ARTICLE
Journal of Cellular Biochemistry 107:171–178 (2009)

Journal of Cellular Biochemistry

Changes in Contraction-Induced Phosphorylation of AMP-Activated Protein Kinase and Mitogen-Activated Protein Kinases in Skeletal Muscle After Ovariectomy

Lindsay M. Wohlers,[1] Sean M. Sweeney,[1] Christopher W. Ward,[2] Richard M. Lovering,[3] and Espen E. Spangenburg[1*]

[1]*Department of Kinesiology, School of Public Health, University of Maryland, College Park, Maryland 21045*
[2]*School of Nursing, University of Maryland, Baltimore, Maryland 21201*
[3]*Department of Physiology, School of Medicine, University of Maryland, Baltimore, Maryland 21201*

ABSTRACT

Recent evidence suggests that ovarian hormones contribute to altered function of skeletal muscle, however the signaling processes thought to regulate muscle function remain undefined in females. Thus, the purpose of this investigation is to determine if ovarian hormone status is critical for contraction-induced activation of AMPK or MAPK in skeletal muscle. Female mice were divided into two groups, ovariectomy (OVX) and SHAM, which were then subjected to in situ isometric contractile protocols. AMPK, ERK 1/2, p38, and JNK phosphorylation were measured in the control and contracting limb. In the in situ protocol, OVX muscles were significantly more resistant to fatigue compared to the SHAM animals. In addition, the muscles from OVX mice demonstrated significantly lower levels of normalized AMPK phosphorylation at rest. AMPK phosphorylation was not increased in the muscles from SHAM mice after the in situ contractile protocol, while the OVX demonstrated significant increases in AMPK phosphorylation. After contraction, normalized ERK2 phosphorylation was significantly higher in the OVX group compared to the SHAM group. Both p38 and JNK phosphorylation increased in response to contraction; but no group differences were detected. A second set of SHAM and OVX animals were subjected to fatigue stimulated under in vitro conditions. Significant increases in AMPK and ERK2 phosphorylation were detected, but no differences were found between groups. In conclusion, removal of the ovaries results in different responses to contraction-induced changes in phosphorylation of AMPK and ERK2 in female mice and suggests hormones secreted from the ovaries significantly impacts cellular signaling in skeletal muscle. J. Cell. Biochem. 107: 171–178, 2009.

KEY WORDS: SIGNALING; FATIGUE; OVARIAN; GENE EXPRESSION; MUSCLE

Repetitive skeletal muscle contraction induces alterations in energetic homeostasis, which can contribute to changes in gene expression by altering activation patterns of signaling proteins in the muscle cell. Although the contraction-induced cellular mechanisms that produce these physiological effects are unclear, recent evidence suggests that AMP-activated kinase (AMPK) and members of the mitogen-activated protein kinase (MAPK) family play an active role in regulating these processes [Kramer and Goodyear, 2007; Osler and Zierath, 2008]. Although prior evidence has demonstrated that muscle contraction is a sufficient stimulus to induce an increase in activation of AMPK or any of the MAPK family members, other evidence has suggested that various chronic conditions can affect the ability of contraction to induce activation of these proteins [Williamson et al., 2003; Thomson and Gordon, 2005; Sriwijitkamol et al., 2006].

AMPK is a heterotrimeric protein composed of one alpha, one beta, and one gamma subunit [Osler and Zierath, 2008]. The variety of different subunits results in a number of different possible combinations of AMPK, however only three appear to be present in skeletal muscle ($\alpha1/\beta2/\gamma1$, $\alpha2/\beta2/\gamma1$, and $\alpha2/\beta2/\gamma3$) [Birk and Wojtaszewski, 2006]. Repetitive muscle contraction can induce increases in AMPK activity by altering the AMP/ATP ratio [Jorgensen et al., 2006], thus activating an allosteric mechanism rendering AMPK a better substrate for upstream kinases and

Grant sponsor: National Institutes of Health Grant; Grant numbers: AR051396, AR053318, K01AR053235, MDA 4278.
*Correspondence to: Dr. Espen E. Spangenburg, PhD, Department of Kinesiology, University of Maryland, College Park, MD 21045. E-mail: espen@umd.edu
Received 19 December 2008; Accepted 29 January 2009 • DOI 10.1002/jcb.22113 • 2009 Wiley-Liss, Inc.
Published online 3 March 2009 in Wiley InterScience (www.interscience.wiley.com).

enhancing phosphorylation on the Thr172 residue of the α-subunit. AMPK activation is thought to affect numerous mechanisms including regulation of gene expression [McGee and Hargreaves, 2008], glucose uptake [Jorgensen et al., 2006], and lipid oxidation [Osler and Zierath, 2008] by the muscle.

Various roles have been ascribed to the MAPK family members, ERK 1/2, p38, and JNK during contraction, with particular emphasis on regulation of gene expression [Kramer and Goodyear, 2007]. For example, p38 activation during contraction is thought to contribute to increased PGC-1α transcription by activating the transcription factor ATF-2 [Yan et al., 2007], which would be predicted to result in a more oxidative phenotype. JNK activation in muscle is thought to play a role in altered gene expression [Ryder et al., 2000; Hilder et al., 2003]. Specifically, JNK1 KO mice have altered glucose and insulin dynamics at rest [Hirosumi et al., 2002]. The role of ERK 1 and 2 in skeletal muscle remains unclear, although recent evidence has suggested that they may contribute to changes in lipid metabolism in skeletal muscle [Kramer and Goodyear, 2007] by affecting hormone sensitive lipase (HSL) [Donsmark et al., 2003] and FAT/CD36 [Turcotte et al., 2005]. The MAPK family clearly has the ability to target gene expression and proteins that can affect metabolic function of skeletal muscle.

Through undefined cellular mechanisms the loss of ovarian hormones (i.e., menopause) affects numerous physiological processes in both striated muscle and other tissues [Campbell and Febbraio, 2002; D'Eon et al., 2005; Moran et al., 2006]. We and others have previously demonstrated that loss of ovarian hormones results in a failure of mechanical loading to induce activation of $p70^{s6k}$ in female rats [McClung et al., 2006; Sitnick et al., 2006; McClung et al., 2007]. Thus, there is a fair amount of evidence documenting that skeletal muscle is a target tissue of ovarian hormones. However, there is no evidence indicating what the role of ovarian hormones is in regulating the activation of various signaling proteins in response to repetitive contractures of skeletal muscle. Thus, the purpose of this investigation was to determine if ovarian hormone status is critical for contraction-induced activation of AMPK or MAPK in skeletal muscle. It was hypothesized that ovariectomy would result in attenuated activation of AMPK and MAPK in response to repetitive contractions in muscle.

METHODS

ANIMALS

Eight-week-old virgin female C57/BL6 (Harlan) mice were used for these studies. Half of the animals were bilaterally ovariectomized (OVX) according to previously described methods [Sitnick et al., 2006] and were returned to their cages for 8 weeks. The SHAM female mice were housed in the same cage to encourage synchronization (The Whitten effect) of the estrous cycle. In our hands, bilateral ovariectomy lowers circulating estrogens down to levels approximately 30% of normal (~4.0 pg/ml) within 24 h of the surgery and remains depressed [Sitnick et al., 2006]. The other half of the animals underwent SHAM surgeries where the animals underwent the same surgical procedure, but did not have their ovaries removed. All experiments were conducted on female mice at ~16 weeks of age. All experiments were approved by the University of Maryland Animal Care and Use Committee.

MUSCLE FATIGUE PROTOCOLS

Contractile fatigue of the tibialis anterior muscle (TA) and extensor digitorum longus (EDL) muscle was induced in a similar manner as previously described [Spangenburg et al., 1998, 2008; Lees et al., 2001; Sakamoto et al., 2005], where we found that approximately an 80% reduction in muscle force production resulted in a 77% reduction in muscle glycogen levels [Lees et al., 2001].

IN SITU

We used four SHAM and four OVX mice to compare fatigue characteristics in a model where muscle blood flow is intact. All mice were tested on the same day. Animals were anesthetized with isoflurane (2% with oxygen flow rate of 0.5 L/min) and surgical anesthesia was confirmed by lack of response to a normally painful stimulus (pinching the foot). With the animal supine, the hindlimb was stabilized and the distal tendon of the TA muscle was released. Its proximal portion was secured in a custom made clamp and attached to a load cell (Grass Instruments FT03) with a suture tie (4.0 coated Vicryl). Contractions of the TA were induced by direct stimulation via the peroneal nerve and single twitches (rectangular pulse, 1 ms) were applied at different muscle lengths to determine the optimal length (resting length L_0, measured with calipers as the distance between the tibial tuberosity and the myotendinous junction). With muscles set at L_0, maximum tetanic force (P_0) was measured (100 Hz) and then fatigue was induced through tetanic contractions (100 Hz for 100 ms) delivered every 2 s for 5 min. Maximal tetanic tension was measured during continuous stimulation and expressed as a percentage of P_0, to provide an index of fatigue. The fatigued and contralateral TA muscles were immediately removed, weighed, and frozen in liquid nitrogen.

IN VITRO

These experiments were performed according to previously described methodology [Spangenburg et al., 2008]. All mice were tested on the same day. Here muscle fatigue was induced in a surgically excised EDL muscle; therefore fatigue occurred without blood flow to the muscle. In brief, one of the EDL muscles was surgically excised with ligatures at each tendon (5-0 silk suture) and mounted in an in-vitro bath between a fixed post and force transducer (Aurora 300B-LR) operated in isometric mode. The muscle was maintained in physiological saline solution (PSS; pH 7.6) containing (in mM) 119 NaCl, 5 KCl, 1 $MgSO_4$, 5 $NaHCO_3$, 1.25 $CaCl_2$, 1 KH_2PO_4, 10 HEPES, 10 dextrose, and maintained at 30°C under aeration with 95 O_2/5 CO_2 (%) throughout the experiment. Resting tension, muscle length and stimulation current was iteratively adjusted for each muscle to obtain optimal twitch force. During a 5 min equilibration, single twitches were elicited at every 30 s with electrical pulses (0.5 ms) via platinum electrodes running parallel to the muscle. Optimal resting tension was determined. Following a 15 min rest period, the muscle was stimulated to fatigue by delivering tetanic trains (100 Hz for 100 ms) every 2 s for 5 min. After the experimental protocol, the muscle length was determined with a digital micrometer, the muscle was trimmed

proximal to the suture connections, weighed, and frozen in liquid nitrogen.

MUSCLE HOMOGENIZATION

The TA or EDL muscles were homogenized on ice in buffer containing 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7{\cdot}H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, 50 μg/ml leupeptin, 50 μg/ml pepstatin, 40 μg/ml aprotinin, 5 mM Na_3VO_4, and 1 mM PMSF as previously described [Spangenburg and McBride, 2006; Spangenburg et al., 2006]. After homogenization, the samples were centrifuged at 13,000*g* for 10 min and the supernatants were stored at −80°C. The protein concentration of the samples was determined in triplicate via the BCA assay (Pierce, Rockford, IL).

AMPK AND MAPK (ERK 1/2, P38, AND JNK) MEASUREMENTS

Measurement of phosphorylation of Thr172 is often used as indicator of activation of AMPK. These measurements appear to supply the most reliable indicator of AMPK activation, since in vitro AMPK activity results in the loss of any allosteric activation [Ponticos et al., 1998]. Seventy-five micrograms of total protein for each sample was loaded onto 7.5% SDS–PAGE gels for AMPK, p38, JNK, and ERK 1/2. All gels were run at 150 V for 1 h to separate proteins and then transferred onto PVDF membranes. To confirm successful transfer of protein and equal loading of lanes the membranes were stained with Ponceau S (not shown). After successful transfer, the membrane was placed in blocking buffer (5% non-fat dry milk) for 1 h at room temperature, serially washed and incubated with primary antibody overnight at 4°C. The primary antibodies were specific to AMPK (Thr172), AMPK (total), ACC (Ser72), p38 (Thr180/Tyr182), p38 total, JNK/SAPK (Thr183/Tyr185), JNK/SAPK (total), ERK 1/2 (Thr202/Tyr204), and ERK 1/2 (total) (Cell Signaling, Boston, MA). After another serial wash with TBS-T (3 × 5 min), the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Enhanced chemiluminescence reagent (Pierce) was used to detect the HRP activity by exposure in a gel documentation system (GeneGnome, SynGene, Frederick, MD) for the appropriate durations to keep the integrated optical densities (IODs) within a linear and non-saturated range for all bands of each membrane. The IODs were quantified using SynGene densitometry software (Frederick, MD).

NON-ESTERIFIED FREE FATTY ACIDS (NEFA) AND GLUCOSE MEASUREMENTS

NEFA was measured in serum isolated from each mouse after a 4-h fast using a colorimetric assay (Wako Diagnostics, Richmond, VA). Blood glucose was measured using a YSI Glucose Analyzer (YSI Life Sciences, Yellow Springs, OH).

STATISTICS

All data are expressed as means ± SE. Statistical significance for all immunoblotting data was determined using a two-way ANOVA; when an interaction was found the test was followed by a Tukey's post-hoc test. A *P*-value of <0.05 was considered significant. Statistical significance for all blood variables, force and fatigue data were determined using a one-way ANOVA; if significance was achieved the test was followed by a Tukey's post hoc test. A *P*-value of <0.05 was considered significant. A post hoc power analysis was conducted upon completion of the experiments and the lowest power level achieved was 0.62, while the majority of experiments achieved a power level of 0.7 or higher.

RESULTS

IN SITU MUSCLE CONTRACTION

Absolute and normalized tetanic force production was not statistically different between groups (Table I). TA muscle mass from the OVX group was significantly larger by 14.3% compared to the SHAM TA muscle mass. We also found that prior to any contractile experiments, OVX animals exhibited significant elevations in plasma NEFA (OVX 0.926 mmol/L vs. SHAM 0.712 mmol/L ($P < 0.05$)) and blood glucose levels (OVX 221.42 mg/dl vs. SHAM 188.1 mg/dl ($P < 0.05$)) compared to the SHAM animals. In the in situ model, the OVX animals were significantly more resistant to the development of fatigue than the SHAM animals (Fig. 1).

There was no significant increase in normalized AMPK phosphorylation in the SHAM animals, but AMPK phosphorylation was significantly increased by 42% after muscle fatigue in the OVX animals (Fig. 2). AMPK phosphorylation in the fatigued muscles from the OVX animals was also significantly elevated compared to the fatigued muscles from the SHAM animals (Fig. 2). Interestingly, the SHAM animals exhibited significantly higher levels of AMPK phosphorylation in the control muscles (no contraction) when compared to the control muscles from the OVX animals (Fig. 2). ACC phosphorylation was significantly increased after the contractions in both the SHAM and OVX animals, but no differences were detected between groups.

Normalized ERK2 phosphorylation was significantly increased after muscle fatigue in the OVX animals and in the SHAM animals (Fig. 3). ERK1 phosphorylation did not appear to be as sensitive to contraction as ERK2 (Fig. 3). Interestingly, in both groups total ERK2 levels significantly decreased in the isolated cytosolic fraction after contraction, significantly more so in the OVX group than the SHAM (quantitative data not shown). Thus, when normalized to total ERK2 the OVX animals exhibited significant increases in ERK2 phosphorylation compared to the Sham animals (Fig. 3).

p38 phosphorylation was significantly increased after muscle fatigue in both the OVX animals and SHAM animals (Fig. 4). There were no differences between groups in normalized p38 phosphorylation before or after contraction (Fig. 4).

TABLE I. Tibialis Anterior (TA) Muscle Force Production and Muscle Mass

	SHAM	SEM	OVX	SEM
P_0 (g)	54.33	4.45	65.60	8.25
P_0 (g/mm^2)	19.83	1.73	19.73	1.85
TA mass (mg)	42.50	1.81	48.72*	2.17

Values are means ± SEM.
*$P < 0.05$.

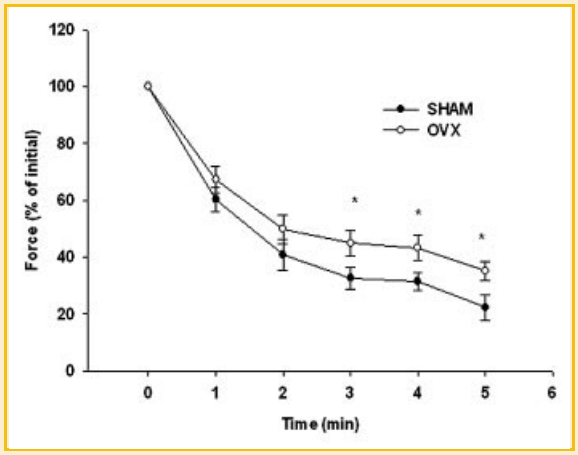

Fig. 1. Tibialis anterior (TA) in situ fatigue measurements in 16 weeks old SHAM and OVX mice. Maximal tetanic contractions were induced every 2 s for 5 min. Fatigue was less in OVX mice (- ○ -) compared to SHAM mice (- ● -) (SHAM n = 4; OVX n = 4). * Indicates statistically different from SHAM mice ($P < 0.05$). No difference was found between the OVX and SHAM muscle using an in vitro protocol (not shown).

JNK phosphorylation was significantly increased after muscle fatigue in the OVX animals and in the SHAM animals (Fig. 5). There were no differences in normalized JNK phosphorylation between groups before or after contraction (Fig. 5).

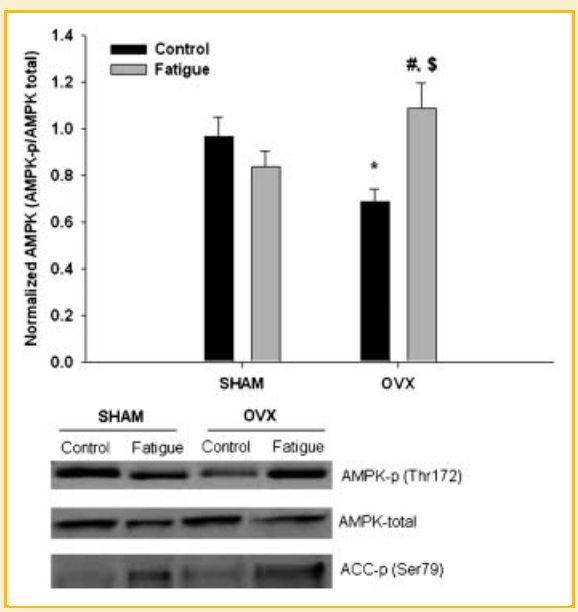

Fig. 2. Effects of repetitive in situ muscle contraction on normalized AMPK (Thr172) and ACC (Ser 79) phosphorylation in SHAM and OVX mice. * Indicates statistically different from SHAM control, $ statistically different from SHAM fatigue, or # statistically different from OVX control ($P < 0.05$). (SHAM n = 4; SHAM fatigue n = 4; OVX n = 4; OVX fatigue n = 4.) Example immunoblots are depicted below the graph.

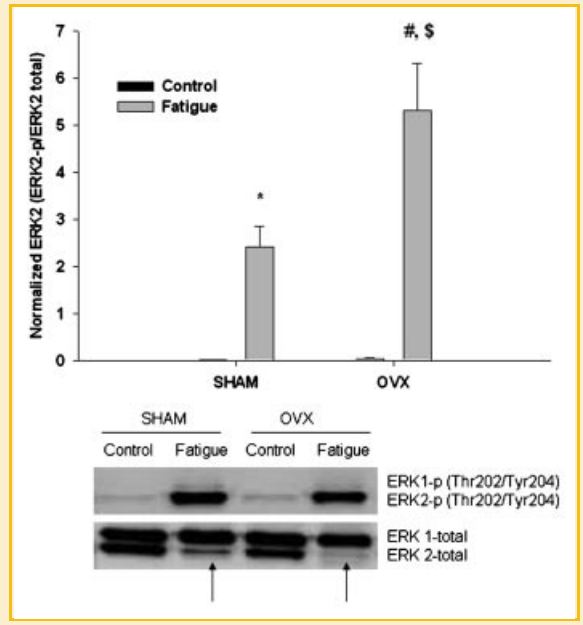

Fig. 3. Effects of repetitive in situ muscle contraction on normalized ERK2 phosphorylation (Thr202/Tyr204) in SHAM and OVX mice. Repetitive contractions resulted in significant increases in normalized levels of ERK2 phosphorylation. Significant decreases in total ERK2 expression occurred after contraction in both groups (see arrows), with greater decreases in total ERK2 in the OVX animals compared to the SHAM animals (quantitative data not shown). * Indicates statistically different from SHAM control or # statistically different from OVX control or $ statistically different from SHAM fatigue ($P < 0.05$). (SHAM n = 4; SHAM fatigue n = 4; OVX n = 4; OVX fatigue n = 4.) Example immunoblots are depicted below the graph.

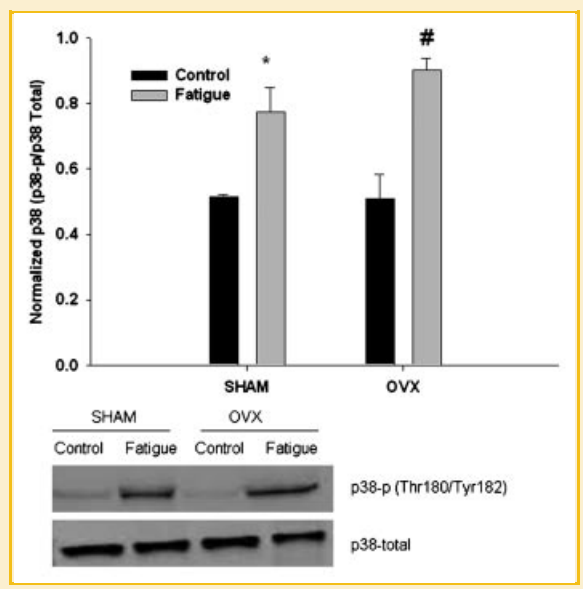

Fig. 4. Effects of repetitive in situ muscle contraction on normalized p38 phosphorylation (Thr180/Tyr182) in SHAM and OVX mice. * Indicates statistically different from SHAM control or # statistically different from OVX control ($P < 0.05$). (SHAM n = 4; SHAM fatigue n = 4; OVX n = 4; OVX fatigue n = 4.) Example immunoblots are depicted below the graph.

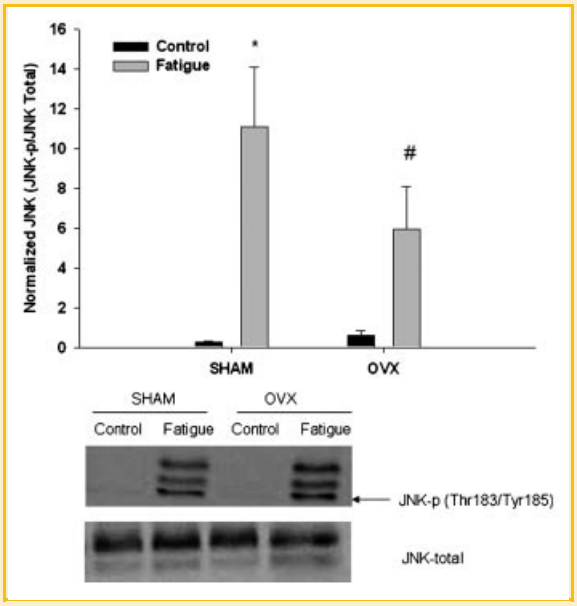

Fig. 5. Effects of repetitive in situ muscle contraction on normalized JNK phosphorylation (Thr183/Tyr185) in SHAM and OVX mice. * Indicates statistically different from SHAM control or # statistically different from OVX control ($P < 0.05$). (SHAM n = 4; SHAM fatigue n = 4; OVX n = 4; OVX fatigue n = 4.) Example immunoblots are depicted below the graph.

IN VITRO MUSCLE CONTRACTION

These data suggest that removal of the ovaries from female mice alters the ability of contraction to induce changes in signaling events. To determine if this effect was mediated by a circulating factor, we employed an in vitro contractile model where the EDL muscle was physically removed from the animal. If there was a circulating factor in the blood that was critical for affecting the signaling mechanisms in the female animal, then removing any exposure to the circulating factor would remove the effect. In the in vivo experiments, blood flow remained intact during contractions, allowing exposure of the muscle to any circulating factor. With the in vitro model, blood flow was not intact, precluding exposure of circulating factors to the muscle. The EDL was utilized for the in vitro experiments since it is not possible to use the TA in an in vitro bath model. However, these muscles are similar in that they contain similar fiber type compositions and both are dorsiflexors [Burkholder et al., 1994]. The same fatigue protocol was used for both the in vitro protocol and the in situ protocol. No differences were detected in fatigue characteristics between the groups (data not shown).

In the in vitro model, normalized AMPK and ERK2 phosphorylation was significantly increased to similar levels in both the SHAM and OVX animals after muscle fatigue was induced (Fig. 6A,B). No significant differences were detected between groups. Since no differences were detected in p38 or JNK phosphorylation in the in situ models, no in vitro data are presented for these signaling proteins.

DISCUSSION

Our findings suggest that ovarian hormones affect the ability of repetitive muscle contraction to induce increases in AMPK and ERK2 phosphorylation. Interestingly, these differences are not discernable in all models of muscle contraction. When the muscle from a SHAM female mouse was fatigued in situ (i.e., blood flow intact), there was no increase in AMPK phosphorylation, whereas in an OVX animal there was a significant increase. At baseline, the OVX group exhibited reduced levels of AMPK phosphorylation compared to the SHAM animals. Also, normalized AMPK and ERK2 phosphorylation was found to be higher after contractions in the OVX animals compared to the SHAM animals. However, when the muscle was removed from the animal and stimulated in vitro, these differences between groups did not occur. These data demonstrate that a circulating factor(s), presumably from the ovaries, affects the ability of contraction to activate AMPK and ERK2 in skeletal muscle.

With respect to AMPK, these data support similar findings in humans, in which females did not increase AMPK activation in response to exercise when compared to men [Roepstorff et al., 2006]. These differences in AMPK phosphorylation between genders appear to be due to minimal changes that occurred in the AMP/ATP ratio in the women during the exercise bout [Roepstorff et al., 2006]. Our findings confirm those results and extend them by indicating that intact blood flow is critical to replicate these findings. However, if the muscle is removed from the female animal and placed in an in vitro system, then AMPK phosphorylation occurs, as would be predicted. It appears that ovarian hormones may contribute to this effect, in that the OVX animals showed an expected contraction-induced increase in AMPK phosphorylation, just as males do [Roepstorff et al., 2006]. It should be noted that the gender effect appears to only be detected in AMPK measured from a cytosolic fraction, since recent data have found no gender differences in AMPK phosphorylation isolated from a nuclear fraction [Vissing et al., 2008].

The reduction in AMPK activation at rest in the OVX animals compared to the SHAM animals could result in a number of molecular or physiological effects in the skeletal muscle. For example, AMPK has been shown to affect gene transcription of a number of targets including GLUT4 [McGee et al., 2008], VEGF [Zwetsloot et al., 2008], or PGC-1α [Irrcher et al., 2008]. Interestingly, previous publications have shown that GLUT4 levels are reduced in OVX animals [Saengsirisuwan et al., 2009] and VEGF levels are reduced in post-menopausal women [Croley et al., 2005], thus it is possible that the changes in basal AMPK activation in the OVX animals may contribute to these alterations in gene expression. It is also equally possible that alterations in AMPK activity in the OVX animals contribute to changes in fiber type, since it has been suggested recently that genetic alterations in AMPK affect fiber type composition of skeletal muscle [Rockl et al., 2007]. However, in our hands, and in the hands of other laboratories, minimal changes have been detected in the fiber types of muscle from OVX animals (data not shown). For example, others have not found significant changes in fiber type percentages based on myosin heavy chain staining of individual fibers when comparing OVX and SHAM animals [McCormick et al., 2004; Piccone et al., 2005; Moran et al., 2007].

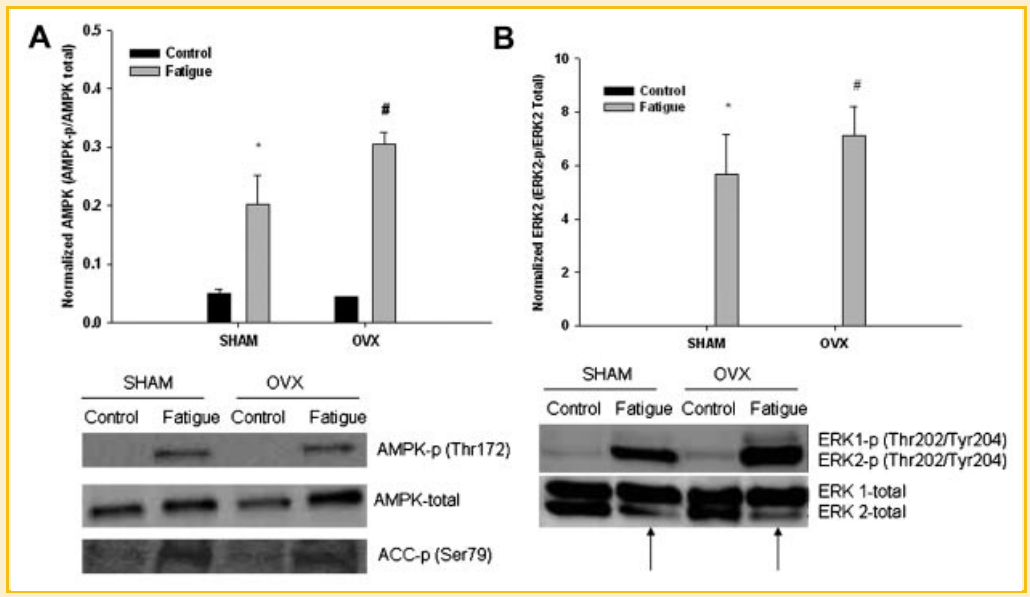

Fig. 6. A,B: Effects of repetitive in vitro muscle contraction on normalized AMPK (Thr172) (A) and normalized ERK2 (Thr202/Tyr204) (B) phosphorylation in SHAM and OVX mice. * Indicates statistically different from SHAM control or # statistically different from OVX control ($P < 0.05$). (SHAM n = 4; SHAM fatigue n = 4; OVX n = 4; OVX fatigue n = 4.) Example immunoblots are depicted underneath the graph.

Changes in circulating ovarian hormones had a clear affect on contraction induced-activation of ERK2 in skeletal muscle. Although the effect was not visible by measurement of absolute levels of ERK2 phosphorylation, we observed a decrease in the total amount of ERK2 in our cytosolic fraction in the OVX animals compared to the SHAM animals. Thus, when normalized to the total amount of ERK2, ERK2 phosphorylation was increased in the OVX animals. The decrease in ERK2 total levels in our cytosolic fraction is likely not due to degradation of the protein, but rather to translocation of ERK to a cellular compartment that is not visible in our cytosolic fraction. There is precedence for ERK2 movement in other tissues with contraction or stretch [Kawamura et al., 2003], but this is the first indirect evidence of translocation in contracting skeletal muscle (although ERK translocation to the nucleus has been shown to be influenced by changes in estrogen levels in other non-muscle tissue) [Chen et al., 2005]. Experiments are currently being pursued to determine if cellular movement of ERK2 does in fact occur in response to muscle contraction. Our data suggest that a decrease in ovarian hormones results in enhanced cytosolic phosphorylation of ERK2 after contraction, although the down-stream consequence of this effect is unclear at the moment.

A number of roles have been defined for the MAPK signaling family in contracting muscle. In particular, there is good evidence that members of the MAPK family contribute to contraction-induced alterations in muscle specific gene expression [Kramer and Goodyear, 2007]. Previous research has suggested that OVX animals exhibit increased tissue inflammation at rest, as measured by elevations in various pro-inflammatory cytokines and subsequent activation of p38 and JNK [Meldrum et al., 2005; Wang et al., 2006]. Thus, it was surprising that we detected no differences in p38 or JNK phosphorylation at rest between the OVX and SHAM animals, since both p38 and JNK are stress activated kinases and sensitive to various inflammatory cytokines. In addition, there is also evidence suggesting that activation of the ERK proteins may contribute to regulation of lipid metabolism during contraction. Activation of ERK signaling is thought to regulate increased mobilization of triacylglycerol by means of increasing the activity of HSL [Donsmark et al., 2003]. We made an effort towards measuring changes in HSL phosphorylation to determine if it was altered in our preparation, however we were unable to successfully quantify it due to a weak signal of HSL. This may in part be due to the fact that we performed our contractile measures in the TA and EDL muscles, where expression of HSL would be predicted to be lower [Langfort et al., 1999]. Furthermore, the inhibition of ERK signaling has been shown to block free fatty acid (FFA) uptake by decreasing CD36/FAT presence at the muscle membrane and decreasing fat oxidation [Raney and Turcotte, 2007]. It is possible that, when compared to SHAM animals, the contracting muscles in the OVX animals are capable of increased triacylglercol mobilization and FFA uptake during repeated contraction. OVX animals did exhibit increased levels of circulating FFAs compared to the SHAM animals. Although untested at this time, we predict that this would result in sparing of stored muscle glycogen and improved fatigue resistance. The enhanced activation of ERK2 may explain why the OVX muscles exhibited increased fatigue resistance in response to in situ contraction, even though AMPK activation levels were similar.

At this time, the mechanism that provided the OVX animals with resistance to in situ muscle fatigue remains unclear; particularly when one considers that the protection was lost in the in vitro stimulation model. Since there were no discernible contractile differences between the SHAM and OVX animals in the in vitro model, it appears that mechanisms that alter EC-coupling are not

affected in this model. However, when in the in situ model (where blood flow is maintained), it is possible that a circulating factor could affect the susceptibility to muscle fatigue. Previous studies have shown that muscle fatigue can be delayed when blood glucose levels are elevated [Helander et al., 2002], which we found to occur in the OVX animal at rest.

In conclusion, these findings suggest that females have a circulating ovarian hormonal factor(s) that can affect the ability of muscle contraction to alter the phosphorylation status of AMPK and ERK2. The differences were consistently found in the in situ model of muscle contraction, where blood flow remains intact, while the differences were lost in the in vitro model, where the muscle is removed from the animal. The in vitro model removes the muscle from exposure to any circulating factors, by placing the muscle in a bath system. Thus when comparing the in situ and in vitro data, we conclude that the skeletal muscle is exposed to a circulating factor, that maybe generated by the ovary, which has an effect on contraction-induced activation of signaling events in the muscle. Together with previously published data, these data indicate that ovarian hormone status is a critical consideration when understanding cellular regulation of muscle processes in females.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant AR051396 (EES), AR053318 (CWW), K01AR053235 (RML), and MDA 4278 (RML). Also, the authors wish to thank Dr. David Thomson for helpful discussion of the data.

REFERENCES

Birk JB, Wojtaszewski JF. 2006. Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. J Physiol 577:1021–1032.

Burkholder TJ, Fingado B, Baron S, Lieber RL. 1994. Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. J Morphol 221:177–190.

Campbell SE, Febbraio MA. 2002. Effect of the ovarian hormones on GLUT4 expression and contraction-stimulated glucose uptake. Am J Physiol Endocrinol Metab 282:E1139–E1146.

Chen JR, Plotkin LI, Aguirre JI, Han L, Jilka RL, Kousteni S, Bellido T, Manolagas SC. 2005. Transient versus sustained phosphorylation and nuclear accumulation of ERKs underlie anti-versus pro-apoptotic effects of estrogens. J Biol Chem 280:4632–4638.

Croley AN, Zwetsloot KA, Westerkamp LM, Ryan NA, Pendergast AM, Hickner RC, Pofahl WE, Gavin TP. 2005. Lower capillarization, VEGF protein, and VEGF mRNA response to acute exercise in the vastus lateralis muscle of aged vs. young women. J Appl Physiol 99:1872–1879.

D'Eon TM, Souza SC, Aronovitz M, Obin MS, Fried SK, Greenberg AS. 2005. Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. J Biol Chem 280:35983–35991.

Donsmark M, Langfort J, Holm C, Ploug T, Galbo H. 2003. Contractions activate hormone-sensitive lipase in rat muscle by protein kinase C and mitogen-activated protein kinase. J Physiol 550:845–854.

Helander I, Westerblad H, Katz A. 2002. Effects of glucose on contractile function [Ca^{2+}]i, and glycogen in isolated mouse skeletal muscle. Am J Physiol Cell Physiol 282:C1306–C1312.

Hilder TL, Tou JC, Grindeland RE, Wade CE, Graves LM. 2003. Phosphorylation of insulin receptor substrate-1 serine 307 correlates with JNK activity in atrophic skeletal muscle. FEBS Lett 553:63–67.

Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. 2002. A central role for JNK in obesity and insulin resistance. Nature 420:333–336.

Irrcher I, Ljubicic V, Kirwan AF, Hood DA. 2008. AMP-activated protein kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells. PLoS ONE 3:e3614.

Jorgensen SB, Richter EA, Wojtaszewski JF. 2006. Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise. J Physiol 574:17–31.

Kawamura S, Miyamoto S, Brown JH. 2003. Initiation and transduction of stretch-induced RhoA and Rac1 activation through caveolae: Cytoskeletal regulation of ERK translocation. J Biol Chem 278:31111–31117.

Kramer HF, Goodyear LJ. 2007. Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. J Appl Physiol 103:388–395.

Langfort J, Ploug T, Ihlemann J, Saldo M, Holm C, Galbo H. 1999. Expression of hormone-sensitive lipase and its regulation by adrenaline in skeletal muscle. Biochem J 340(Pt 2): 459–465.

Lees SJ, Franks PD, Spangenburg EE, Williams JH. 2001. Glycogen and glycogen phosphorylase associated with sarcoplasmic reticulum: Effects of fatiguing activity. J Appl Physiol 91:1638–1644.

McClung JM, Davis JM, Wilson MA, Goldsmith EC, Carson JA. 2006. Estrogen status and skeletal muscle recovery from disuse atrophy. J Appl Physiol 100:2012–2023.

McClung JM, Davis JM, Carson JA. 2007. Ovarian hormone status and skeletal muscle inflammation during recovery from disuse in rats. Exp Physiol 92:219–232.

McCormick KM, Burns KL, Piccone CM, Gosselin LE, Brazeau GA. 2004. Effects of ovariectomy and estrogen on skeletal muscle function in growing rats. J Muscle Res Cell Motil 25:21–27.

McGee SL, Hargreaves M. 2008. AMPK and transcriptional regulation. Front Biosci 13:3022–3033.

McGee SL, van Denderen BJ, Howlett KF, Mollica J, Schertzer JD, Kemp BE, Hargreaves M. 2008. AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. Diabetes 57:860–867.

Meldrum DR, Wang M, Tsai BM, Kher A, Pitcher JM, Brown JW, Meldrum KK. 2005. Intracellular signaling mechanisms of sex hormones in acute myocardial inflammation and injury. Front Biosci 10:1835–1867.

Moran AL, Warren GL, Lowe DA. 2006. Removal of ovarian hormones from mature mice detrimentally affects muscle contractile function and myosin structural distribution. J Appl Physiol 100:548–559.

Moran AL, Nelson SA, Landisch RM, Warren GL, Lowe DA. 2007. Estradiol replacement reverses ovariectomy-induced muscle contractile and myosin dysfunction in mature female mice. J Appl Physiol 102:1387–1393.

Osler ME, Zierath JR. 2008. Minireview: Adenosine 5'-monophosphate-activated protein kinase regulation of Fatty Acid oxidation in skeletal muscle. Endocrinology 149:935–941.

Piccone CM, Brazeau GA, McCormick KM. 2005. Effect of oestrogen on myofibre size and myosin expression in growing rats. Exp Physiol 90: 87–93.

Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D. 1998. Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. EMBO J 17:1688–1699.

Raney MA, Turcotte LP. 2007. Evidence for the regulation of contraction-induced fatty acid oxidation via extracellular signal-regulated kinase 1/2 activation independent of changes in fatty acid uptake. Metabolism 56: 1192–1200.

Rockl KS, Hirshman MF, Brandauer J, Fujii N, Witters LA, Goodyear LJ. 2007. Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates muscle fiber type shift. Diabetes 56:2062–2069.

Roepstorff C, Thiele M, Hillig T, Pilegaard H, Richter EA, Wojtaszewski JF, Kiens B. 2006. Higher skeletal muscle alpha2AMPK activation and lower energy charge and fat oxidation in men than in women during submaximal exercise. J Physiol 574:125–138.

Ryder JW, Fahlman R, Wallberg-Henriksson H, Alessi DR, Krook A, Zierath JR. 2000. Effect of contraction on mitogen-activated protein kinase signal transduction in skeletal muscle. Involvement of the mitogen- and stress-activated protein kinase 1. J Biol Chem 275:1457–1462.

Saengsirisuwan V, Pongseeda S, Prasannarong M, Vichaiwong K, Toskulkao C. 2009. Modulation of insulin resistance in ovariectomized rats by endurance exercise training and estrogen replacement. Metabolism 58:38–47.

Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A, Alessi DR. 2005. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. EMBO J 24:1810–1820.

Sitnick M, Foley AM, Brown M, Spangenburg EE. 2006. Ovariectomy prevents the recovery of atrophied gastrocnemius skeletal muscle mass. J Appl Physiol 100:286–293.

Spangenburg EE, McBride TA. 2006. Inhibition of stretch-activated channels during eccentric muscle contraction attenuates p70S6K activation. J Appl Physiol 100:129–135.

Spangenburg EE, Ward CW, Williams JH. 1998. Effects of lactate on force production by mouse EDL muscle: Implications for the development of fatigue. Can J Physiol Pharmacol 76:642–648.

Spangenburg EE, Brown DA, Johnson MS, Moore RL. 2006. Exercise increases SOCS-3 expression in rat skeletal muscle: Potential relationship to IL-6 expression. J Physiol 572:839–848.

Spangenburg EE, Le Roith D, Ward CW, Bodine SC. 2008. A functional insulin-like growth factor receptor is not necessary for load-induced skeletal muscle hypertrophy. J Physiol 586:283–291.

Sriwijitkamol A, Ivy JL, Christ-Roberts C, DeFronzo RA, Mandarino LJ, Musi N. 2006. LKB1-AMPK signaling in muscle from obese insulin-resistant Zucker rats and effects of training. Am J Physiol Endocrinol Metab 290: E925–E932.

Thomson DM, Gordon SE. 2005. Diminished overload-induced hypertrophy in aged fast-twitch skeletal muscle is associated with AMPK hyperphosphorylation. J Appl Physiol 98:557–564.

Turcotte LP, Raney MA, Todd MK. 2005. ERK1/2 inhibition prevents contraction-induced increase in plasma membrane FAT/CD36 content and FA uptake in rodent muscle. Acta Physiol Scand 184:131–139.

Vissing K, McGee SL, Roepstorff C, Schjerling P, Hargreaves M, Kiens B. 2008. Effect of sex differences on human MEF2 regulation during endurance exercise. Am J Physiol Endocrinol Metab 294:E408–E415.

Wang M, Tsai BM, Reiger KM, Brown JW, Meldrum DR. 2006. 17-beta-Estradiol decreases p38 MAPK-mediated myocardial inflammation and dysfunction following acute ischemia. J Mol Cell Cardiol 40:205–212.

Williamson D, Gallagher P, Harber M, Hollon C, Trappe S. 2003. Mitogen-activated protein kinase (MAPK) pathway activation: Effects of age and acute exercise on human skeletal muscle. J Physiol 547:977–987.

Yan Z, Li P, Akimoto T. 2007. Transcriptional control of the Pgc-1alpha gene in skeletal muscle in vivo. Exerc Sport Sci Rev 35:97–101.

Zwetsloot KA, Westerkamp LM, Holmes BF, Gavin TP. 2008. AMPK regulates basal skeletal muscle capillarization and VEGF expression, but is not necessary for the angiogenic response to exercise. J Physiol 586:6021–6035.