanalyzer as described above. Labeled cRNA samples that passed this second round of quality control were then hybridized to Human Ref-8 BeadChips (Illumina) according to the manufacturer's instructions (approximately 23,000 genes), using equipment specified by the manufacturer (Illumina). Briefly, 850 ng biotin-labeled cRNA in 11.3 µl nuclease-free water was adjusted to 34 µl through the addition of 22.7 µl of 5:3 HybE1 buffer/formamide. The sample was heated at 65°C for 5 min, allowed to cool to room temperature, and then immediately added to a single array of an 8-array Human Ref-8 BeadChip. Once all 8 samples were added to each BeadChip, it was sealed in a Hyb Cartridge and incubated for 16 h at 55°C with rotation in an Illumina hybridization oven (rotation setting 5). Following overnight hybridization, BeadChips were moved to a slide rack and serially washed using gentle rotation in glass staining dishes filled with a) 250 ml Illumina Wash Buffer×5 min, b) 250 ml 100% ethanol×10 min, c) 250 ml Illumina Wash Buffer×2 min. BeadChips were then blocked for 10 min in 4 ml Block E1 buffer (Illumina), followed by staining for 10 min in 1 µg/ml Streptavidin-Cy3 conjugate (GE Healthcare) in Block E1 buffer. Stained BeadChips were finally washed using gentle rotation in a glass staining dish filled with 250 ml Illumina Wash Buffer×5 min. BeadChips were dried by centrifugation at 280 *g* for 4 min and stored in a light-tight box until reading.

Array Reading. Processed arrays were read using a BeadStation array reader (Illumina) according to the manufacturer's instructions.

Gene ontology analysis. In the lists of genes that were significantly differentially expressed with exercise in our study, we carried out gene ontology (GO) analysis to determine the relative enrichment of genes with common or related functionalities to gain insight into biological processes mediated by E2 or exercise. This was carried out using the web interface driven GoMiner tool using an FDR of 5% [44]. Genes were also referenced to their biological functions and canonical pathways with Ingenuity Pathway Analysis (IPA) software. This software identifies functions and pathways most significant to the data set in two ways: the number of differentially expressed genes included in a pathway or function and calculation of a *p*-value using a Fisher's Exact Test to determine the probability of the association of the data set.

Real-time RT-PCR analysis. Changes in gene expression relative to baseline values were measured using real-time reverse transcription-polymerase chain reaction (RT-PCR). Regulator of calcineurin 1 (RCAN1) and capping protein (actin filament) muscle Z-line, alpha 1 (CAPZA1) were selected for analysis because of their roles in growth and sarcomerogenesis [34]. Hemeoxygenase 1 (HMOX1) was chosen for analysis because of its role in stress management [45].

The selected housekeeping gene was β2-microglobulin. Its constant expression following eccentric exercise has been shown in previous work [43], and was confirmed for the current study. The efficiencies of all primers were tested and determined to be greater than 98%. The primer and probe sequences for these genes can be found in Table 1.

RT-PCR was completed using a TaqMan® real-time method. The primers and a probe to each target gene were designed based on the cDNA sequence in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez/?db=gene) with primer 3 designer (http://frodo.wi.mit.edu/primer3-0.4.0/input.htm). All target gene probes were labeled with FAM at their 5′ ends and BHQ-1 at their 3′ ends. Duplex RT-PCR was performed on an iCycler real-time PCR system (Bio-Rad Laboratories, Hercules, CA) in the One-step TaqMan® RT-PCR Master Mix Reagents (Roche, Branchburg, New Jersey) according to the manufacture's instruction with target gene primers, target probe, housekeeping gene primers and housekeeping gene probe in the same reaction [46]. Determination of significant gene expression change was done as previously described [46]. The genes of interest were normalized to the housekeeping gene, β2-microglobulin by following the standard method. Briefly, C_T values of the housekeeping gene were subtracted from the C_T values of the gene of interest giving a δC_T. This is equivalent to the $\log_2$ difference between endogenous control and target gene [47]. Values were then normalized to baseline, $\delta\delta C_T$. All samples were run in triplicate, fluorescence

emission was detected using FAM and Tamra filters, and C_T was automatically calculated.

Western blotting. Muscle biopsy samples were homogenized and prepared for polyacrylamide gel electrophoresis using methods previously described [48]. Briefly, frozen skeletal muscle tissue samples (25-35 mg) were hand homogenized in 25 µl of phosphate buffer (50 mM Kpi, 5 mM EDTA, 0.5 mM DTT, 1.15%KCl (w/v)) per milligram of tissue. A protease inhibitor cocktail (Sigma, St. Louis, Missouri) was added to the phosphate buffer immediately prior to use at a ratio of 1:1,000. Samples were centrifuged at 600 *g* for 10 min at 4°C and the supernatant aliquoted for analyses. Protein concentrations of each sample were determined using the method described by Lowry et al [49].

Samples were loaded on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. Membranes were blocked with 5% BSA (wt/vol) in Tris-buffered saline with 0.1% Tween (vol/vol) (TBST) and incubated in primary antibody: RND3 (Abcam, Cambridge, MA; ab50316, 1:1000); FOS (Abcam; ab16902, 1:1000); total p38MAPK (Cell Signaling Technology, Danvers, MA; no. 9212, 1:1000); p38MAPK (Thr180/Tyr182) (Cell Signaling Technology; no. 9215, 1:1000); total GSK-3β (Cell Signaling Technology; no. 9315, 1:1000); GSK-3β (Ser 9) (Cell Signaling Technology; no. 9323, 1:1000), β-actin (BD Biosciences, Mississauga, ON; no. 612657, 1:10000). After washing in TBST,

membranes were incubated in either HRP-linked anti-rabbit or anti-mouse IgG secondary antibody (Amersham Biosciences, Piscataway, NJ; no. NA934V, 1:6000), washed with TBST and developed using ECL (Amersham Biosciences; model no. RPN2106). Membranes were exposed to x-ray film (Biomax XAR; Kodak, Rochester, New York), the film was scanned with a Dell 920 scanner at 300 DPI and saved in TIF file format. Using Image J v1.40g software (National Institutes of Health, Bethesda, Maryland), background noise was removed and bands in the region of interest were selected for analysis. Individual profile plots were generated and area under the curve measured in arbitrary units (AU).

Statistical and Bioinformatics Analysis. Student's unpaired t-tests were used to determine differences in subject characteristics and total work. A 2-way repeated measures ANOVA (supplementation protocol x time) was used to assess differences in creatine kinase, estrogen concentration, testosterone concentration, protein levels and the linear $2^{-\delta\delta CT}$ data set for gene expression measured with RT-PCR using computerized software (Statistica, Statsoft). When statistical significance was achieved, Tukey's honestly significance difference post-hoc test was used to determine the significance among the means. STATISTICA for Windows 5.0 (Statsoft, Tulsa OK) was used to perform t-tests and ANOVAs. The threshold for significance was set at $P \leq 0.05$. Data are presented as mean ± SEM unless otherwise indicated.

Gene array data analyses were done comparing baseline, post supplementation, 3 and 48 hours post exercise using simple paired t-test on $\log_2$ expression ratios. Genes were ranked by p-value and the inference reported following adjustment for multiple testing using the FDR and the Benjamini and Hochberg method. Among those genes with an adjusted q-value (based on FDR) of <0.05, we used hierarchical clustering (based on the HOPACH algorithm) to find groups of genes with similar profiles across the subjects.

Results

Subject and work characteristics.

CON and EXP groups were not different in age, weight, height, body fat percentage, average thigh cross-sectional area or total work completed (Table 2). All subjects completed the required 150 eccentric contractions.

E2 and testosterone concentration were affected by supplementation with E2.

Following 8 days of supplementation serum E2 concentration increased by 146% ($P < 0.001$) and testosterone concentration was reduced by 26% ($P = 0.01$) in the EXP group (Table 3). Both hormone concentrations remained unchanged in the CON group.

Microarray data identifies altered mRNA expression during recovery from eccentric exercise that is not affected by E2. Eccentric exercise significantly increased the early mRNA expression of 310 genes at 3H. DNA microarray analyses did not identify differential mRNA expression of any gene as a result of E2 at any time (we could not reject the global null that E2 was independent of mRNA expression of all genes represented on the chip). For this reason, microarray data at each timepoint was collapsed between groups, increasing the sample size to 18 subjects. Mean fold change for genes with ratios greater than 1 at 3H was 2.3 ± 0.1. In addition, 301 genes had ratios less than 1 at 3H with a mean fold change of 0.8 ± 0.01. By 48H, all genes had expression values that were not different from baseline. The complete data set is freely available at GEO (accession no. GSE19062).

Of the genes differentially expressed at 3H, we identified 25 that participate in two signaling cascades for muscle growth and adaptation: ras homologue gene family, member A (RHOA) and nuclear factor of activated T-cells (NFAT) (Table 4). Other regulators of muscle growth and remodelling not directly involved in RHOA or NFAT signaling that were also highly induced included: ATF3, MYC, XIRP1, HBEGF and DNAJB4 (Table 4).

IPA identified several biological functions related to muscle growth and remodelling identified by the number of differentially expressed genes included in

the function and the calculation of a P-value using a Fisher's Exact Test with a threshold for significance set at $P \leq 0.05$. They included: cancer ($P < 0.05$, 233 molecules), gene expression ($P < 0.05$, 146 molecules), cell assembly and organization ($P < 0.05$, 69 molecules), cell morphology ($P < 0.05$, 59 molecules) and skeletal and muscular system development and function ($P < 0.05$, 50 molecules). In the same manner, IPA also identified three canonical pathways related to gene expression and growth/proliferation: ILK signaling ($P = 0.005$, 16/186 molecules), RAN signaling ($P = 0.005$, 4/23 molecules) and PI3K/AKT signaling ($P = 0.006$, 12/136 molecules).

RT-PCR analysis provides additional genes involved in actin dynamics and regulation of RhoA and NFAT signaling. Targeted real time RT-PCR was conducted on several genes selected *a priori* for their involvement in recovery from skeletal muscle damage. Confirming the identification of two of its transcript variants in the microarray, RCAN1 mRNA content was highly elevated at 3H (16.6-fold, $P < 0.001$) (Fig. 1A). Also induced was HMOX1 at both 3H (3.9-fold, $P = 0.009$) and 48H (3.5-fold, $P = 0.002$) (Fig. 1B).

Expression of CAPZA1, a regulator of the growth of actin filaments, was increased at 48H (1.8-fold, $P = 0.04$) (Fig 2).

Signaling proteins and protein content are affected by eccentric exercise.

Phosphorylation (Thr^{180}/Tyr^{182}) of p38MAPK was significantly lower at 3H (0.77-fold, $P = 0.07$) and 48H (0.73-fold, $P = 0.005$) as a result of exercise with no effect of E2 (Fig. 3A). Phosphorylation (Ser^{9}) of GSK-3β was not affected by either exercise or E2 (Fig.3B).

Two species highly expressed at 3H by the microarray were selected for western blot analysis. RND3 was significantly higher at both 3H (1.34-fold, $P = 0.02$) and 48H (1.39-fold, $P < 0.01$) (Fig. 4A). FOS was significantly higher at 48 hours following exercise (1.16-fold, $P < 0.05$) (Fig. 4B).

Discussion

The sex hormone E2 displays anti-oxidant and membrane stabilizing properties that could protect skeletal muscle from the effects of exercise induced muscle damage and influence genetic expression patterns [50,51]. Using microarray, real time RT-PCR and protein analyses, we have identified that 8 days of E2 supplementation did not affect the myofibre transcriptome in men. However, a single bout of eccentric exercise did induce differential mRNA transcription in the hypertrophic signaling pathways RhoA and nuclear factor of activated T-cells (NFAT) (Fig. 5), changes in the phosphorylation status of related signaling proteins and the protein quantities of two of the upregulated genes.

Of the genes affected early after exercise, one of the greatest inductions was observed in the novel actin binding protein striated muscle activator of Rho signaling (STARS). This protein is a muscle-specific transducer of cytoskeletal signaling in cardiac and skeletal muscle that responds to calcineurin activation and biomechanical stress [52,53,54,55]. STARS stimulates growth through a mechanism requiring actin polymerization and Rho GTPase activation, increasing serum response factor (SRF)-mediated gene transcription (Fig. 6) [54,55,56,57]. Originally identified in cardiac muscle, STARS mRNA content increases more than 3-fold following the hypertrophic signaling of pressure overload [52,53]. More recently, Lamon *et al.* identified a 3.4-fold increase in STARS mRNA in human skeletal muscle following 8 weeks of resistance training [58]. Our measurement of more than a 10-fold increase suggests that this gene is very important for the early signaling for growth and remodelling following eccentric exercise.

A downstream target of STARS associated with skeletal muscle hypertrophy and adaptation is the Rho GTPase, RhoA [58,59]. Rho GTPases are a family of small signaling G proteins that interact with effector proteins to regulate actin cytoskeleton, cell cycle progression and gene transcription [3,60,61,62]. These molecular signals switch to an active GTP bound state under the control of Rho GEFs (guanidine exchange factors), and return to their inactive GDP bound state

by Rho GAPs (GTPase-activating proteins) [60,63,64]. For the first time, our array analysis has identified increased expression of two GEFs (ARHGEF7 and ARHGEF12) and reduced expression of a GAP (ARHGAP24) that specifically regulate RhoA [60,65]. These gene expression modifications, if translated into altered protein quantities, could increase the potential for RhoA activation.

Although increased activity of RhoA protein is necessary for myogenesis induction, it must be downregulated before myotube formation can proceed [66,67]. This is achieved by RND3, another negative regulator of RhoA activity whose upregulation is an essential step of myoblast fusion [62,68,69]. Our damaging exercise protocol resulted in an early induction of this gene at 3H and elevated protein content by 48H. Cell culture experiments have identified that in the presence of growth factors, RND3 mRNA increases of ~1.7-fold result in greater protein content within 30 h [62]. This in turn inhibits RhoA activity and promotes myotube formation and elongation [62].

Once activated, RhoA signaling is associated with myogenesis and actin remodelling in various cell types through regulation of genes that include DIAPH1 [70,71], CORO1C [72], FLNB [73] and CAPZA1 [74]. Through SRF activation, RhoA and STARS also mediate the induction of actin proteins ACTA2 [75], ACTN1 [76] and members of the AP1 transcription factor complex: FOS [76,77], FOSB [77] and JUND [78]. Each of these genes was induced by eccentric

exercise at 3H, as identified by our gene array and targeted real time RT-PCR analysis and our data indicate that the increased transcription of FOS was effectively translated into protein, increasing levels significantly by 48H. Although the number of studies investigating these genes after exercise is few, some support can be found for the upregulation of select downstream targets. Following eccentric exercise, the largest induction occurs with the transcription factor FOS 2-8 hours post-exercise (23 to 38-fold increases) [10,79]. Resistance training results in a 2.7-fold increase in ACTN1 after 8 weeks [58]. Thirty minutes of high intensity running increased the expression of FOS (7.0-fold) FOSB (17.8-fold) and JUND (7.6-fold) [80]. Given that the mRNA levels for STARS, associated regulatory and transcription factors and downstream targeted genes were all significantly elevated 3 h after exercise, it appears that STARS signaling through a RhoA/SRF pathway is important for early skeletal muscle remodelling following damaging exercise.

A second calcineurin influenced signaling pathway identified in our microarray analysis to be transcriptionally active was nuclear factor of activated T-cells (NFAT). NFAT proteins exist in the cytoplasm of cells in a phosphorylated and inactive state [81]. The influx of calcium following sustained contraction or damage increases the binding of calcineurin to NFAT, dephosphorylating conserved serine residues and promoting translocation of NFAT into the nucleus [81,82,83,84]. Once inside the nucleus, NFAT cooperatively binds to DNA with

transcription factors AP1 and MAF initiating the transcription of prohypertrophic genes (Fig. 7) [85]. Our microarray analysis also identified a significant induction of the genes NFATc1 and MAF at 3H. Along with the greater expression of the AP1 complex components FOS, FOSB and JUND, an increased abundance of NFATc1 and MAF could improve signaling by NFAT.

The NFAT pathway interacts with mitogen activated protein kinases (MAPK) and glycogen synthase kinase-3β (GSK-3β) for coordination of the hypertrophic response [84,86]. p38MAPK and GSK-3β act as a negative regulators of cardiac hypertrophy, rephosphorylating NFAT and promoting its export from the nucleus [83,86,87]. Although GSK-3β mRNA content was higher in the microarray, its activity did not change and would not have affected NFAT nuclear residence. GoMiner analysis identified MAPK regulation as a transcriptionally active biological process through the induction of four related kinases (MAPKAPK2, MAPK6, MAP3K6 and MAP3K8), three related phosphatases (PP2CA, DUSP8 and DUSP16) and one surface receptor (ADRB2). Western blotting confirmed that p38MAPK phosphorylation status was lower after exercise, reaching significance by 48H. Lower activity of p38MAPK would assist in the transcriptional activity of NFAT, and may also occur to inhibit the induction of apoptosis and necrosis [2].

It should be noted that molecules that function in the recovery and repair of skeletal muscle through other mechanisms were also induced by our exercise protocol. Similar to other reports, ATF3 [10], MYC [10,34], XIRP1 [88], HBEGF [10] and DNAJB4 [10,34,35] were highly up-regulated (6.1 to 28.4-fold) early after exercise. In addition, the altered mRNA content of members of the signaling pathways ILK, RAN and PI3K/AKT identified them as being transcriptionally active. Their respective functions in actin cytoskeleton remodelling [89], transport across the nuclear envelope for gene expression [90] and protein synthesis [91] relate to the top biological functions returned by gene ontology analysis. Interestingly, the biological function that contained the greatest number of molecules was cancer (233 molecules), likely due to similar alterations to the regulation of cellular growth experienced with both exercise and cancer.

Maintained muscle contractions and exercise-induced damage to the sarcoplasmic reticulum and sarcolemma may result in the accumulation of excess calcium, known as Ca^{2+} overload [5,92]. As a signaling molecule, Ca^{2+} binds to calcineurin, activating both STARS [52] and NFAT [81,82,83,84,93]. Unrestrained calcineurin activity can be regulated in skeletal and cardiac muscle by the inhibitors regulator of calcineurin 1 (RCAN1, aka MCIP1, DSCR1) [81,94,95,96,97] and hemeoxygenase 1 (HMOX1) [98]. Increased mRNA content of RCAN1 was identified in microarray and targeted real time RT-PCR, confirming a previous gene expression profile that also identified an increase

following exercise (3.8-fold) [34]. RT-PCR also identified significant increases in HMOX1 at both time points after exercise, similar to the 8-11-fold induction following 5 days of resistance training [99]. Together, the upregulation of these two calcineurin inhibitors identifies the importance of regulating the elevated calcineurin activity that occurred following unaccustomed eccentric exercise [97].

These results indicate that E2 supplementation does not affect the transcriptional pattern in skeletal muscle following eccentric exercise in men. However, the stress of a single bout of exercise induced a transcriptional response in two signaling pathways, STARS/RhoA/AP1 and NFAT/AP1, providing important insights for future research into the early hypertrophic response.

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FIGURE LEGENDS

Figure 1. Expression fold changes in mRNA expression of genes in muscle from baseline after exercise protocol. Graph A – RCAN1 (N = 18). Graph B – HMOX1 (N = 18). 3H = 3 hours post exercise, 48H = 48 hours post exercise. **Significant difference vs. baseline when collapsed across supplementation ($P < 0.01$). ***Significant difference vs. baseline when collapsed across supplementation ($P < 0.001$).

Figure 2. Expression fold changes in mRNA expression of CAPZA1 in muscle from baseline after exercise protocol. 3H = 3 hours post exercise, 48H = 48 hours post exercise. N = 18. *Significant difference vs. baseline when collapsed across supplementation ($P < 0.05$).

Figure 3. Fold change of phosphorylated/total ratio of signaling pathways from baseline after eccentric exercise. BL = baseline, 3H = 3 hours post exercise, 48H = 48 hours post exercise. Graph A – p38MAPK (Thr ^{180}/Tyr ^{182}) (N = 18). Graph B – GSK-3β (Ser^{9}) (N = 18). Values are mean ± S.E.M. **Significant difference vs. baseline when collapsed across supplementation ($P < 0.01$).

Figure 4. Western blot analysis of RND3 and FOS in skeletal muscle after eccentric exercise. BL = baseline, 3H = 3 hours post exercise, 48H = 48 hours post exercise. Graph A – RND3 (N = 18). Graph B – FOS (N = 14). Values are

mean ± S.E.M. *Significant difference vs. baseline when collapsed across supplementation ($P < 0.05$).

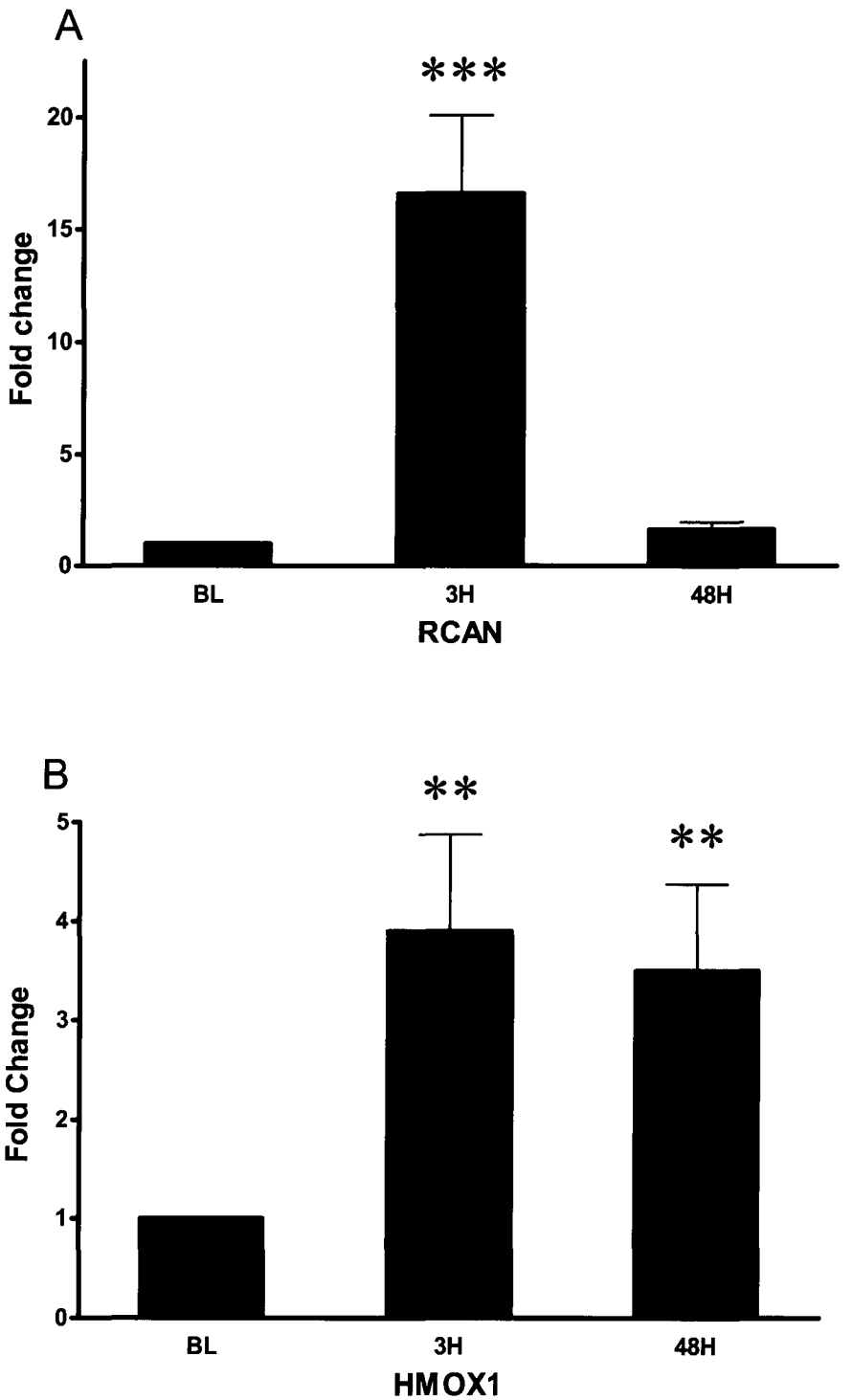
Figure 5. Schematic representation of the transcriptionally active pathways following exercise induced muscle damage. Eccentric exercise promoted greater expression of targets within the STARS/RhoA/AP1 and NFAT/AP1 signaling pathways for hypertrophy and actin biogenesis and organization.

Figure 6. Regulatory and downstream targets of STARS transcriptionally active following a single bout of eccentric exercise. RCAN – regulator of calcineurin; HMOX1 – hemeoxygenase 1; ARHGEF7 and ARHGEF12 – Rho guanine nucleotide exchange factor 7 and 12; ARHGAP24 – Rho GTPase activating protein 24; RND3 – Rho family GTPase 3; DIAPH1 – diaphanous homologue 1; CORO1C – Coronin, actin binding protein 1; FLNB – Filamin B, beta; CAPZA1 – capping protein (actin filament) muscle Z-line alpha 1; ACTA2 – actin, alpha 2, smooth muscle, aorta; ACTN1 – actinin, alpha 1; AP1 – activator protein 1; FOS – FBJ murine osteosarcoma viral oncogene homologue; FOSB – FBJ murine osteosarcoma viral oncogene homologue B; JUND – Jun D proto-oncogene.

Figure 7. Regulatory and downstream targets of NFAT transcriptionally active following a single bout of eccentric exercise. p38MAPK – p38 mitogen

activated protein kinase; GSK-3β – glycogen synthase kinase 3 beta; MAF – v-maf musculoaponeurotic fibrosarcoma oncogene homologue (avian).

Figure 1.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HMOX1

Figure 2.

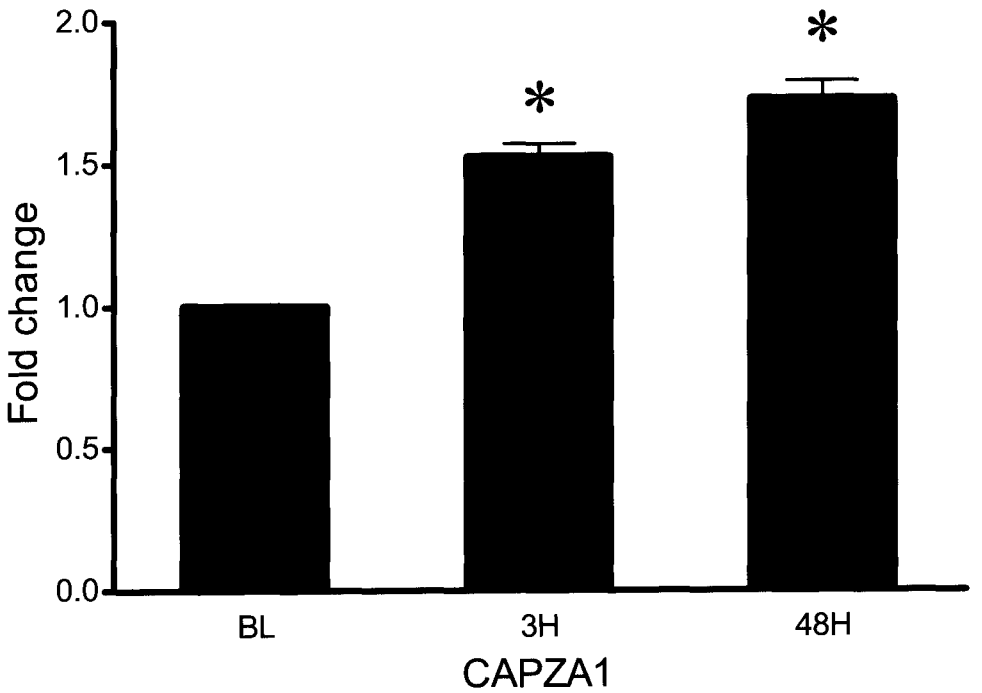

Figure 3.

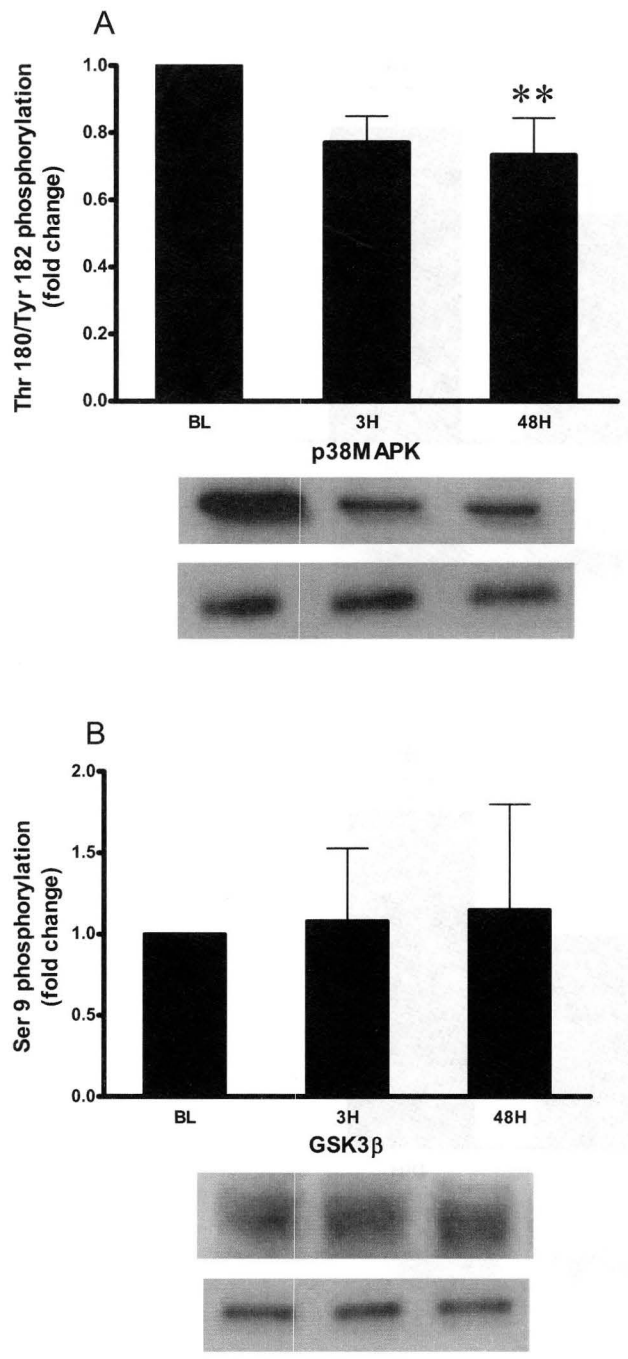
A
Thr 180/Tyr 182 phosphorylation (fold change)
1.0
0.8
0.6
0.4
0.2
0.0
**
BL
3H
48H
p38MAPK
B
Ser 9 phosphorylation (fold change)
2.0
1.5
1.0
0.5
0.0
BL
3H
48H
GSK3β

Figure 4.

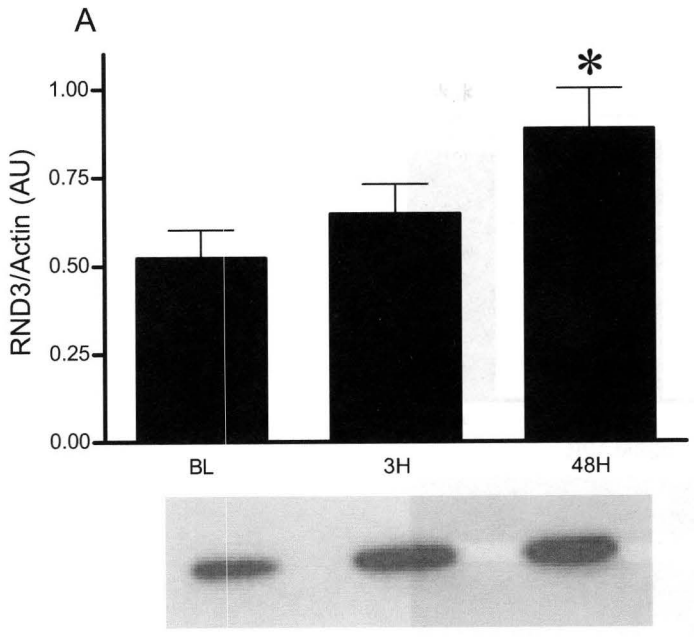
A
RND3/Actin (AU)
1.00
0.75
0.50
0.25
0.00
*
BL
3H
48H
B
FOS/Actin (AU)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
BL
3H
48H

Figure 5.

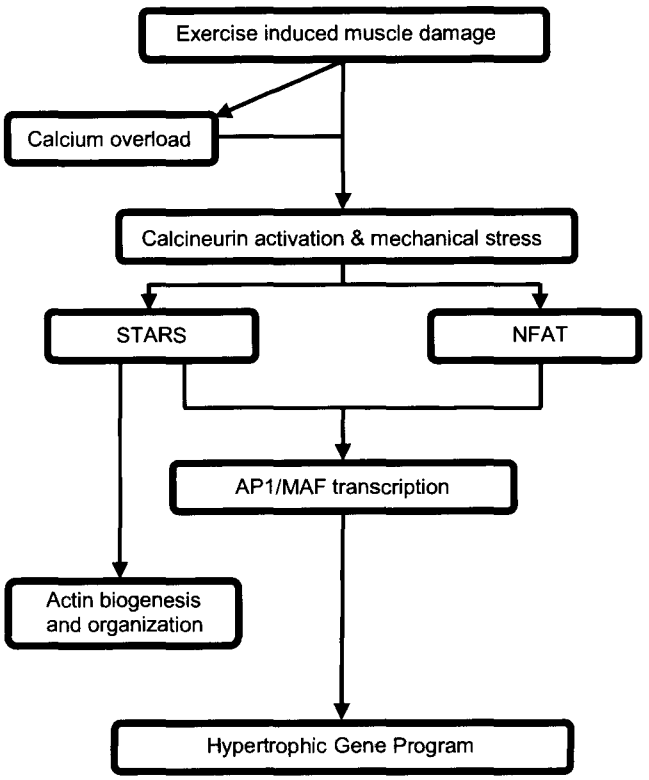
Exercise induced muscle damage
Calcium overload
Calcineurin activation & mechanical stress
STARS
NFAT
AP1/MAF transcription
Actin biogenesis and organization
Hypertrophic Gene Program

Figure 6.

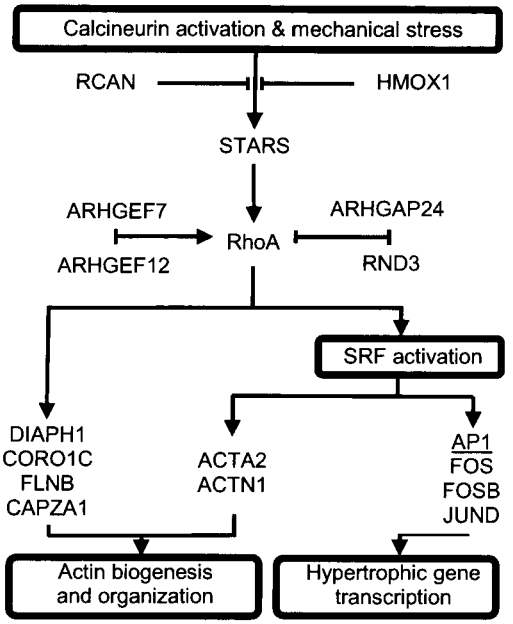
Calcineurin activation & mechanical stress
RCAN
HMOX1
STARS
ARHGEF7
ARHGAP24
RhoA
ARHGEF12
RND3
SRF activation
DIAPH1
CORO1C
FLNB
CAPZA1
ACTA2
ACTN1
AP1
FOS
FOSB
JUND
Actin biogenesis and organization
Hypertrophic gene transcription

Figure 7.

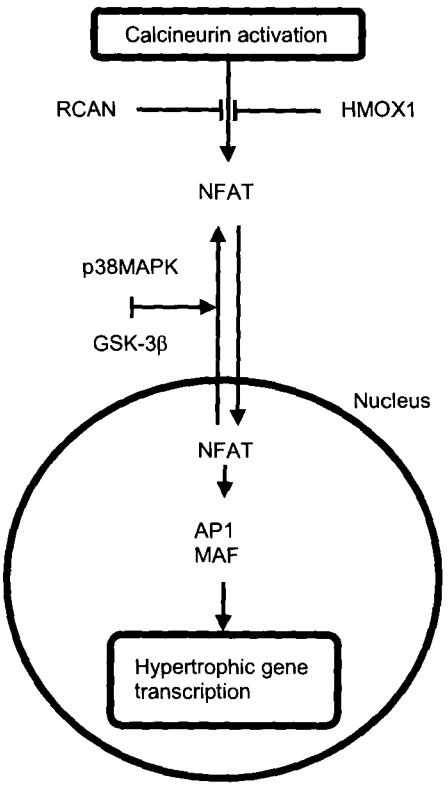
Calcineurin activation
RCAN
HMOX1
NFAT
p38MAPK
GSK-3β
Nucleus
NFAT
AP1
MAF
Hypertrophic gene transcription

Table 1. Primer and probe sequences for calcineurin regulation, actin dynamics and housekeeping genes.

Gene	Left Primer	Right Primer	Probe
RCAN1	gacaaggacatcacctttcagt	tcatttcctttcccagaaactc	caaacgagtcagaataacttcagcaaccc
HMOX1	tctccgatgggtccttacac	cctgcattcacatggcataa	ctaagccaactgtcgccaccagaaa
CAPZA1	ccttccaagcacttctggtact	gagggagaaggatgaatgtgt	atccaccaacacctaaagaggctatgc
β2M	ggctatccagcgtactccaa	gatgaaacccagacacatagca	tcaggtttactcaacgtcatccagcagag

Table 2. Subject and eccentric exercise trial characteristics.

	CON	EXP
No. of subjects	9	9
Age, yr	21.1 ± 0.8	20.9 ± 0.9
Height, cm	181.6 ± 2.0	180.9 ± 1.4
Weight, kg	73.4 ± 3.8	80.4 ± 4.6
Body fat, %	14.7 ± 1.7	20.1 ± 2.4
Quadriceps CSA, cm^2	74.4 ± 3.3	78.5 ± 4.1
Work, kJ	24.9 ± 2.9	25.1 ± 1.3

Values are means ± SEM

Table 3. Serum hormone concentrations after 8d of supplementation with either placebo (CON) or E2 (EXP).

Estradiol (pg/ml)	BL	PS
CON	36.4 ± 2.5	38.4 ± 2.8
EXP	38.8 ± 4.0	95.4 ± 11.7***
Testosterone (nmol/L)	BL	PS
CON		14.6 ± 2.0
EXP		10.8 ± 2.1**

Values are mean ± SEM **P < 0.01. ***P < 0.001. N = 9/group.

Table 4. Fold change of gene expression after 3 hours of recovery from eccentric exercise using DNA microarray analysis (n = 18).

Categories and Gene Names	Accession Number	Fold change at 3H	Potential relevant function
Calcineurin regulation			
regulator of calcineurin 1 (RCAN1), transcript variant 3	NM_203418.1	5.6	Calcinuerin regulation
regulator of calcineurin 1 (RCAN1), transcript variant 2	NM_203417.1	3.9	Calcinuerin regulation
NFAT			
nuclear factor of activated T-cells, (NFATC1), transcript variant 1	NM_172390.1	1.3	calcineurin transcription/regulation
glycogen synthase kinase 3 beta (GSK3B)	NM_002093.2	1.3	calcineurin transcription/regulation
MAPK regulation			
Activation			
mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), transcript variant 2	NM_032960.2	1.2	MAPKKK cascade
adrenergic, beta-2-, receptor, surface (ADRB2)	NM_000024.3	2.5	activation of MAPK activity
mitogen-activated protein kinase 6 (MAPK6)	NM_002748.2	1.3	MAP kinase activity
mitogen-activated protein kinase kinase kinase 6 (MAP3K6)	NM_004672.3	1.4	protein serine/threonine kinase activity
mitogen-activated protein kinase kinase kinase 8 (MAP3K8)	NM_005204.2	11.1	protein serine/threonine kinase activity
Inactivation			
protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform (PPP2CA)	NM_002715.2	1.5	inactivation of MAPK activity
dual specificity phosphatase 16 (DUSP16)	NM_030640.1	1.8	inactivation of MAPK activity
dual specificity phosphatase 8 (DUSP8)	NM_004420.1	1.4	inactivation of MAPK activity
RHOA			
striated muscle activator of Rho-dependant signaling (STARS)	NM_139166.2	10.1	positive regulation of Rho protein signal transduction
Rho family GTPase 3 (RND3)	NM_005168.3	5.4	small GTPase mediated signal transduction
Rho guanine nucleotide exchange factor (GEF) 7 (ARHGEF7), transcript variant 2	NM_145735.1	1.3	RHOA positive regulation
Rho guanine nucleotide exchange factor (GEF) 12 (ARHGEF12)	NM_015313.1	1.2	RHOA positive regulation
Rho GTPase activating protein 24 (ARHGAP24)	NM_031305.1	0.8	RHOA negative regulation
Actin cytoskeleton and transcription factor activity			
actinin, alpha 1 (ACTN1)	NM_001102.2	1.4	actin cytoskeleton
diaphanous homolog 1 (Drosophila) (DIAPH1)	NM_005219.2	1.9	actin cytoskeleton organization and biogenesis
coronin, actin binding protein, 1C (CORO1C)	NM_014325.2	1.5	actin binding/cytoskeleton
filamin B, beta (actin binding protein 278) (FLNB)	NM_001457.1	1.5	actin binding/cytoskeleton
actin, alpha 2, smooth muscle, aorta (ACTA2)	NM_001613.1	1.7	actin filament
jun D proto-oncogene (JUND)	NM_005354.2	1.9	activated transcription factor component
v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS)	NM_005252.2	14.8	activated transcription factor component
FBJ murine osteosarcoma viral oncogene homolog B (FOSB)	NM_006732.1	3.7	activated transcription factor component
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian) (MAFF), transcript variant 1	NM_012323.2	2.6	regulation of transcription

Other regulators of muscle growth and remodelling			
activating transcription factor 3 (ATF3), transcript variant 3	NM_001030287.1	28.4	transcription factor activity
Xin actin-binding repeat containing 1 (XIRP1)	NM_194293.2	13.6	actin cytoskeleton organization
v-myc myelocytomatosis viral oncogene homolog (avian) (MYC)	NM_002467.3	11.0	transcription factor activity
heparin-binding EGF-like growth factor (HBEGF)	NM_001945.1	7.0	growth factor activity
DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4)	NM_007034.3	6.1	heat shock protein binding

CHAPTER 4: GENERAL DISCUSSION

4.0 Introduction

The sex hormone 17β-estradiol (E2) may influence several indices of exercise-induced muscle damage, inflammation and repair. Although the majority of support for the attenuation of muscle damage and inflammation has occurred in mice, some research has identified similar attenuations when women are compared to men. The study outlined in this thesis was designed to identify what effect elevated E2 serum concentration would have on anti-oxidative capacity, membrane stability and gene expression in skeletal muscle following a single bout of eccentric exercise in humans. Our investigation suggests that a physiologically relevant change in circulating E2 does not improve anti-oxidant status or membrane fluidity but attenuates the inflammatory response, possibly through altered eNOS activity. High density oligonucleotide based microarray analysis has also identified novel regulation of two hypertrophic pathways involved in the repair and recovery of exercise-induced muscle damage in skeletal muscle, contributing to the understanding of gene expression following resistance exercise.

4.1 Within normal physiological concentrations, E2 did not improve anti-oxidant status or membrane stability, but attenuated the inflammatory response.

Based on the primary hypothesis, a major contribution from this thesis was that increased circulating E2 did not alter anti-oxidant capacity, appearance of the muscle protein creatine kinase in serum or mRNA content of genes related to oxidative stress defense and membrane homeostasis. These findings were surprising given the proposed anti-oxidant and membrane stabilizing properties of E2, that were suggested in earlier studies to attenuate post-exercise muscle damage and inflammation (1, 2). This may be due to wide range in concentrations used in different research models. In vivo, normal serum concentrations of E2 in women range from ~150–750 pM (3). Our supplementation protocol successfully increased E2 while remaining within normal physiological values (144.6 ± 10.3 → 350.1 ± 42.9 pM). However, the concentrations used for inhibition of lipid peroxidation and improved membrane fluidity are 10–20 μM (4) and 4–40 μM (5, 6), respectively.

Similar to previous studies, E2 attenuated the increase in neutrophil density typically found in skeletal muscle following eccentric exercise (7-10). Without improved anti-oxidant capacity or membrane stability, the chemical attraction of circulating neutrophils must have been reduced by an alternative mechanism. E2 also affects the phosphorylation status of endothelial nitric oxide synthase (eNOS) downstream of ERK and Akt activity, completely attenuating neutrophil infiltration in mice (11, 12). However, activation (phosphorylation state) of both ERK and Akt both return to baseline by 15 minutes following

vascular injury, much sooner than our earliest post-exercise biopsy making it impossible to prove or refute this possibility (11, 13, 14).

E2 did not affect the level of skeletal muscle macrophage infiltration. The 2-3-fold increase in cells positively stained for macrophages is similar to what has been found previously found previously (15-18). Although neutrophils release chemokines for the recruitment of macrophages, they are not the only cells with this capacity. Microarray analysis indicates that myogenic precursor cells release the chemotactic factors MP-derived chemokine, monocyte chemoattractant protein-1, fractalkine and VEGF (19). The myogenic precursor cells likely us these macrophages to stimulate proliferation as well as rescue damaged cells from apoptosis (19, 20).

4.2 E2 did not affect mRNA expression

A second critical finding from this thesis is that E2 did not alter the myofibre transcriptome. Muscle biopsies taken after 8 days of supplementation were analyzed using targeted RT-PCR and microarray analysis and indicated that E2 did not affect mRNA expression. Estrogens can influence growth, differentiation and function of muscle tissue and recent studies have identified different expression of genes involved in metabolism and growth regulation when comparing men and women (21-23). Trifunctional protein β and lipoprotein

lipase, genes related to fat metabolism, are more abundant in women and may be involved in the physiological differences that promote greater oxidation of fat during exercise (21). Additionally, two negative regulators of muscle growth have higher mRNA content in women than men. Growth factor receptor-bound 10 interferes with hypertrophic signaling by insulin-like growth factor 1 (IGF-1) and activin A receptor IIB promotes myostatin signaling for attenuated muscle growth (22). Taken together, these results suggest that differential gene expression between men and women may result from factors beyond E2 alone, including variations in body composition, X-chromosome genes, and/or other sex hormones (i.e., progesterone).

4.3 Microarray analysis identified that a single bout of eccentric exercise alters early mRNA expression in skeletal muscle.

The use of microarray analysis has provided a list of 611 differentially expressed genes, all occurring 3 h after exercise (Appendix 1). Since E2 supplementation did not differentially affect the expression of any genes, the data across each timepoint was collapsed, resulting in the largest number of subjects (N = 18) ever used for eccentric exercise transcriptome analysis. Of the 611 affected genes, 310 were up-regulated and 301 were down-regulated. As we have shown previously (24), all genes returned to baseline values by 48 h post-exercise.

Analysis of the microarray dataset revealed that a regulator of actin dynamics was one of the top 10 up-regulated genes. Striated activator of Rho signaling (STARS) is a relatively novel protein, recently identified for its increased expression following resistance exercise training in skeletal muscle (25). Activated by mechanical stress and the calcium dependant phosphatase calcineurin, STARS regulates actin dynamics and gene transcription through the

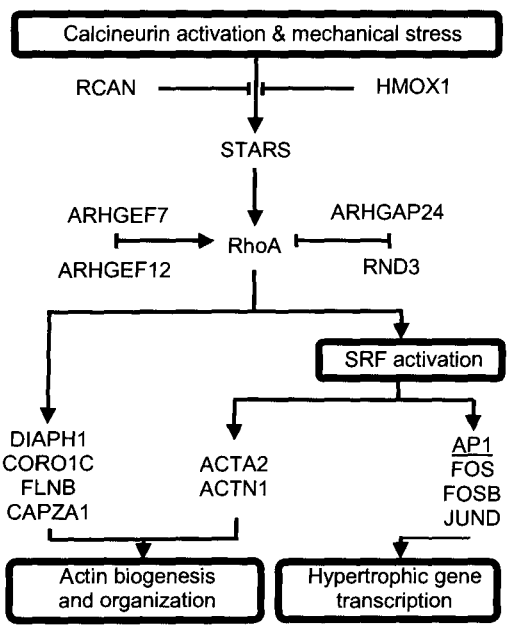

Figure 1. Regulators and downstream targets of the actin binding protein striated muscle activator of Rho signaling (STARS) identified by microarray following a single bout of eccentric exercise.

small G-protein RhoA (25-30). Further investigation of the dataset also identified four regulators of RhoA signaling (RND3, ARHGEF7, ARHGEF12 and

ARHGAP24) and nine downstream targets of RhoA and STARS (DIAPH1, CORO1C, FLNB, CAPZA1, ACTA2, ACTN1, FOS, FOSB and JUND). As a result, a pathway between the structural and chemical stresses following eccentric exercise and the transcriptional active genes involved in muscle cell repair and remodelling can be mapped (Fig. 1).

Calcineurin also activates the transcription factor NFAT, resulting in its migration to the nucleus and increased transcriptional activity (31-34). Further investigation of the microarray dataset revealed that not only was NFATc1 induced, but two negative regulators of NFAT signaling (p38MAPK and GSK-3β) were also affected (Fig. 2). Eight genes that encode proteins involved in the regulation of p38MAPK were altered, along with the induction of GSK-3β. Western blotting was done to determine if the phosphorylation of either of these proteins was changed following exercise. While GSK-3β remained unchanged, the activity of p38MAPK was significantly lower by 48H, possibly allowing longer nuclear residence by NFAT and greater transcriptional activity.

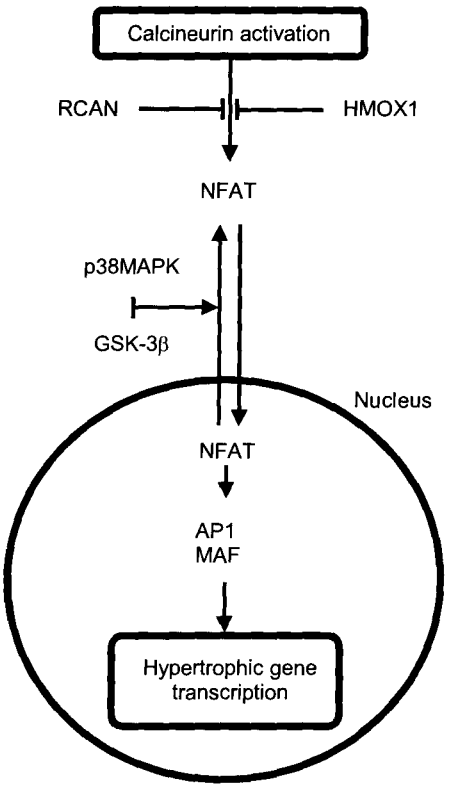

Figure 2. Regulation and downstream targets of nuclear factor of activated T-cells (NFAT) identified by microarray following a single bout of exercise.

Together the transcriptional activation of the signaling pathways STARS/RhoA/AP1 and NFAT/AP1 indicates that both are very important for early hypertrophic signaling for growth and remodelling after a single bout of unaccustomed eccentric exercise (Fig. 3).

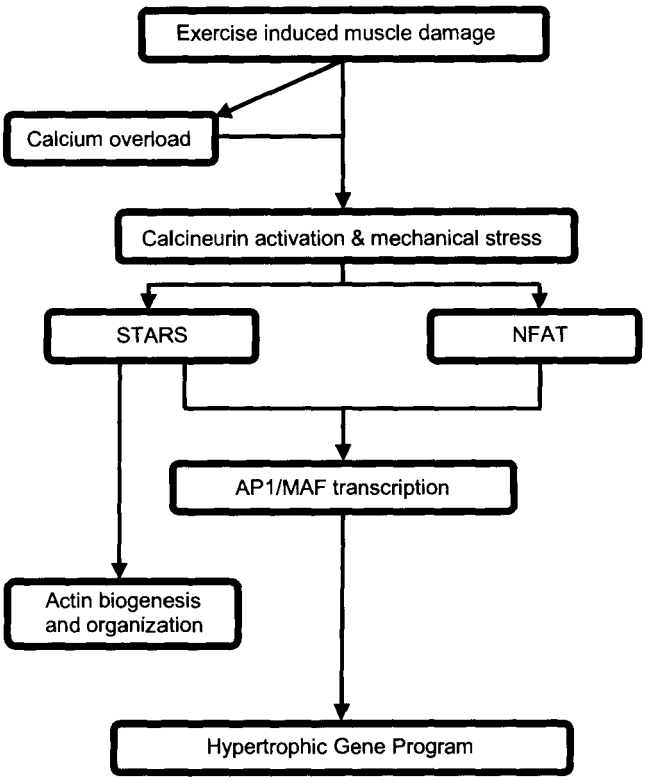

Figure 3. Schematic representation of the transcriptionally active pathways following exercise induced muscle damage. Eccentric exercise promoted greater expression of targets within the STARS/RhoA/AP1 and NFAT/AP1 signaling pathways for hypertrophy and actin biogenesis and organization.

4.4 Gene ontology analysis identifies additional significant biological functions and transcriptionally active pathways following a single bout of eccentric exercise in skeletal muscle.

Using gene ontology, genes can be clustered into canonical or common pathways based on their cellular functions and biological processes. Ingenuity Pathway Analysis (IPA) software modeled the interactions occurring following

exercise, reporting the genes with the greatest change in expression and the important biological functions and signaling pathways. The top canonical pathways related to gene expression and cellular growth were integrin-linked kinase (ILK), PI3K/Akt, Ran and Nrf2-mediated oxidative stress response, identified by the number of molecules differentially expressed included in these pathways as well as the probability of the association of these pathways to the dataset.

Two of these pathways, ILK ($P = 0.005$, 16/186 molecules) and PI3K/Akt ($P = 0.006$, 12/136 molecules), positively influence the cellular growth and repair of exercise-induced damage. The focal adhesion protein ILK functions to remodel the actin cytoskeleton for maintenance of mechanical stability in skeletal muscle (35). Loss of this protein in mice reduces cell contractility and alters cell structure, resulting in a muscular dystrophy phenotype (35). PI3K/AKT signaling is also important for muscle hypertrophy, promoting protein synthesis and inhibiting protein degradation (36). Activated by mechanical stimulation and feeding, this pathway acts through mammalian target of rapamycin (mTOR) to phosphorylate downstream members of the protein synthesis (36, 37).

Transforming growth factor β (TGFβ) is a modulator of proliferation and apoptotic signalling, suppressing cellular growth (38). This cytokine uses the Smad cytoplasmic effector proteins for transport across the nuclear envelope,

negatively regulating the expression of a variety of target genes for actin cytoskeleton remodelling and muscle hypertrophy (39, 40). The small GTPase Ran ($P = 0.005$, 4/23 molecules) regulates the nucleocytoplasmic transport of these transcription factors (39). TGFβ has recently been identified as an enhancer of the transcription factor NF-E2 related factor 2 (Nrf2) ($P = 0.006$, 16/185 molecules). Elevated oxidative stress dissociates Nrf2 from its inhibitor, Keap1, allowing it to migrate to the nucleus and bind to antioxidant response elements (ARE), inducing the expression of genes that enhance antioxidant capacity (41).

4.5 Targeted RT-PCR confirms that genes involved in antioxidant status, growth, membrane remodelling and ER stress are influenced by exercise but not E2.

Based on previous research, four categories were selected *a priori* for RT-PCR analysis of mRNA expression: antioxidant defense, membrane homeostasis, endoplasmic reticulum (ER) stress and growth. Within each category, genes were chosen for their response to exercise and their potential to identify differences that may have resulted from the attenuated exercise-induced muscle damage and oxidative stress associated with E2 exposure.

Three protective enzymes were selected as part of skeletal muscle's natural antioxidant defense system, superoxide dismutase 1 (SOD1), SOD2 and

catalase (42). Continuously neutralizing reactive oxygen species (ROS) generated by exercise and inflammation, these enzymes exist throughout the myofibre in concentrations adaptable to the amount of ROS present (42, 43). SOD1 and

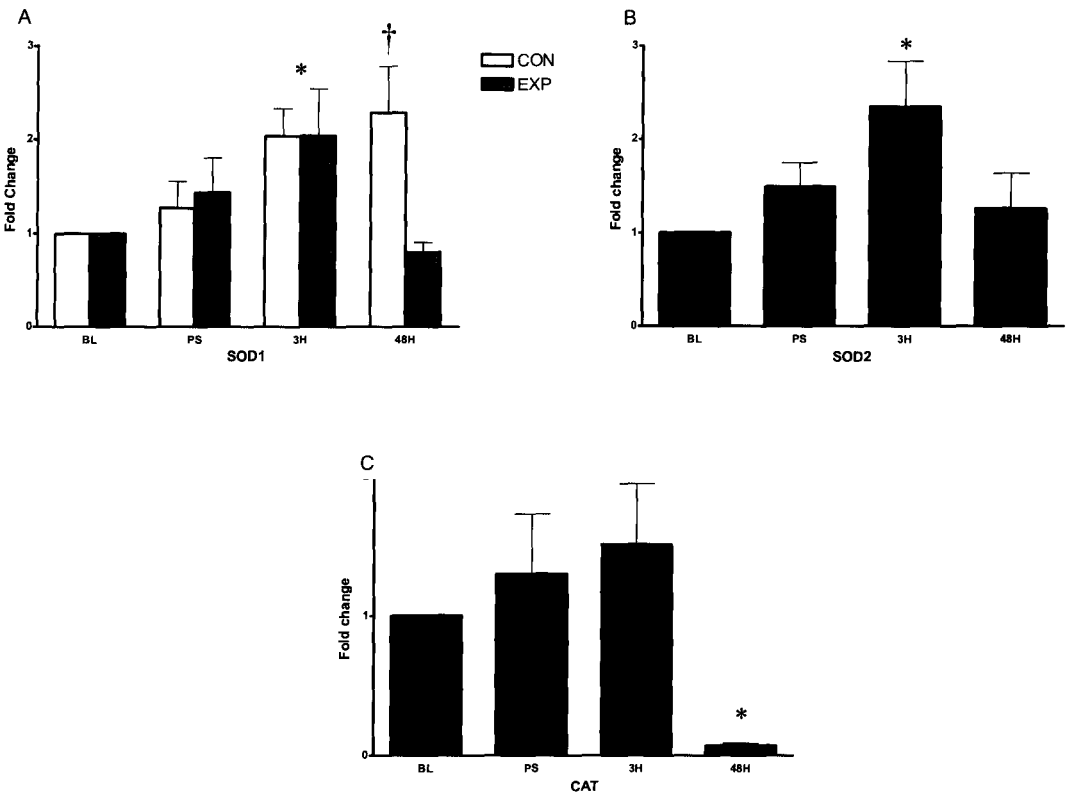

Figure 4. Expression fold changes in mRNA expression of antioxidant defence genes in muscle from baseline after exercise protocol. Graph A – SOD1 (CON = 9; EXP = 9). Graph B – SOD2 (N = 18). Graph C – Catalase (N = 18). PS = post supplementation, 3H = 3 hours post exercise, 48H = 48 hours post exercise. *Significant difference vs. baseline when collapsed across supplementation ($P < 0.05$). †Significant difference between conditions in same timepoint ($P < 0.05$). ***Significant difference vs. baseline when collapsed across supplementation ($P < 0.001$).

SOD2 were both elevated at 3H, similar to the pattern observed in the microarray (Fig. 4). Of the genes measured, SOD1 was the only one to interact with E2,

returning to baseline levels at 48H. Without a difference observed immediately following supplementation or exercise, this may be related to the attenuation of neutrophils measured at the same timepoint and a reduced source of signaling ROS. Catalase was dramatically down-regulated at 48H but was not affected by E2.

Genes controlling cholesterol and lipid homeostasis may become transcriptionally active following exercise-induced damage to assist in the repair of the phospholipid cell membrane (24). Forkhead transcription factor 1 (FOXO1) is an insulin-regulated transcription factor responsible for the regulation of myoblast differentiation and increased glucose and lipid production (44). Caveolin-1 is a lipid binding protein in the plasma membrane necessary for the production of caveola (45), used for cholesterol transport across plasma membrane (46). Sterol regulatory element binding proteins (SREBPs) transcriptionally activate enzymes required for endogenous cholesterol, fatty acids (FA), and triglyceride and phospholipids synthesis (47). Three hours after eccentric exercise each of these genes was significantly induced (Fig. 5), confirming their early expression identified by previous work (24).

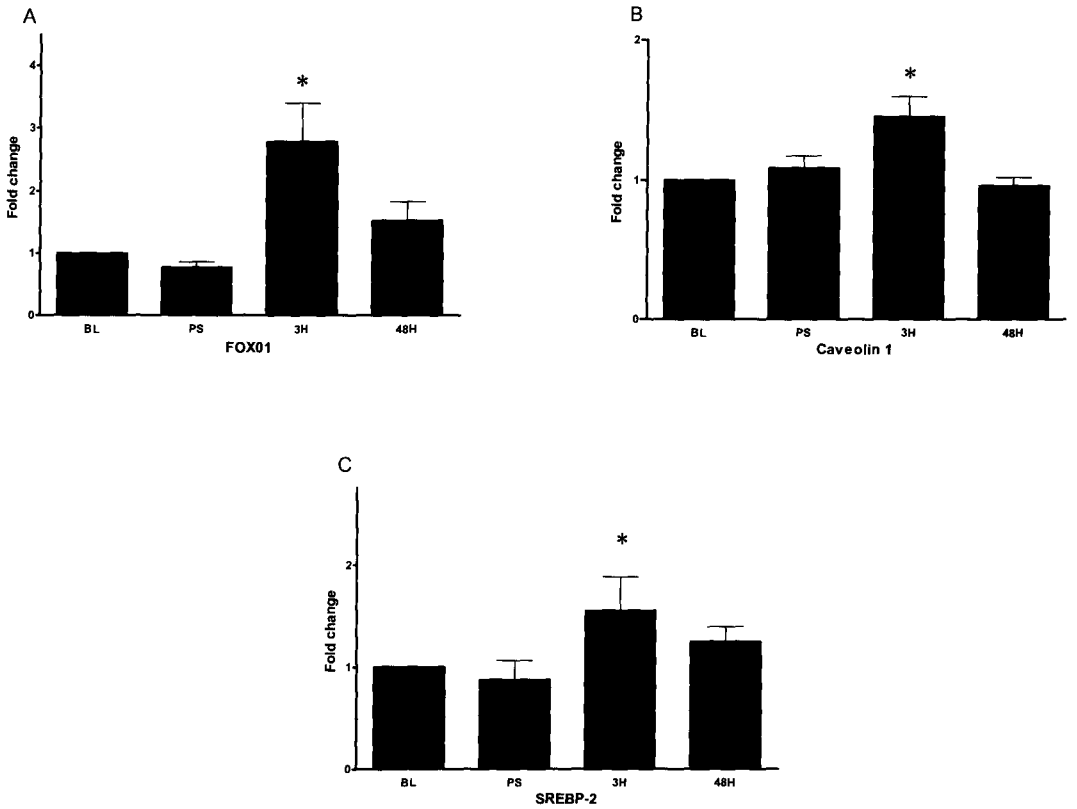

Figure 5. Expression fold changes in mRNA expression of membrane homeostasis genes in muscle from baseline after exercise protocol (N = 18). Graph A – FOX01. Graph B – Caveolin 1. Graph C – SREBP-2. PS = post supplementation, 3H = 3 hours post exercise, 48H = 48 hours post exercise. *Significant difference vs. baseline when collapsed across supplementation ($P < 0.05$).

The endoplasmic reticulum (ER) provides a specialized environment for post-translational modification and folding of proteins (48). Properly folded proteins leave the ER, are secreted and proceed to their end point, while malfolded/unfolded proteins are disposed of by an ER associated protein degradation machine (48). Disequilibrium between load and folding capacity is

known as ER stress. Four species of heat shock proteins (HSP) that aid in protein-protein interaction were selected as markers of ER stress: glucose related protein 78 (GRP78, HSPA5), hemeoxygenase 1 (HMOX1, HSP32), HSP78 and HSP27. All were upregulated at 3H, three of which (GRP78, HMOX1 and HSP78)

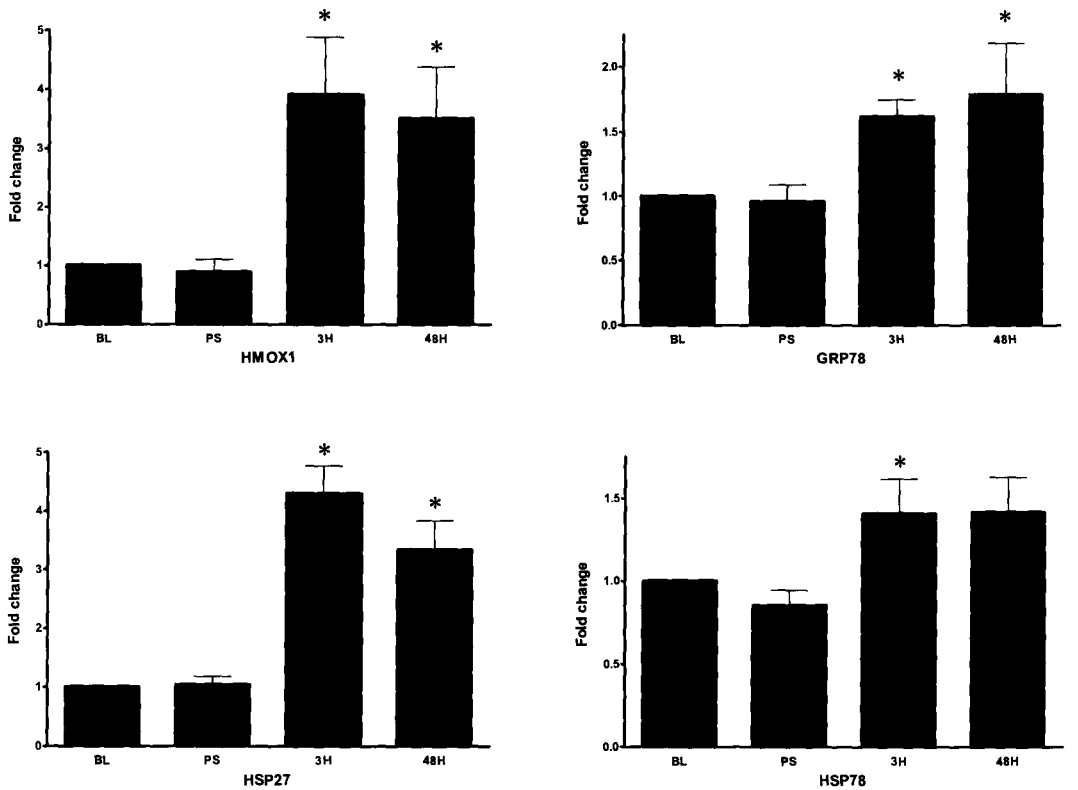

Figure 6. Expression fold changes in mRNA expression of ER stress genes in muscle from baseline after exercise protocol (N = 18). Graph A – HMOX1. Graph B – GRP78. Graph C – HSP27. Graph D – HSP78. PS = post supplementation, 3H = 3 hours post exercise, 48H = 48 hours post exercise. *Significant difference vs. baseline when collapsed across supplementation ($P < 0.05$).

remained higher at 48H (Fig. 6). Only HSP27 has been previously investigated following eccentric exercise, supporting our measurements with 234% greater expression after 48 h (49).

The final category of interest seen in the microarray analysis was the regulation of myocyte growth. Regulator of calcineurin 1 (RCAN1) is a member of the MCIP family of calcineurin inhibitors that is upregulated during muscle differentiation (50, 51). MCIP1 is activated by oxidative and calcium stress (52), and has been shown to activate slow fibre gene expression and mediate fast to slow fibre-type conversion (53, 54). Dystrophia myotonica-protein kinase (DMPK) is predominantly expressed in heart and skeletal muscle however its biological role in skeletal muscle is not well defined (55). It may regulate myogenesis as myoblast cultures from patients with MDI show delayed differentiation, possible due to the lack of DMPK (56). Capping protein (muscle Z-line) α1 (CAPZA1) is crucial for myofibrillogenesis anchoring the thin actin filament to the Z-disc by direct interaction with α-actinin (57). Alteration in the mRNA expression of these genes may coincide with sarcomere damage, as they are involved in repair, assembly and differentiation of muscle fibres. Our findings are similar to previous work (24) showing elevations in the mRNA abundance for RCAN at 3H, DMPK at 48H and CAPZA1 at 48H (Fig. 7).

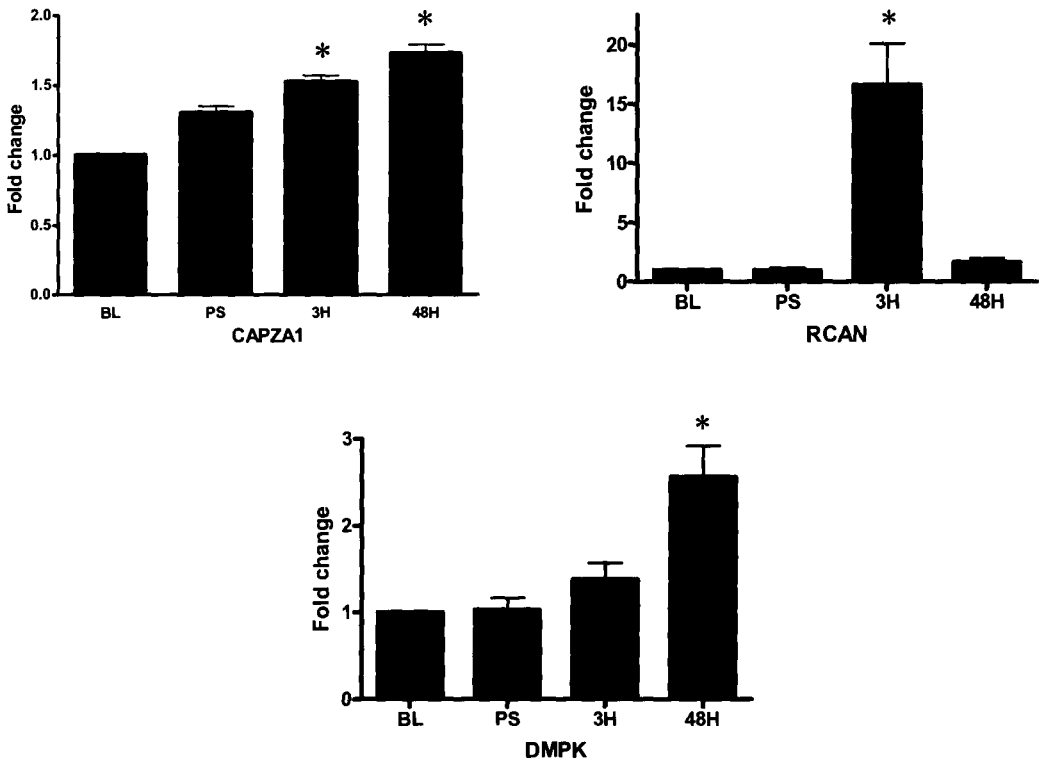

Figure 7. Expression fold changes in mRNA expression of growth genes in muscle from baseline after exercise protocol (N = 18). Graph A – CAPZA1. Graph B – RCAN. Graph C – DMPK. PS = post supplementation, 3H = 3 hours post exercise, 48H = 48 hours post exercise. *Significant difference vs. baseline when collapsed across supplementation ($P < 0.05$).

4.6 Intense eccentric exercise did not increase signaling through key remodelling pathways.

As mentioned in this and previous chapters, key signaling pathways regulating muscle plasticity for adaptive remodelling following exercise include Akt, GSK3β and the family of MAPK proteins (58, 59). Activation of the

Akt/PKB pathway phosphorylates mammalian target of rapamycin (mTOR), a key regulator of protein synthesis (60). Extra-cellular signal-regulated kinase 1/2 (ERK1/2) is a member of the MAPK family of signaling proteins that phosphorylate transcription factors for muscle remodelling (60). Although resistance exercise commonly increases signaling through these pathways we did not detect a change in the phosphorylation status of Akt, mTOR, GSK3β and

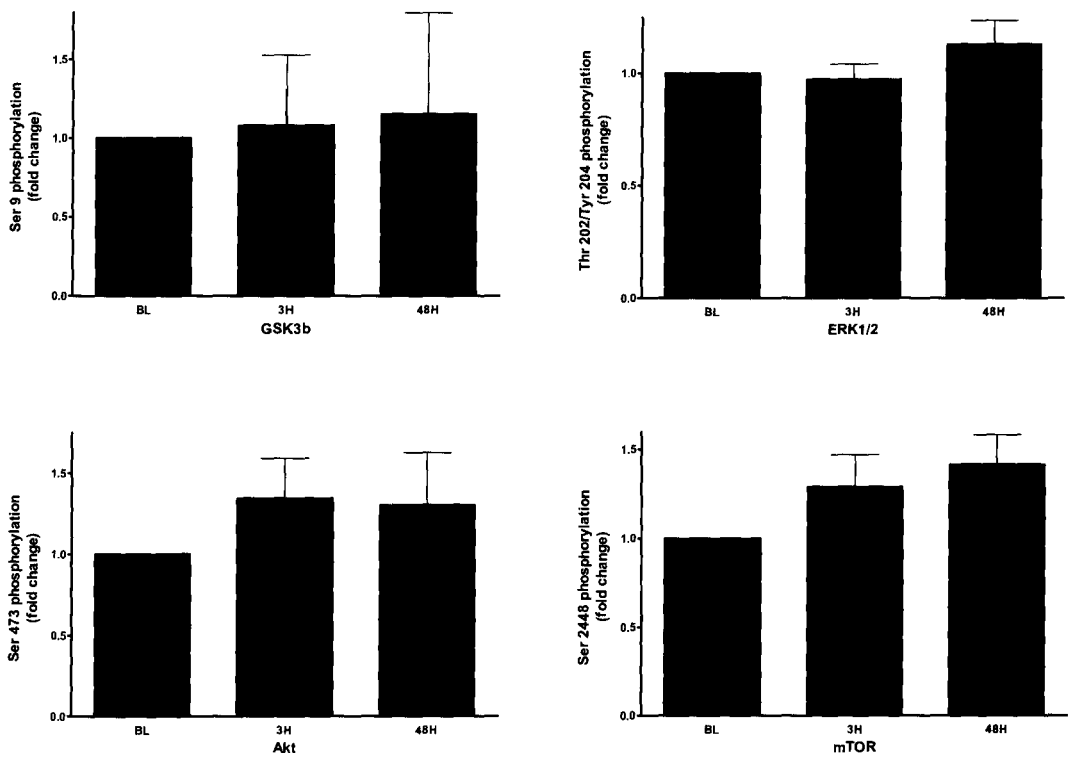

Figure 8. Fold change of phosphorylated/total ratio of signaling pathways from baseline after eccentric exercise (N = 18). BL = baseline, 3H = 3 hours post exercise, 48H = 48 hours post exercise. Graph A – GSK-3β (Ser^{9}). Graph B – ERK1/2 (Thr^{202}/Tyr^{204}). Graph C – Akt/PKB (Ser^{473}). Graph D – mTOR (Ser^{2448}). Values are mean ± S.E.M.

ERK1/2 (Fig. 8), likely a result of the timing of the biopsies. Akt and mTOR are highest 10 minutes following exercise and had likely returned to baseline levels (61) Additionally, the high levels of damage and proteolysis associated with intense eccentric exercise may result in a lack of change of delayed response (60, 62).

4.6 Conclusions and future directions.

We have investigated the effects of the sex hormone E2 on markers of exercise-induced muscle damage and found that the initial influence may not be to affect antioxidant capacity or membrane stability but to influence infiltration of inflammatory neutrophils. Increased circulating E2 did not improve the total antioxidant capacity, attenuate CK efflux or affect gene transcription in men. In addition, our analysis of the early global gene changes following an acute bout of eccentric exercise has identified novel transcriptional activation in two signaling pathways, STARS/RhoA/AP1 and NFAT, adding to our understanding of the repair and recovery mechanisms.

The research presented in this thesis has several limitations. We selected a ten day E2 supplementation protocol to replicate a previous study that did not report significant side effects (63). Although similar to the length of time used in murine research, the lack of effect we observed may be a result of lower E2

exposure. CK activity as a measure of membrane damage is highly variable, a property evident in our measurements taken after 48 h. Even though blood CK levels have routinely been reported as different with E2, a more direct marker of membrane damage may provide different results. Our choice to measure the early signaling mechanisms 3 h after exercise is later than the peak phosphorylation changes for molecules such as eNOS, Akt and p38MAPK. Increasing the number of biopsies taken immediately following the cessation of exercise would determine if our observations were a result of transient signalling by these molecules. Finally, we selected a highly strenuous eccentric exercise bout as our stimulus of muscle damage. The protective effects of E2 may have been unable to compensate for the large amount of damage incurred and may have been more evident with a different exercise model.

In conclusion, there are several experiments needed to address the limitations of the work presented in this thesis and continue to investigate the proposed hypotheses. First, an experiment measuring the effect of altered E2 exposure over a longer period of time is needed to clearly define if it is solely responsible for differences between men and women. This would likely involve a comparison between two groups of women of similar age, body composition and activity levels, one with normal E2 exposure and another with reduced E2 exposure. The reduced E2 exposure group could include women that are post-menopausal, participating in anti-estrogen therapy (i.e., Tamoxifen for breast

cancer) or athletes experiencing exercise-associated amenorrhea. Using only women and limiting differences in age, fat and muscle mass and levels of physical activity, the confounding factors associated with comparing men and women would be lessened.

Second, a greater number of muscle biopsies over the first several minutes following exercise would confirm if active signaling mechanisms were missed with our protocol. Several signaling molecules that attenuate neutrophil infiltration after E2 exposure and regulate hypertrophic gene transcription have transient peak activities and return to baseline levels shortly after exercise. To do this the number of biopsies would have to be increased to include not only a greater number of collections in the hours immediately following exercise, but possibly points during exercise between sets.

Third, further information about the transcriptionally active pathways identified by microarray and RT-PCR is needed. This could be achieved using cell culture or animal models to identify: (1) specific upstream signals induced by mechanical disruption for STARS activation; (2) if the downstream signals for actin biogenesis and remodelling are directly promoted by RhoA or are they also regulated by SRF and AP1; (3) if this pathway is also activated by calcium overload resulting from endurance exercise or is the mechanical damage the more important signal. Together, these experiments would determine the importance of

the STARS/RhoA/AP1 signaling pathway and the dominant mechanisms of activation and action.

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APPENDIX

Gene name	Definition	Fold change	Accession number
AADAT	aminoadipate aminotransferase, transcript variant 2	0.8	NM_182662.1
AASDH	2-aminoadipic 6-semialdehyde dehydrogenase	0.8	NM_181806.2
ABC1	amplified in breast cancer 1	0.8	NM_022070.3
ABCA12	ATP-binding cassette, sub-family A (ABC1), member 12, transcript variant 2	1.2	NM_015657.3
ABCC5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5, transcript variant 2	1.4	NM_001023587.1
ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	1.3	NM_002940.1
ABHD10	abhydrolase domain containing 10	0.8	NM_018394.1
ABHD6	abhydrolase domain containing 6	0.8	NM_020676.4
ABLIM3	actin binding LIM protein family, member 3	0.8	NM_014945.1
ABRA	actin-binding Rho activating protein	10.1	NM_139166.2
ACSL3	acyl-CoA synthetase long-chain family member 3, transcript variant 1	1.2	NM_004457.3
ACTA2	actin, alpha 2, smooth muscle, aorta	1.7	NM_001613.1
ACTN1	actinin, alpha 1	1.4	NM_001102.2
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.0	NM_006988.3
ADFP	adipose differentiation-related protein	1.4	NM_001122.2
ADRB2	adrenergic, beta-2-, receptor, surface	2.5	NM_000024.3
AF5L	TAF5-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65kDa, transcript variant 2	1.4	NM_001025247.1
AFF3	AF4/FMR2 family, member 3, transcript variant 1	0.8	NM_002285.2
AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	1.2	NM_020132.3
AKAP10	A kinase (PRKA) anchor protein 10, nuclear gene encoding mitochondrial protein	0.8	NM_007202.2
ALKBH4	alkB, alkylation repair homolog 4 (E. coli)	0.9	NM_017621.2
ALS2CR13	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 13	0.6	NM_173511.2
AMD1	adenosylmethionine decarboxylase 1, transcript variant 2	2.0	NM_001033059.1
AMD1	adenosylmethionine decarboxylase 1, transcript variant 1	1.7	NM_001634.4
ANKFY1	ankyrin repeat and FYVE domain containing 1, transcript variant 1	0.7	NM_016376.2
ANKRD37	ankyrin repeat domain 37	4.5	NM_181726.1
ANKRD40	ankyrin repeat domain 40	1.2	NM_052855.2
ANKRD49	ankyrin repeat domain 49	0.8	NM_017704.2
ANXA2	annexin A2, transcript variant 2	1.8	NM_001002857.1
APAF1	apoptotic peptidase activating factor, transcript variant 5	0.8	NM_181869.1
ARHGAP24	Rho GTPase activating protein 24	0.8	NM_031305.1
ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12	1.2	NM_015313.1
ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7, transcript variant 2	1.3	NM_145735.1
ARID5B	AT rich interactive domain 5B (MRF1-like)	3.8	NM_032199.1
ARL3	ADP-ribosylation factor-like 3	0.8	NM_004311.2
ARMC7	armadillo repeat containing 7	0.9	NM_024585.2
ARRDC2	arrestin domain containing 2, transcript variant 1	0.5	NM_015683.1
ASB5	ankyrin repeat and SOCS box-containing 5	3.2	NM_080874.2
ATF3	activating transcription factor 3, transcript variant 3	28.4	NM_001030287.1
ATM	ataxia telangiectasia mutated (includes complementation groups A, C and D), transcript variant 1	0.8	NM_000051.3
ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide, transcript variant 1	1.7	NM_000701.6

ATP6V1A	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	0.8	NM_001690.2
AUTS2	autism susceptibility candidate 2	2.0	NM_015570.1
AVIL	advillin	0.6	NM_006576.2
AXUD1	AXIN1 up-regulated 1	5.0	NM_033027.2
AZIN1	antizyme inhibitor 1, transcript variant 1	1.5	NM_015878.4
B3GNT5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	2.1	NM_032047.3
BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1, transcript variant 3	1.1	NM_001011545.1
BAG3	BCL2-associated athanogene 3	2.5	NM_004281.3
BCL2L14	BCL2-like 14 (apoptosis facilitator), transcript variant 4	1.1	NM_138723.1
BMP2K	BMP2 inducible kinase, transcript variant 2	1.3	NM_017593.3
BRD8	bromodomain containing 8, transcript variant 3	0.8	NM_183359.1
BTG2	BTG family, member 2	5.8	NM_006763.2
BTN3A3	butyrophilin, subfamily 3, member A3, transcript variant 1	0.8	NM_006994.3
C10orf57	chromosome 10 open reading frame 57	0.7	NM_025125.2
C10orf65	chromosome 10 open reading frame 65	1.9	NM_138413.2
C10orf88	chromosome 10 open reading frame 88	0.8	NM_024942.1
C12orf22	chromosome 12 open reading frame 22	1.4	NM_030809.1
C12orf30	chromosome 12 open reading frame 30	1.2	NM_024953.2
C12orf60	chromosome 12 open reading frame 60	0.8	NM_175874.2
C13orf10	chromosome 13 open reading frame 10	0.9	NM_022118.3
C13orf8	chromosome 13 open reading frame 8	0.7	NM_032436.1
C14orf102	chromosome 14 open reading frame 102, transcript variant 1	0.7	NM_017970.2
C14orf147	chromosome 14 open reading frame 147	1.5	NM_138288.2
C14orf28	chromosome 14 open reading frame 28	0.7	NM_001017923.1
C14orf93	chromosome 14 open reading frame 93	0.8	NM_021944.1
C15orf5	chromosome 15 open reading frame 5	1.3	NM_030944.1
C16orf50	chromosome 16 open reading frame 50	1.1	NM_032269.3
C16orf58	chromosome 16 open reading frame 58	0.8	NM_022744.1
C17orf44	chromosome 17 open reading frame 44	0.8	NM_173621.1
C17orf71	chromosome 17 open reading frame 71	0.8	NM_018149.5
C18orf21	chromosome 18 open reading frame 21	0.8	NM_031446.3
C1orf156	chromosome 1 open reading frame 156	0.7	NM_033418.1
C1orf162	chromosome 1 open reading frame 162	0.7	NM_174896.2
C1orf174	chromosome 1 open reading frame 174	0.9	NM_207356.1
C1orf51	chromosome 1 open reading frame 51	0.5	NM_144697.2
C1orf85	chromosome 1 open reading frame 85	0.8	NM_144580.1
C21orf81	chromosome 21 open reading frame 81	0.7	NM_153750.1
C2orf11	chromosome 2 open reading frame 11	0.6	NM_144629.1
C3orf38	chromosome 3 open reading frame 38	0.9	NM_173824.2
C3orf40	chromosome 3 open reading frame 40	1.3	NM_144635.3
C3orf59	chromosome 3 open reading frame 59	0.8	NM_178496.2
C5orf4	chromosome 5 open reading frame 4, transcript variant 1	0.8	NM_016348.1
C6orf155	chromosome 6 open reading frame 155	1.4	NM_024882.1
C6orf166	chromosome 6 open reading frame 166	1.3	NM_018064.2
C6orf75	chromosome 6 open reading frame 75, transcript variant 1	1.2	NM_001031712.1
C8orf76	chromosome 8 open reading frame 76	0.8	NM_032847.1
C9orf61	chromosome 9 open reading frame 61	0.7	NM_004816.2

C9orf72	chromosome 9 open reading frame 72, transcript variant 1	1.3	NM_018325.1
C9orf76	chromosome 9 open reading frame 76	0.7	NM_024945.1
C9orf85	chromosome 9 open reading frame 85, transcript variant 1	0.8	NM_182505.3
CA4	carbonic anhydrase IV	0.7	NM_000717.2
CAPS2	calcyphosine 2	0.8	NM_032606.2
CASZ1	castor homolog 1, zinc finger (Drosophila)	1.7	NM_017766.2
CCDC28A	coiled-coil domain containing 28A	0.7	NM_015439.2
CCDC66	coiled-coil domain containing 66	0.7	NM_001012506.1
CCL2	chemokine (C-C motif) ligand 2	3.6	NM_002982.3
CCRN4L	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	1.8	NM_012118.2
CD34	CD34 antigen, transcript variant 2	0.8	NM_001773.1
CDC37L1	CDC37 cell division cycle 37 homolog (S. cerevisiae)-like 1	1.6	NM_017913.2
CDC42BPB	CDC42 binding protein kinase beta (DMPK-like)	1.1	NM_006035.2
CDCA3	cell division cycle associated 3	0.9	NM_031299.3
CDO1	cysteine dioxygenase, type 1	0.8	NM_001801.2
CDRT15	CDRT15 protein	0.9	NM_001007530.1
CEBPZ	CCAAT/enhancer binding protein zeta	1.1	NM_005760.2
CHD1	chromodomain helicase DNA binding protein 1	1.4	NM_001270.2
CHKB	choline kinase beta, transcript variant 1	0.8	NM_005198.3
CHORDC1	cysteine and histidine-rich domain (CHORD)-containing 1	2.4	NM_012124.1
CIR	CBF1 interacting corepressor, transcript variant 1	0.7	NM_004882.3
CMYA1	cardiomyopathy associated 1	13.6	NM_194293.2
CNNM3	cyclin M3, transcript variant 1	0.8	NM_017623.3
COQ10B	coenzyme Q10 homolog B (yeast)	1.6	NM_025147.2
CORO1C	coronin, actin binding protein, 1C	1.5	NM_014325.2
CPEB4	cytoplasmic polyadenylation element binding protein 4	2.4	NM_030627.1
CPNE1	copine I, transcript variant 3	1.3	NM_003915.2
CPXM	carboxypeptidase X (M14 family)	1.1	NM_019609.3
CRSP3	cofactor required for Sp1 transcriptional activation, subunit 3, 130kDa, transcript variant 1	0.8	NM_004830.2
CRY1	cryptochrome 1 (photolyase-like)	2.5	NM_004075.2
CRYZL1	crystallin, zeta (quinone reductase)-like 1, transcript variant 1	0.8	NM_005111.5
CSNK1A1	casein kinase 1, alpha 1, transcript variant 2	1.3	NM_001892.4
CSNK1D	casein kinase 1, delta, transcript variant 2	1.3	NM_139062.1
CSRP1	cysteine and glycine-rich protein 1	1.4	NM_004078.1
CSTF3	cleavage stimulation factor, 3' pre-RNA, subunit 3, 77kDa, transcript variant 3	1.5	NM_001033506.1
CSTF3	cleavage stimulation factor, 3' pre-RNA, subunit 3, 77kDa, transcript variant 2	1.3	NM_001033505.1
CTAGE5	CTAGE family, member 5, transcript variant 4	0.6	NM_203356.1
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	1.3	NM_001511.1
CXorf38	chromosome X open reading frame 38	0.8	NM_144970.1
CYFIP2	cytoplasmic FMR1 interacting protein 2	1.2	NM_014376.1
CYLD	cylindromatosis (turban tumor syndrome)	1.4	NM_015247.1
CYR61	cysteine-rich, angiogenic inducer, 61	8.0	NM_001554.3
DACH1	dachshund homolog 1 (Drosophila), transcript variant 1	0.6	NM_080759.3
DBP	D site of albumin promoter (albumin D-box) binding protein	0.7	NM_001352.2
DDX21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	1.4	NM_004728.2
DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	1.7	NM_004396.2

DFFB	DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase), transcript variant 2	0.9	NM_001004286.1
DFNA5	deafness, autosomal dominant 5	2.0	NM_004403.1
DGKD	diacylglycerol kinase, delta 130kDa, transcript variant 2	2.7	NM_152879.2
DHRS8	dehydrogenase/reductase (SDR family) member 8	0.7	NM_016245.2
DIAPH1	diaphanous homolog 1 (Drosophila)	1.9	NM_005219.2
DKFZp451A211	DKFZp451A211 protein	10.0	NM_001003399.1
DKFZP564O0523	hypothetical protein DKFZp564O0523	0.8	NM_032120.1
DKFZP586D0919	hepatocellularcarcinoma-associated antigen HCA557a, transcript variant 2	0.8	NM_206914.1
DMRTC1	DMRT-like family C1	1.2	NM_033053.1
DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	3.1	NM_018602.2
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	6.1	NM_007034.3
DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5	1.8	NM_012266.3
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6, transcript variant 2	2.5	NM_005494.2
DNAL4	dynein, axonemal, light polypeptide 4	0.8	NM_005740.2
DOC1	downregulated in ovarian cancer 1, transcript variant 2	1.3	NM_014890.1
DOLPP1	dolichyl pyrophosphate phosphatase 1	0.7	NM_020438.3
DSC2	desmocollin 2, transcript variant Dsc2b	0.8	NM_004949.2
DSCR1	Down syndrome critical region gene 1, transcript variant 3	5.6	NM_203418.1
DSCR1	Down syndrome critical region gene 1, transcript variant 2	3.9	NM_203417.1
DST	dystonin, transcript variant 1	1.4	NM_183380.1
DTNBP1	dystrobrevin binding protein 1, transcript variant 2	0.8	NM_183040.1
DUSP14	dual specificity phosphatase 14	2.3	NM_007026.1
DUSP16	dual specificity phosphatase 16	1.8	NM_030640.1
DUSP8	dual specificity phosphatase 8	1.4	NM_004420.1
DYRK1A	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A, transcript variant 5	1.3	NM_130438.1
DYSFIP1	dysferlin interacting protein 1 (toonin)	3.2	NM_001007533.1
E2F3	E2F transcription factor 3	1.2	NM_001949.2
EBF	early B-cell factor	0.7	NM_024007.2
EDG3	endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	1.3	NM_005226.2
EFNA1	ephrin-A1, transcript variant 1	0.8	NM_004428.2
EGLN1	egl nine homolog 1 (C. elegans)	1.7	NM_022051.1
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	2.7	NM_004836.3
EIF2C3	eukaryotic translation initiation factor 2C, 3, transcript variant 2	1.1	NM_177422.1
EIF4A1	eukaryotic translation initiation factor 4A, isoform 1	1.6	NM_001416.1
EIF4ENIF1	eukaryotic translation initiation factor 4E nuclear import factor 1	0.7	NM_019843.2
ELL2	elongation factor, RNA polymerase II, 2	1.8	NM_012081.3
ERRFI1	ERBB receptor feedback inhibitor 1	4.7	NM_018948.2
ESRRG	estrogen-related receptor gamma, transcript variant 1	1.3	NM_001438.2
ETAA16	ETAA16 protein	0.8	NM_019002.2
ETF1	eukaryotic translation termination factor 1	1.5	NM_004730.1
EVA1	epithelial V-like antigen 1, transcript variant 1	2.3	NM_005797.2
FAM20C	family with sequence similarity 20, member C	1.2	NM_020223.1
FAM46C	family with sequence similarity 46, member C	0.6	NM_017709.2
FBXL14	F-box and leucine-rich repeat protein 14	0.9	NM_152441.1
FBXO3	F-box protein 3, transcript variant 2	1.7	NM_033406.2
FBXO32	F-box protein 32, transcript variant 1	0.6	NM_058229.2
FBXO34	F-box protein 34	1.3	NM_017943.2

FBXO8	F-box protein 8	0.8	NM_012180.2
FBXW7	F-box and WD-40 domain protein 7 (archipelago homolog, Drosophila), transcript variant 1	1.7	NM_033632.2
FBXW7	F-box and WD-40 domain protein 7 (archipelago homolog, Drosophila), transcript variant 3	1.5	NM_001013415.1
FCHO2	FCH domain only 2	0.8	NM_138782.1
FCN3	ficolin (collagen/fibrinogen domain containing) 3 (Hakata antigen), transcript variant 2	1.7	NM_173452.1
FGF9	fibroblast growth factor 9 (glia-activating factor)	1.8	NM_002010.1
FILIP1	filamin A interacting protein 1	2.3	NM_015687.2
FLJ11021	similar to splicing factor, arginine/serine-rich 4, transcript variant 1	1.2	NM_023012.4
FLJ11171	hypothetical protein FLJ11171	0.7	NM_018348.4
FLJ12788	hypothetical protein FLJ12788	0.8	NM_022492.2
FLJ13910	hypothetical protein FLJ13910	1.5	NM_022780.2
FLJ14803	hypothetical protein FLJ14803	0.7	NM_032842.2
FLJ20125	hypothetical protein FLJ20125	0.7	NM_017676.1
FLJ20232	hypothetical protein FLJ20232	0.8	NM_019008.4
FLJ21125	hypothetical protein FLJ21125	0.8	NM_024627.4
FLJ38451	FLJ38451 protein	0.9	NM_175872.3
FLJ38663	hypothetical protein FLJ38663	0.7	NM_152269.1
FLJ90396	hypothetical protein LOC163049	0.7	NM_153358.1
FLNB	filamin B, beta (actin binding protein 278)	1.5	NM_001457.1
FLRT3	fibronectin leucine rich transmembrane protein 3, transcript variant 1	2.9	NM_013281.2
FNDC3B	fibronectin type III domain containing 3B	1.2	NM_022763.2
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	14.8	NM_005252.2
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	3.7	NM_006732.1
FOSL2	FOS-like antigen 2	1.2	NM_005253.3
FOXO1A	forkhead box O1A (rhabdomyosarcoma)	1.6	NM_002015.2
FRMD3	FERM domain containing 3	2.7	NM_174938.3
FSD2	fibronectin type III and SPRY domain containing 2	0.8	NM_001007122.1
FUK	fucokinase	0.8	NM_145059.2
FUSIP1	FUS interacting protein (serine/arginine-rich) 1, transcript variant 1	1.5	NM_006625.3
FYTTD1	forty-two-three domain containing 1, transcript variant 2	2.7	NM_001011537.1
FZD4	frizzled homolog 4 (Drosophila)	0.7	NM_012193.2
GABARAPL1	GABA(A) receptor-associated protein like 1	1.9	NM_031412.2
GADD45A	growth arrest and DNA-damage-inducible, alpha	4.1	NM_001924.2
GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	1.6	NM_004482.2
GALNTL2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	0.7	NM_054110.2
GATAD2A	GATA zinc finger domain containing 2A	1.4	NM_017660.2
GEM	GTP binding protein overexpressed in skeletal muscle, transcript variant 1	3.1	NM_005261.2
GNL3	guanine nucleotide binding protein-like 3 (nucleolar), transcript variant 1	1.2	NM_014366.4
GOLPH3L	golgi phosphoprotein 3-like	0.6	NM_018178.3
GPR124	G protein-coupled receptor 124	0.9	NM_032777.6
GPR177	G protein-coupled receptor 177, transcript variant 2	0.9	NM_001002292.1
GPR30	G protein-coupled receptor 30, transcript variant 1	0.7	NM_001031682.1
GPSM2	G-protein signalling modulator 2 (AGS3-like, C. elegans)	2.4	NM_013296.3
GRPEL2	GrpE-like 2, mitochondrial (E. coli)	0.8	NM_152407.2
GSK3B	glycogen synthase kinase 3 beta	1.3	NM_002093.2

GSPT2	G1 to S phase transition 2	0.8	NM_018094.2
GTF2H4	general transcription factor IIH, polypeptide 4, 52kDa	0.9	NM_001517.4
GUSBL1	glucuronidase, beta-like 1	0.7	NM_001033523.1
HBEGF	heparin-binding EGF-like growth factor	7.0	NM_001945.1
HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1, transcript variant 3	0.8	NM_001010990.1
HEY1	hairy/enhancer-of-split related with YRPW motif 1	1.9	NM_012258.2
HEYL	hairy/enhancer-of-split related with YRPW motif-like	2.4	NM_014571.2
HIPK3	homeodomain interacting protein kinase 3	1.2	NM_005734.2
HIST1H2AL	histone 1, H2al	1.2	NM_003511.2
HIST1H2BA	histone 1, H2ba	1.1	NM_170610.2
HIST2H2BE	histone 2, H2be	1.5	NM_003528.2
HLA-DMB	major histocompatibility complex, class II, DM beta	0.8	NM_002118.3
HMFN0839	hypothetical protein MGC11324	2.6	NM_032717.3
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	0.9	NM_000859.1
HNRPA0	heterogeneous nuclear ribonucleoprotein A0	1.3	NM_006805.3
HNRPA3	heterogeneous nuclear ribonucleoprotein A3	1.2	NM_194247.1
HNRPAB	heterogeneous nuclear ribonucleoprotein A/B, transcript variant 1	1.3	NM_031266.2
HOMER1	homer homolog 1 (Drosophila)	1.6	NM_004272.3
HOXA5	homeobox A5	0.7	NM_019102.2
HOXA9	homeo box A9, transcript variant 1	0.6	NM_152739.2
HOXC6	homeobox C6, transcript variant 2	0.7	NM_153693.1
HOXC9	homeobox C9	0.8	NM_006897.1
HPS6	Hermansky-Pudlak syndrome 6	0.7	NM_024747.4
HSPA1A	heat shock 70kDa protein 1A	4.5	NM_005345.4
HSPA1B	heat shock 70kDa protein 1B	7.1	NM_005346.3
HSPB8	heat shock 22kDa protein 8	2.2	NM_014365.2
HYLS1	hydrolethalus syndrome 1	0.7	NM_145014.1
IFRD1	interferon-related developmental regulator 1, transcript variant 1	5.5	NM_001550.2
IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3	3.5	NM_006547.2
IL4R	interleukin 4 receptor, transcript variant 1	1.7	NM_000418.2
IL6R	interleukin 6 receptor, transcript variant 2	1.5	NM_181359.1
IL6R	interleukin 6 receptor, transcript variant 1	1.3	NM_000565.2
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa, transcript variant 1	0.9	NM_001017915.1
IPLA2(GAMMA)	intracellular membrane-associated calcium-independent phospholipase A2 gamma	1.6	NM_015723.2
IRF1	interferon regulatory factor 1	1.6	NM_002198.1
IRS2	insulin receptor substrate 2	4.8	NM_003749.2
ISG20L1	interferon stimulated exonuclease gene 20kDa-like 1	2.3	NM_022767.2
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	2.0	NM_002205.2
IXL	intersex-like (Drosophila)	0.9	NM_017592.1
JAK2	Janus kinase 2 (a protein tyrosine kinase)	1.7	NM_004972.2
JMJD1A	jumonji domain containing 1A	0.8	NM_018433.3
JMJD1C	jumonji domain containing 1C	1.9	NM_004241.2
JOSD1	Josephin domain containing 1	1.3	NM_014876.3
JOSD3	Josephin domain containing 3	1.6	NM_024116.1
JRK	jerky homolog (mouse)	1.2	NM_003724.1
JUND	jun D proto-oncogene	1.9	NM_005354.2
KBTBD3	kelch repeat and BTB (POZ) domain containing 3, transcript variant 1	0.7	NM_152433.2

KBTBD5	kelch repeat and BTB (POZ) domain containing 5	5.4	NM_152393.2
KBTBD7	kelch repeat and BTB (POZ) domain containing 7	0.7	NM_032138.3
KBTBD8	kelch repeat and BTB (POZ) domain containing 8	6.4	NM_032505.1
KIAA0157	KIAA0157	0.8	NM_032182.2
KIAA0240	KIAA0240	0.8	NM_015349.1
KIAA0564	KIAA0564 protein, transcript variant 2	1.3	NM_001009814.1
KIAA0690	KIAA0690	1.7	NM_015179.2
KIAA0961	zinc finger protein KIAA0961	0.7	NM_014898.1
KIAA1128	KIAA1128	1.2	NM_018999.1
KIAA1217	KIAA1217	1.2	NM_019590.2
KIAA1370	KIAA1370	0.7	NM_019600.1
KIAA1443	KIAA1443	0.8	NM_020834.1
KIF25	kinesin family member 25, transcript variant 1	0.7	NM_030615.1
KIF25	kinesin family member 25, transcript variant 2	0.6	NM_005355.2
KLF5	Kruppel-like factor 5 (intestinal)	2.6	NM_001730.3
KLF6	Kruppel-like factor 6, transcript variant 2	3.2	NM_001300.4
KLF6	Kruppel-like factor 6, transcript variant 1	2.9	NM_001008490.1
KLHL13	kelch-like 13 (Drosophila)	0.9	NM_033495.2
KLHL24	kelch-like 24 (Drosophila)	0.7	NM_017644.3
LCAT	lecithin-cholesterol acyltransferase	1.3	NM_000229.1
LIFR	leukemia inhibitory factor receptor	0.8	NM_002310.3
LIMK2	LIM domain kinase 2, transcript variant 2a	1.1	NM_005569.3
LIMS1	LIM and senescent cell antigen-like domains 1	1.9	NM_004987.3
LIMS3	LIM and senescent cell antigen-like domains 3	2.2	NM_033514.1
LIPT1	lipoyltransferase 1, transcript variant 3	0.8	NM_145197.1
LIX1L	Lix1 homolog (mouse) like	1.9	NM_153713.1
LMCD1	LIM and cysteine-rich domains 1	2.0	NM_014583.2
LOC126295	hypothetical protein LOC126295	0.8	NM_173480.1
LOC133619	hypothetical protein MGC12103	0.8	NM_130809.2
LOC153222	adult retina protein	0.7	NM_153607.1
LOC153364	similar to metallo-beta-lactamase superfamily protein	0.8	NM_203406.1
LOC168850	hypothetical protein LOC168850	1.4	NM_176814.3
LOC283537	hypothetical protein LOC283537	0.7	NM_181785.1
LOC339977	similar to hypothetical protein MGC38937	0.7	NM_001024611.1
LOC400451	hypothetical gene supported by AK075564; BC060873	0.8	NM_207446.1
LOC401431	hypothetical gene LOC401431	0.8	NM_001008745.1
LOC492311	similar to bovine IgA regulatory protein	0.7	NM_001007189.1
LOC90580	hypothetical protein BC011833	0.8	NM_138358.2
LOH3CR2A	loss of heterozygosity, 3, chromosomal region 2, gene A	3.7	NM_013343.1
LONRF3	LON peptidase N-terminal domain and ring finger 3, transcript variant 2	1.7	NM_024778.4
LPXN	leupaxin	0.8	NM_004811.1
LRP11	low density lipoprotein receptor-related protein 11	0.9	NM_032832.3
LRRC37B	leucine rich repeat containing 37B	0.7	NM_052888.2
LRRC8B	leucine rich repeat containing 8 family, member B	1.4	NM_015350.1
MAD2L1BP	MAD2L1 binding protein, transcript variant 2	0.8	NM_014628.2
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian), transcript variant 1	2.6	NM_012323.2
MAN1B1	mannosidase, alpha, class 1B, member 1	0.7	NM_016219.2

KBTBD5	kelch repeat and BTB (POZ) domain containing 5	5.4	NM_152393.2
KBTBD7	kelch repeat and BTB (POZ) domain containing 7	0.7	NM_032138.3
KBTBD8	kelch repeat and BTB (POZ) domain containing 8	6.4	NM_032505.1
KIAA0157	KIAA0157	0.8	NM_032182.2
KIAA0240	KIAA0240	0.8	NM_015349.1
KIAA0564	KIAA0564 protein, transcript variant 2	1.3	NM_001009814.1
KIAA0690	KIAA0690	1.7	NM_015179.2
KIAA0961	zinc finger protein KIAA0961	0.7	NM_014898.1
KIAA1128	KIAA1128	1.2	NM_018999.1
KIAA1217	KIAA1217	1.2	NM_019590.2
KIAA1370	KIAA1370	0.7	NM_019600.1
KIAA1443	KIAA1443	0.8	NM_020834.1
KIF25	kinesin family member 25, transcript variant 1	0.7	NM_030615.1
KIF25	kinesin family member 25, transcript variant 2	0.6	NM_005355.2
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KLF6	Kruppel-like factor 6, transcript variant 1	2.9	NM_001008490.1
KLHL13	kelch-like 13 (Drosophila)	0.9	NM_033495.2
KLHL24	kelch-like 24 (Drosophila)	0.7	NM_017644.3
LCAT	lecithin-cholesterol acyltransferase	1.3	NM_000229.1
LIFR	leukemia inhibitory factor receptor	0.8	NM_002310.3
LIMK2	LIM domain kinase 2, transcript variant 2a	1.1	NM_005569.3
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LIPT1	lipoyltransferase 1, transcript variant 3	0.8	NM_145197.1
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LOC133619	hypothetical protein MGC12103	0.8	NM_130809.2
LOC153222	adult retina protein	0.7	NM_153607.1
LOC153364	similar to metallo-beta-lactamase superfamily protein	0.8	NM_203406.1
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LOC283537	hypothetical protein LOC283537	0.7	NM_181785.1
LOC339977	similar to hypothetical protein MGC38937	0.7	NM_001024611.1
LOC400451	hypothetical gene supported by AK075564; BC060873	0.8	NM_207446.1
LOC401431	hypothetical gene LOC401431	0.8	NM_001008745.1
LOC492311	similar to bovine IgA regulatory protein	0.7	NM_001007189.1
LOC90580	hypothetical protein BC011833	0.8	NM_138358.2
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LRP11	low density lipoprotein receptor-related protein 11	0.9	NM_032832.3
LRRC37B	leucine rich repeat containing 37B	0.7	NM_052888.2
LRRC8B	leucine rich repeat containing 8 family, member B	1.4	NM_015350.1
MAD2L1BP	MAD2L1 binding protein, transcript variant 2	0.8	NM_014628.2
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian), transcript variant 1	2.6	NM_012323.2
MAN1B1	mannosidase, alpha, class 1B, member 1	0.7	NM_016219.2

MAP3K6	mitogen-activated protein kinase kinase kinase 6	1.4	NM_004672.3
MAP3K8	mitogen-activated protein kinase kinase kinase 8	11.1	NM_005204.2
MAPK6	mitogen-activated protein kinase 6	1.3	NM_002748.2
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2, transcript variant 2	1.2	NM_032960.2
MAPRE1	microtubule-associated protein, RP/EB family, member 1	1.5	NM_012325.1
MARCKS	myristoylated alanine-rich protein kinase C substrate	0.7	NM_002356.4
MAT2A	methionine adenosyltransferase II, alpha	1.8	NM_005911.4
MED28	mediator of RNA polymerase II transcription, subunit 28 homolog (yeast)	1.4	NM_025205.3
MEOX1	mesenchyme homeo box 1, transcript variant 1	2.0	NM_004527.2
MGC12981	hypothetical protein MGC12981	0.8	NM_032357.2
MGC33584	hypothetical protein MGC33584	0.8	NM_173680.2
MGC40499	PRotein Associated with Tlr4	0.8	NM_152755.1
MGC52110	hypothetical protein MGC52110	0.8	NM_001008215.1
MGC5297	hypothetical protein MGC5297	0.8	NM_024091.2
MGC75360	similar to zinc finger protein 29	3.9	NM_001004309.1
MGC88387	similar to HSPC296	0.9	NM_001001683.1
MIZF	MBD2-interacting zinc finger, transcript variant 1	0.9	NM_015517.3
MKL2	MKL/myocardin-like 2	0.8	NM_014048.3
MKRN2	makorin, ring finger protein, 2	1.2	NM_014160.3
MLR2	ligand-dependent corepressor	0.7	NM_032440.1
MRPL43	mitochondrial ribosomal protein L43, nuclear gene encoding mitochondrial protein, transcript variant 2	0.8	NM_176792.1
MSI2	musashi homolog 2 (Drosophila), transcript variant 2	1.5	NM_170721.1
MXD4	MAX dimerization protein 4	0.7	NM_006454.2
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	11.0	NM_002467.3
MYH9	myosin, heavy polypeptide 9, non-muscle	1.6	NM_002473.3
MYO10	myosin X	0.5	NM_012334.1
NDEL1	nudE nuclear distribution gene E homolog like 1 (A. nidulans), transcript variant 2	1.2	NM_030808.3
NDN	necdin homolog (mouse)	0.8	NM_002487.2
NDRG3	NDRG family member 3, transcript variant 2	0.7	NM_022477.2
NEDD4	neural precursor cell expressed, developmentally down-regulated 4, transcript variant 2	1.7	NM_198400.1
NES	nestin	1.6	NM_006617.1
NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1, transcript variant 1	1.3	NM_172390.1
NFIA	nuclear factor I/A	0.9	NM_005595.1
NFIL3	nuclear factor, interleukin 3 regulated	6.4	NM_005384.2
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta, transcript variant 2	2.3	NM_001005474.1
NFXL1	nuclear transcription factor, X-box binding-like 1	0.7	NM_152995.3
NFYC	nuclear transcription factor Y, gamma	0.9	NM_014223.2
NIPA1	non imprinted in Prader-Willi/Angelman syndrome 1	0.7	NM_144599.3
NOL10	nucleolar protein 10	1.4	NM_024894.1
NOP5/NOP58	nucleolar protein NOP5/NOP58	1.5	NM_015934.3
NPAL3	NIPA-like domain containing 3	0.8	NM_020448.2
NR4A1	nuclear receptor subfamily 4, group A, member 1, transcript variant 3	2.8	NM_173158.1
NRAP	nebulin-related anchoring protein, transcript variant 1	1.5	NM_006175.3
NUAK1	NUAK family, SNF1-like kinase, 1	1.2	NM_014840.2
NUDT4	nudix (nucleoside diphosphate linked moiety X)-type motif 4, transcript variant 1	2.0	NM_019094.3

NUP62	nucleoporin 62kDa, transcript variant 1	0.8	NM_153719.2
OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A, transcript variant 2	1.8	NM_022837.1
OR5R1	olfactory receptor, family 5, subfamily R, member 1	1.1	NM_001004744.1
OSBPL2	oxysterol binding protein-like 2, transcript variant 2	0.8	NM_144498.1
OSGEPL1	O-sialoglycoprotein endopeptidase-like 1	0.8	NM_022353.1
OSTbeta	organic solute transporter beta	0.9	NM_178859.2
OXSM	3-oxoacyl-ACP synthase, mitochondrial	0.8	NM_017897.1
P2RY6	pyrimidinergic receptor P2Y, G-protein coupled, 6, transcript variant 2	1.1	NM_176798.1
P4HA1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I, transcript variant 1	1.8	NM_000917.2
P4HA1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I, transcript variant 2	1.6	NM_001017962.1
PAGE4	P antigen family, member 4 (prostate associated)	0.8	NM_007003.2
PARS2	prolyl-tRNA synthetase (mitochondrial)(putative)	0.8	NM_152268.2
PCDH17	protocadherin 17	0.9	NM_014459.2
PCF11	PCF11, cleavage and polyadenylation factor subunit, homolog (S. cerevisiae)	0.6	NM_015885.2
PCGF5	polycomb group ring finger 5	1.2	NM_032373.2
PDCD6	programmed cell death 6	0.8	NM_013232.2
PDIK1L	PDLIM1 interacting kinase 1 like	0.6	NM_152835.1
PDRG1	p53 and DNA damage regulated 1	0.8	NM_030815.2
PER1	period homolog 1 (Drosophila)	0.7	NM_002616.1
PFAAP5	phosphonoformate immuno-associated protein 5	2.3	NM_014887.1
PGLS	6-phosphogluconolactonase	0.8	NM_012088.2
PHGDH	phosphoglycerate dehydrogenase	0.8	NM_006623.2
PHLDA1	pleckstrin homology-like domain, family A, member 1	3.6	NM_007350.2
PIGV	phosphatidylinositol glycan, class V	0.7	NM_017837.2
PIK4CB	phosphatidylinositol 4-kinase, catalytic, beta polypeptide	1.3	NM_002651.1
PKIG	protein kinase (cAMP-dependent, catalytic) inhibitor gamma, transcript variant 3	1.3	NM_181804.1
PLEKHC1	pleckstrin homology domain containing, family C (with FERM domain) member 1	1.2	NM_006832.1
PLEKHO1	pleckstrin homology domain containing, family O member 1	1.5	NM_016274.3
PODN	podocan	0.9	NM_153703.3
PON3	paraoxonase 3	0.9	NM_000940.1
PPFIBP2	PTPRF interacting protein, binding protein 2 (liprin beta 2)	0.8	NM_003621.1
PPM1B	protein phosphatase 1B (formerly 2C), magnesium-dependent, beta isoform, transcript variant 5	3.8	NM_001033557.1
PPM1B	protein phosphatase 1B (formerly 2C), magnesium-dependent, beta isoform, transcript variant 4	1.5	NM_001033556.1
PPM1D	protein phosphatase 1D magnesium-dependent, delta isoform	1.6	NM_003620.2
PPM1M	protein phosphatase 1M (PP2C domain containing)	0.9	NM_144641.1
PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	3.2	NM_014330.2
PPP2CA	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	1.5	NM_002715.2
PPP2CB	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform, transcript variant 1	1.7	NM_004156.2
PPP2CB	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform, transcript variant 2	1.5	NM_001009552.1
PPP2R5A	protein phosphatase 2, regulatory subunit B (B56), alpha isoform	1.6	NM_006243.2
PPRC1	peroxisome proliferative activated receptor, gamma, coactivator-related 1	1.9	NM_015062.3
PRNP	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia), transcript variant 2	1.5	NM_183079.1
PRSS2	protease, serine, 2 (trypsin 2), transcript variant 1	1.1	NM_002770.2

PSD2	pleckstrin and Sec7 domain containing 2	1.1	NM_032289.1
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2), transcript variant 1	0.9	NM_002800.4
PTPLAD1	protein tyrosine phosphatase-like A domain containing 1	0.8	NM_016395.1
PTPN1	protein tyrosine phosphatase, non-receptor type 1	1.5	NM_002827.2
PTPN14	protein tyrosine phosphatase, non-receptor type 14	1.5	NM_005401.3
PVR	poliovirus receptor	2.2	NM_006505.2
RAB12	RAB12, member RAS oncogene family	0.7	NM_001025300.1
RAB15	RAB15, member RAS onocogene family	3.9	NM_198686.1
RAB22A	RAB22A, member RAS oncogene family	0.7	NM_020673.2
RAE1	RAE1 RNA export 1 homolog (S. pombe), transcript variant 1	0.9	NM_003610.3
RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1, transcript variant 2	1.2	NM_198679.1
RASL11B	RAS-like, family 11, member B	1.9	NM_023940.2
RBBP6	retinoblastoma binding protein 6, transcript variant 3	1.7	NM_032626.5
RBBP9	retinoblastoma binding protein 9, transcript variant 1	0.8	NM_006606.2
RBM12	RNA binding motif protein 12, transcript variant 1	1.9	NM_006047.4
RBM33	RNA binding motif protein 33	0.5	NM_001008408.1
RBM4B	RNA binding motif protein 4B	0.7	NM_031492.2
RCBTB2	regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2	0.9	NM_001268.2
RCL1	RNA terminal phosphate cyclase-like 1	1.5	NM_005772.2
RDH11	retinol dehydrogenase 11 (all-trans and 9-cis)	0.9	NM_016026.2
RDH14	retinol dehydrogenase 14 (all-trans and 9-cis)	0.7	NM_020905.1
RFP2	ret finger protein 2, transcript variant 4	0.7	NM_001007278.1
RFWD2	ring finger and WD repeat domain 2, transcript variant 1	1.1	NM_022457.5
RHOJ	ras homolog gene family, member J	0.7	NM_020663.2
RICTOR	rapamycin-insensitive companion of mTOR	1.4	NM_152756.3
RND3	Rho family GTPase 3	5.4	NM_005168.3
RNF144	ring finger protein 144	0.8	NM_014746.2
RNF26	ring finger protein 26	0.8	NM_032015.3
RNPC2	RNA-binding region (RNP1, RRM) containing 2, transcript variant 3	1.2	NM_184237.1
RPUSD2	RNA pseudouridylate synthase domain containing 2	0.6	NM_152260.1
RQCD1	RCD1 required for cell differentiation1 homolog (S. pombe)	1.2	NM_005444.1
RRAD	Ras-related associated with diabetes	3.7	NM_004165.1
RRAS2	related RAS viral (r-ras) oncogene homolog 2	1.6	NM_012250.3
RUNDC1	RUN domain containing 1	0.7	NM_173079.1
SAT	spermidine/spermine N1-acetyltransferase	2.0	NM_002970.1
SCHIP1	schwannomin interacting protein 1	1.5	NM_014575.1
SCPEP1	serine carboxypeptidase 1	0.8	NM_021626.1
SCYL3	SCY1-like 3 (S. cerevisiae), transcript variant 1	0.7	NM_020423.4
SDC4	syndecan 4 (amphiglycan, ryudocan)	4.8	NM_002999.2
SEC61A1	Sec61 alpha 1 subunit (S. cerevisiae)	1.4	NM_013336.3
SEC63D1	SEC63 domain containing 1	0.9	NM_198550.1
SENP5	SUMO1/sentrin specific peptidase 5	1.3	NM_152699.2
SEPP1	selenoprotein P, plasma, 1	0.7	NM_005410.2
SETMAR	SET domain and mariner transposase fusion gene	0.9	NM_006515.1
SFRS3	splicing factor, arginine/serine-rich 3	1.6	NM_003017.3
SH3BGRL2	SH3 domain binding glutamic acid-rich protein like 2	0.8	NM_031469.1
SIRT1	sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae)	1.4	NM_012238.3

SIRT4	sirtuin (silent mating type information regulation 2 homolog) 4 (S. cerevisiae)	0.6	NM_012240.1
SIX1	sine oculis homeobox homolog 1 (Drosophila)	0.6	NM_005982.1
SLC15A4	solute carrier family 15, member 4	1.5	NM_145648.1
SLC19A2	solute carrier family 19 (thiamine transporter), member 2	1.5	NM_006996.1
SLC1A7	solute carrier family 1 (glutamate transporter), member 7	0.6	NM_006671.3
SLC27A1	solute carrier family 27 (fatty acid transporter), member 1	0.6	NM_198580.1
SLC35A5	solute carrier family 35, member A5	0.7	NM_017945.2
SLC38A2	solute carrier family 38, member 2	1.5	NM_018976.3
SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	2.1	NM_003486.5
SLC9A8	solute carrier family 9 (sodium/hydrogen exchanger), member 8	0.8	NM_015266.1
SLCO2A1	solute carrier organic anion transporter family, member 2A1	1.5	NM_005630.1
SLCO5A1	solute carrier organic anion transporter family, member 5A1	1.4	NM_030958.1
SLITL2	slit-like 2 (Drosophila)	1.8	NM_138440.1
SMARCAL1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a-like 1	0.7	NM_014140.2
SMG1	PI-3-kinase-related kinase SMG-1	1.6	NM_015092.3
SMO	smoothened homolog (Drosophila)	0.9	NM_005631.3
SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A	1.5	NM_006714.2
SNAPC4	small nuclear RNA activating complex, polypeptide 4, 190kDa	0.8	NM_003086.1
SNF1LK	SNF1-like kinase	3.2	NM_173354.2
SNF1LK2	SNF1-like kinase 2	1.6	NM_015191.1
SNX2	sorting nexin 2	0.8	NM_003100.2
SOCS6	suppressor of cytokine signaling 6	1.3	NM_004232.2
SORBS1	sorbin and SH3 domain containing 1, transcript variant 1	1.7	NM_006434.1
SOX18	SRY (sex determining region Y)-box 18	0.6	NM_018419.2
SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	1.5	NM_000346.2
SPATA7	spermatogenesis associated 7	0.8	NM_018418.1
SPATS2	spermatogenesis associated, serine-rich 2	0.9	NM_023071.1
SPEN	spen homolog, transcriptional regulator (Drosophila)	1.6	NM_015001.2
SPPL2A	signal peptide peptidase-like 2A	1.4	NM_032802.2
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian), transcript variant 1	1.1	NM_005417.3
ST5	suppression of tumorigenicity 5, transcript variant 3	1.4	NM_213618.1
ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1, transcript variant 3	0.6	NM_173217.1
ST6GALNAC6	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1, 3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 6	0.9	NM_013443.3
ST8SIA1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	0.9	NM_003034.2
STRN	striatin, calmodulin binding protein	1.3	NM_003162.2
STX12	syntaxin 12	1.2	NM_177424.1
STXBP6	syntaxin binding protein 6 (amisyn)	0.7	NM_014178.6
SUDS3	suppressor of defective silencing 3 homolog (S. cerevisiae)	1.2	NM_022491.2
SUHW4	suppressor of hairy wing homolog 4 (Drosophila), transcript variant 3	1.2	NM_001002844.1
SYNPO2	synaptopodin 2	1.6	NM_133477.1
TADA1L	transcriptional adaptor 1 (HFI1 homolog, yeast)-like	0.7	NM_053053.2
TBCC	tubulin-specific chaperone c	0.8	NM_003192.1
TBPL1	TBP-like 1	0.8	NM_004865.2
TBX3	T-box 3 (ulnar mammary syndrome), transcript variant 2	1.4	NM_016569.3
TCEAL1	transcription elongation factor A (SII)-like 1), transcript variant 3	0.9	NM_001006640.1
TCF15	transcription factor 15 (basic helix-loop-helix)	0.7	NM_004609.3

TEF	thyrotrophic embryonic factor	0.7	NM_003216.2
TES	testis derived transcript (3 LIM domains), transcript variant 2	1.2	NM_152829.1
TESK2	testis-specific kinase 2	0.8	NM_007170.1
TFB2M	transcription factor B2, mitochondrial	0.9	NM_022366.1
TGDS	TDP-glucose 4,6-dehydratase	0.8	NM_014305.1
TGFB2	transforming growth factor, beta 2	2.0	NM_003238.1
THAP9	THAP domain containing 9	0.8	NM_024672.2
THBD	thrombomodulin	3.1	NM_000361.2
THUMPD2	THUMP domain containing 2	1.2	NM_025264.2
TIAM2	T-cell lymphoma invasion and metastasis 2, transcript variant 2	1.8	NM_001010927.2
TICAM2	toll-like receptor adaptor molecule 2	0.6	NM_021649.3
TLE4	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)	1.4	NM_007005.3
TLOC1	translocation protein 1	0.8	NM_003262.3
TMCC3	transmembrane and coiled-coil domain family 3	0.9	NM_020698.1
TMEM115	transmembrane protein 115	0.8	NM_007024.4
TMEM49	transmembrane protein 49	1.3	NM_030938.2
TMEM88	transmembrane protein 88	0.8	NM_203411.1
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	10.3	NM_016639.1
TNFSF12	tumor necrosis factor (ligand) superfamily, member 12, transcript variant 1	0.8	NM_003809.2
TNKS1BP1	tankyrase 1 binding protein 1, 182kDa	1.3	NM_033396.1
TNS1	tensin 1	1.6	NM_022648.3
TP53INP2	tumor protein p53 inducible nuclear protein 2	1.5	NM_021202.1
TRIM54	tripartite motif-containing 54, transcript variant 2	1.7	NM_187841.1
TRIM55	tripartite motif-containing 55, transcript variant 3	2.1	NM_184086.1
TRIM68	tripartite motif-containing 68	0.8	NM_018073.5
TRIO	triple functional domain (PTPRF interacting)	1.1	NM_007118.2
TSC22D2	TSC22 domain family, member 2	2.1	NM_014779.2
TSPYL2	TSPY-like 2	1.2	NM_022117.1
TTC5	tetratricopeptide repeat domain 5	0.9	NM_138376.1
TUBE1	tubulin, epsilon 1	0.8	NM_016262.3
TUFT1	tuftelin 1	0.7	NM_020127.1
UBL3	ubiquitin-like 3	1.2	NM_007106.2
UBQLN1	ubiquilin 1, transcript variant 2	1.3	NM_053067.1
UBQLN1	ubiquilin 1, transcript variant 1	1.2	NM_013438.3
UBXD6	UBX domain containing 6	0.9	NM_005671.1
USP2	ubiquitin specific peptidase 2, transcript variant 1	1.5	NM_004205.3
USP28	ubiquitin specific peptidase 28	1.5	NM_020886.2
USP54	ubiquitin specific peptidase 54	2.0	NM_152586.2
UTP14C	UTP14, U3 small nucleolar ribonucleoprotein, homolog C (yeast)	0.7	NM_021645.4
UTS2	urotensin 2, transcript variant 1	1.1	NM_021995.1
VGLL2	vestigial like 2 (Drosophila), transcript variant 2	3.1	NM_153453.1
VGLL2	vestigial like 2 (Drosophila), transcript variant 1	2.1	NM_182645.2
WDR1	WD repeat domain 1, transcript variant 1	2.1	NM_017491.3
WDR20	WD repeat domain 20, transcript variant 1	0.7	NM_181291.1
WDR24	WD repeat domain 24	0.9	NM_032259.1
WDR33	WD repeat domain 33, transcript variant 2	1.6	NM_001006622.1
WDR67	WD repeat domain 67	0.8	NM_145647.1
WDSUB1	WD repeat, sterile alpha motif and U-box domain containing 1	0.8	NM_152528.1

WNT5A	wingless-type MMTV integration site family, member 5A	0.7	NM_003392.3
XYLT2	xylosyltransferase II	0.8	NM_022167.1
YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	1.3	NM_005433.3
YIPF5	Yip1 domain family, member 5, transcript variant 2	0.7	NM_030799.6
YWHAQ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	1.4	NM_006826.2
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, transcript variant 1	1.3	NM_003406.2
ZBTB3	zinc finger and BTB domain containing 3	0.7	NM_024784.2
ZBTB41	zinc finger and BTB domain containing 41	0.8	NM_194314.2
ZC3H12A	zinc finger CCCH-type containing 12A	1.7	NM_025079.1
ZCCHC7	zinc finger, CCHC domain containing 7	0.8	NM_032226.1
ZCCHC9	zinc finger, CCHC domain containing 9	0.8	NM_032280.1
ZFP161	zinc finger protein 161 homolog (mouse)	0.8	NM_003409.2
ZFP2	zinc finger protein 2 homolog (mouse)	0.8	NM_030613.2
ZFP3	zinc finger protein 3 homolog (mouse)	0.7	NM_153018.1
ZMYM6	zinc finger, MYM-type 6	0.8	NM_007167.2
ZNF133	zinc finger protein 133 (clone pHZ-13)	0.7	NM_003434.3
ZNF142	zinc finger protein 142 (clone pHZ-49)	0.8	NM_005081.2
ZNF175	zinc finger protein 175	0.7	NM_007147.2
ZNF195	zinc finger protein 195	0.8	NM_007152.1
ZNF2	zinc finger protein 2, transcript variant 1	0.8	NM_021088.2
ZNF20	zinc finger protein 20 (KOX 13)	0.7	NM_021143.1
ZNF200	zinc finger protein 200, transcript variant 3	0.8	NM_198088.1
ZNF207	zinc finger protein 207, transcript variant 1	1.3	NM_003457.2
ZNF212	zinc finger protein 212	0.8	NM_012256.2
ZNF214	zinc finger protein 214	0.8	NM_013249.1
ZNF224	zinc finger protein 224	0.8	NM_013398.1
ZNF295	zinc finger protein 295	2.1	NM_020727.3
ZNF297B	zinc finger protein 297B	2.1	NM_014007.2
ZNF30	zinc finger protein 30 (KOX 28)	0.6	NM_194325.1
ZNF307	zinc finger protein 307	0.7	NM_019110.3
ZNF319	zinc finger protein 319	0.8	NM_020807.1
ZNF323	zinc finger protein 323, transcript variant 1	0.7	NM_030899.2
ZNF323	zinc finger protein 323, transcript variant 2	0.7	NM_145909.1
ZNF350	zinc finger protein 350	0.8	NM_021632.3
ZNF364	zinc finger protein 364	2.2	NM_014455.1
ZNF403	zinc finger protein 403	0.7	NM_024835.2
ZNF415	zinc finger protein 415	0.7	NM_018355.2
ZNF425	zinc finger protein 425	0.8	NM_001001661.1
ZNF433	zinc finger protein 433	0.9	NM_152602.1
ZNF491	zinc finger protein 491	0.8	NM_152356.2
ZNF518	zinc finger protein 518	0.8	NM_014803.2
ZNF521	zinc finger protein 521	0.8	NM_015461.1
ZNF539	zinc finger protein 539	0.8	NM_203282.1
ZNF541	zinc finger protein 541	0.8	NM_032255.1
ZNF545	zinc finger protein 545	0.8	NM_133466.1
ZNF564	zinc finger protein 564	0.7	NM_144976.2
ZNF573	zinc finger protein 573	0.8	NM_152360.2

ZNF585A	zinc finger protein 585A, transcript variant 2	0.8	NM_199126.1
ZNF586	zinc finger protein 586	1.4	NM_017652.1
ZNF613	zinc finger protein 613, transcript variant 1	0.8	NM_001031721.1
ZNF615	zinc finger protein 615	0.7	NM_198480.2
ZNF616	zinc finger protein 616	0.7	NM_178523.3
ZNF627	zinc finger protein 627	0.8	NM_145295.2
ZNF658	zinc finger protein 658	0.6	NM_033160.4
ZNF658B	zinc finger protein 658B	0.8	NM_001032297.1
ZNF679	zinc finger protein 679	0.7	NM_153363.1
ZNF684	zinc finger protein 684	0.6	NM_152373.2
ZNF721	zinc finger protein 721	0.8	NM_133474.1
ZNF77	zinc finger protein 77 (pT1)	0.7	NM_021217.1
ZNF84	zinc finger protein 84 (HPF2)	0.9	NM_003428.2
ZSCAN2	zinc finger and SCAN domain containing 2, transcript variant 1	0.8	NM_181877.3
ZYX	zyxin, transcript variant 1	1.5	NM_003461.4