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PRINCIPAL INVESTIGATOR: Amber Lynn Pond

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Amber L. Pond and Gregory Howard Hockerman

E-Mail: apond@siumed.edu; gregh@purdue.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Southern IL University
1135 Lincoln Drive
Carbondale, IL 62901

Purdue University
575 Stadium Mall Drive
West Lafayette, IN 47907

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14. ABSTRACT

The ERG1A potassium channel is up-regulated in atrophic skeletal muscle and increases proteolysis when it is ectopically expressed in muscle. We have shown that, when it is expressed in cultured C2C12 myotubes, ERG1A increases the basal intracellular calcium concentration; however, the mechanism by which this occurs and the consequences of this are not known. Thus, we proposed to investigate the mechanism by which ERG1A increases intracellular calcium and the downstream effect of this on calpain enzyme-mediated proteolysis. We have now completed all Major Tasks. Major Task 1. We have determined that the increase in intracellular calcium is not a consequence of ERG1A modulation of L-type calcium channel gene expression or protein abundance nor modulation of L-type calcium channel conductance; nor is it a result of modulation of store operated calcium entry (SOCE). However, our data shows that the source of the HERG-induced calcium increase is intracellular stores through modulation of both IP3 signaling and ryanodine receptor channel activity. Major Task 2. We have determined that ERG1A does increase calpain activity mainly as a result of the increased calcium concentration and also a decrease in calpastatin protein abundance. Major Task 3. We prepared samples for Next Generation Sequencing which was completed. We viewed this large set of data and denoted certain ERG1-modulated gene sets of interest which has included members of both the IP3 and RYR1 signaling cascades, the retinol pathway, and modulators of reactive oxygen species. We have confirmed modulation of genes encoding members of the RYR and retinol pathways.

15. SUBJECT TERMS

ETHER-A-GOGO-RELATED GENE; skeletal muscle; atrophy; intracellular calcium concentration; calpain enzymes

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INTRODUCTION:

Skeletal muscle atrophy is a loss of muscle mass and function that can rapidly ensue with the muscle disuse that often accompanies an injury or illness. It complicates the healing and recovery processes and interferes with an affected individual's ability to perform necessary duties. Current pharmacological therapies to combat atrophy are inadequate. Indeed, exercise and good nutrition are the most beneficial treatments; however, not all ill or injured individuals can exercise because of limitations imposed by the injury or illness. Thus, development of additional effective treatments for preserving muscle tissue and promoting muscle growth is important and this will require greater knowledge of the cellular mechanisms contributing to atrophy. The ERG1A potassium channel has been shown to modulate proteolysis in skeletal muscle atrophy, however, the mechanism is not known. The goal of this project is to explore the role of the ERG1A channel in skeletal muscle atrophy, specifically the mechanism by which it increases intracellular calcium concentration (i.e., $[Ca^{++}]i$) and calpain activity. Through this work, we hope to open an area of research which will lead to discovery of new pharmacological targets for atrophy treatment.

KEYWORDS:

skeletal muscle; atrophy; ether-a-gogo related gene (ERG1A); potassium channel; intracellular calcium concentration; calcium signaling; calpastatin; calpain enzymes; calcium channel; Cav1.1

ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Explore the mechanism responsible for the calcium signal increase that occurs in cultured C2C12 myotubes in response to HERG1A transfection. *~100% Completed by end of the second no cost extension period.*

Subtask 1.A (Hockerman and Pond Labs*): Determine the source of the HERG1A-mediated increase in intracellular calcium levels using Fura-2 assays and specific pharmacological treatments. *The Pond lab worked on the FURA-2 studies so that the Hockerman lab could focus more on the single cell calcium imaging (subtask 1.A) and calcium current density studies (subtask 1.B.1 below). *100% Completed within second no cost extension period (2020-2021).*

Subtask 1.B.1 (Hockerman): Determine if there are HERG1A-mediated changes in Ca^{2+} current density using electrophysiology and specific pharmacological treatments. *100% Completed in second no cost extension period (2020-2021).*

Subtask 1.B.2 (Pond Lab): Determine if there are HERG1A-mediated changes in L-type calcium channel expression using quantitative PCR. Determine if there are changes in L-type channel protein abundances where indicated using immunoblot. *100% Completed in the first year (2018-2019).*

Subtask 1.C (Hockerman): Determine the effect of HERG1A on IP_3 levels in myotubes using an IP-ONE time-resolved fluorescence (TRF) assay kit (Cisbio). *100% Completed in second no cost extension (2020-2021).*

Major Task 2: Determine the effect of HERG1A on the activities and expression levels of proteolytic enzymes calpain 1 and 2 and the expression of calpastatin and calpain 3 in cultured C2C12 myotubes. ***100% Completed in first year (2018-2019) and published in Skeletal Muscle (see Appendix).***

Subtask 2.A (Pond): Determine the effect of HERG1A on calpain activity in C2C12 myotubes using the Calpain-Glo assay system (ProMega; Madison, WI). *100% Completed in the first year.*

Subtask 2.B (Pond): Determine the effect of HERG1A on expression of genes encoding Calpains 1, 2 and 3 and calpastatin in C2C12 myotubes using quantitative PCR. Where possible and indicated immunoblot will be used to quantify calpain and calpastatin proteins**.** *100% Completed in the first year.*

Major Task 3 (Pond): Determine the effect of HERG1A on gene expression in cultured C2C12 myotubes using qPCR arrays. ***100% Completed in second no cost extension period (2020-2021).***
Per DOD science reviewer recommendation, we performed Next Generation Sequencing through the Purdue University Genomics facility rather than doing the PCR arrays. This has generated MUCH more data for our use and we needed more time than originally planned to analyze and interpret this data. We have determined which genes we believe will be the most important to pursue and have used some appropriate primers with quantitative PCR to verify some of the RNAseq data. Indeed, we have found modulation of some genes contributing to IP3 and RYR1 signaling cascades as well as to the retinol pathway and reactive oxygen species signaling.

What was accomplished under these goals?

Progress Report: We are pleased to report that all Major Tasks have been 100% completed (in the time frames described above).

Major Task 1.

Subtask 1.A (Hockerman and Pond Labs): Using fura-2 calcium assays and the HERG blocker astemizole, we determined in year one that HERG expression causes an increase in basal intracellular calcium levels (Whitmore et al., 2020; see Appendix) as well as an increase in calcium levels induced by depolarization with 100 mM KCl (Pond & Hockerman Report 2019). We also explored the source of the intracellular calcium and showed that the increase in calcium is not a consequence of HERG modulation of L-type calcium channel function (Pond & Hockerman Report 2019). This was additionally supported by data showing that expression of Cav 1.X L-type calcium channel genes is not significantly affected at 48 hours post transduction nor is Cav1.X L-type channel protein abundances (Pond & Hockerman Report 2019; see Subtask 1.B.2 also). We also showed that the source of the calcium is intracellular stores because the HERG-mediated increase in intracellular calcium is sensitive to the calcium re-uptake inhibitor thapsigargin (Pond & Hockerman Report 2019).

In the second no cost extension period (2020-2021), we explored potential contributions to the HERG-mediated increase in calcium by *IP3 signaling*. Fura imaging experiments demonstrated that HERG-expression in myotubes more than doubled ($p<0.05$) the intracellular calcium concentration in response to treatment with bethanechol (Pond & Hockerman Report 2021), a reagent known to induce IP3 signaling through muscarinic receptor activation (Powell et al., 2001). Further, we showed that the HERG-induced increase was not affected by the phospholipase C inhibitor U73122, although it was inhibited by

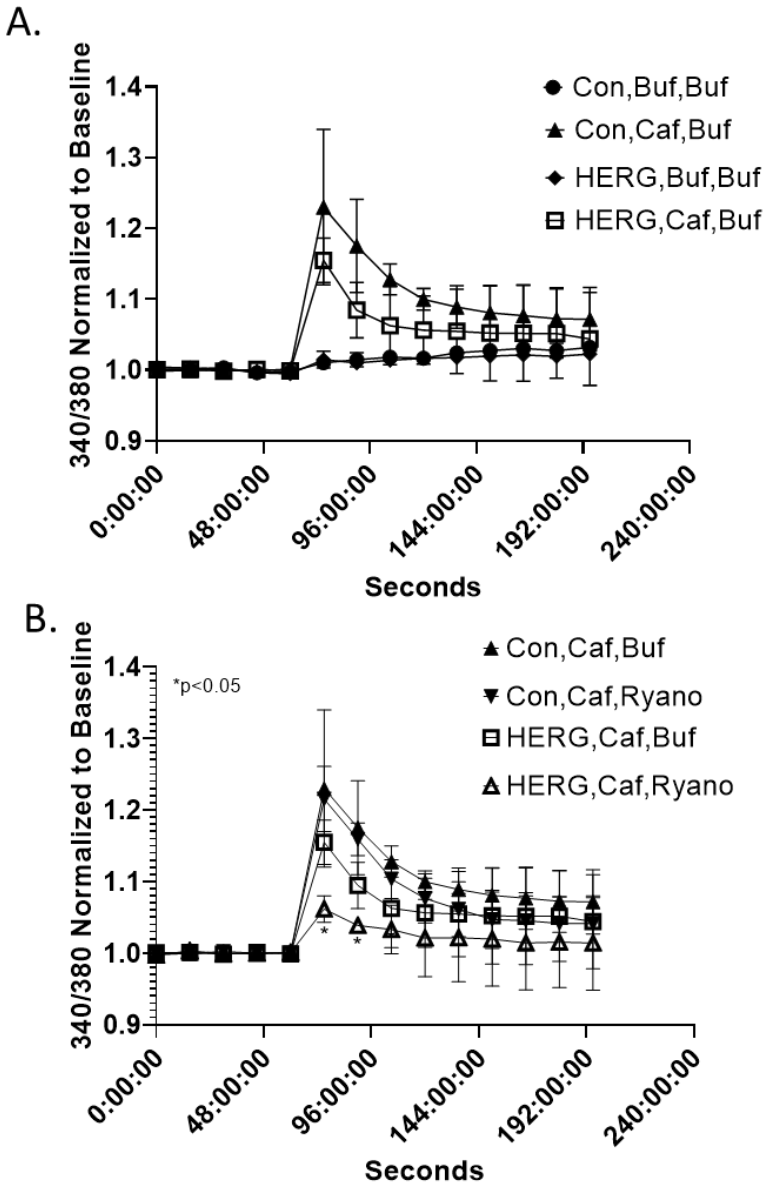

Figure 1. HERG modulates ryanodine receptors to produce an increase in intracellular calcium concentration. A. Caffeine induces calcium release in control and HERG expressing myotubes. B. Ryanodine significantly inhibits the HERG-induced calcium release in myotubes.

xestospongin C, a known IP3 receptor 1 (IP3R1) antagonist (Pond & Hockerman Report 2021). These data strongly suggest that, although HERG does not affect phospholipase C activity, the calcium increase could be, at least in part, a response to HERG-modulation of the IP3 receptor.

More interestingly, during the <u>first no cost extension period (2019-2020)</u>, we explored the effect of ryanodine and initially reported (Pond & Hockerman Report 2020) that the HERG1A-mediated increase in intracellular calcium likely does not result from ryanodine receptor activation. [Ryanodine receptors are found in the sarcoplasmic reticulum (SR) of skeletal muscle cells and interact with dihydropyridine receptors/Cav1.1 channels to release calcium from the SR into the surrounding cytoplasm. Ryanodine molecules will block or activate this calcium release depending upon the dose (Woo et al., 2020).] However, because our RNAseq data showed modulation of calsequestrin 1 (CaSeq1), known to modulate RYR1, we revisited our FURA work, carefully first performing a broad dose response curve with ryanodine to ensure our concentration of drug would block (rather than activate) RYR1 proteins. We found a triphasic calcium response to dose in our C2C12 myotubes. Thus, we repeated the fura experiments with a more appropriate dose of ryanodine (90 uM final) shown to BLOCK the receptors of our myotubes. That is, we treated HERG1A-transduced (and control-transduced) myotubes with ryanodine to block RYR1 and measured its effect on the intracellular calcium released in response to the RYR1 activator, caffeine. Our results indicate that caffeine induces a calcium response in HERG-expressing and control cells (Figure 1A). More impressive is the fact that the HERG-induced increase in $[Ca^{++}]i$ is inhibited by ryanodine when it is compared to non-ryanodine treated HERG-expressing cells (Figure 1B; $p<0.05$, $n=3$). Indeed, $[Ca^{++}]i$ is more greatly inhibited by ryanodine in HERG-expressing cells when compared to all transduced cells (HERG and control) with the difference being statistically significant at 15, 30, and 45 seconds post injection (Figure 1B); this indicates that HERG induces calcium release through modulation of the skeletal

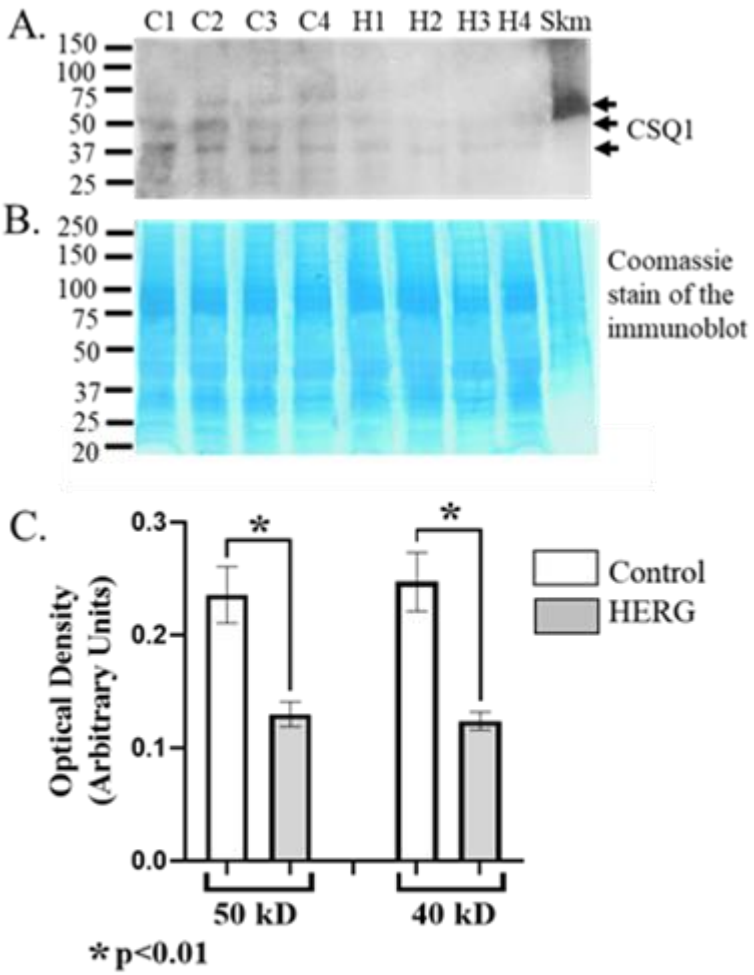

Figure 2. HERG decreases calsequestrin protein abundance after 48 hours of expression. A. Immunoblot of control and HERG-expressing myotubes. B. Coomassie stain of the immunoblot confirms that equal amounts of protein were loaded into the lanes. C. Relative abundance of calsequestrin protein as measured by optical density.

muscle RYR1. To strengthen this result, we followed up on the exciting RNAseq data which suggested that HERG modulates expression of calsequestrin 1 (CaSeq1), a molecule known to act as a calcium sensor to inhibit RYR1 activity (thereby, lowering calcium release) at higher calcium concentrations (Woo et al., 2020). Thus, by lowering CaSeq1 levels, HERG would indeed effectively activate RYR1 and increase intracellular calcium concentration. Indeed, our data demonstrate that HERG expression in myotubes results in a 44.9% decrease in 50 kD CaSeq1 and a 49.8% decrease in 40 kD CaSeq1 protein abundances (Figure 2; $p<0.05$, $n=8$). This novel discovery reveals that HERG is a player in RYR modulation. This information has the potential to affect multiple tissues and numerous disease states.

Here, we also report that the source of the increase in $[Ca^{++}]i$ is not store operated channel entry (SOCE; Figure 3). Store operated calcium entry is the process by which emptying the endoplasmic reticulum (ER) results in an influx of calcium across the plasma membrane through calcium selective cation channels. Reuptake of calcium into the ER by SERCA inhibits SOCE activity (Hogan & Rao 2015). We assayed SOCE using fura dyes to detect calcium and thapsigargin to block SERCA and thereby empty the ER. We then measured calcium influx into the cells by overwhelming the extracellular environment with calcium with and without the SOCE blocker, 2-APB (Figure 3A). The data show that, although block of SOCE does lower calcium concentration (influx) in control cells, it does not do so in HERG-expressing cells (Figure 3B); thus, HERG does not modulate SOCE.

Subtask 1.B.1 (Hockerman Lab). The exploration of the effects of HERG on calcium current density have been completed. HERG expression in C2C12 myotubes did not significantly change the L-type current density (Pond & Hockerman Report 2021). However, we originally reported that analysis of recordings found that the transient T-type currents (observed at the beginning of traces) were of a larger amplitude, relative to the L-type current amplitude (within the same trace) in myotubes expressing HERG (Pond &

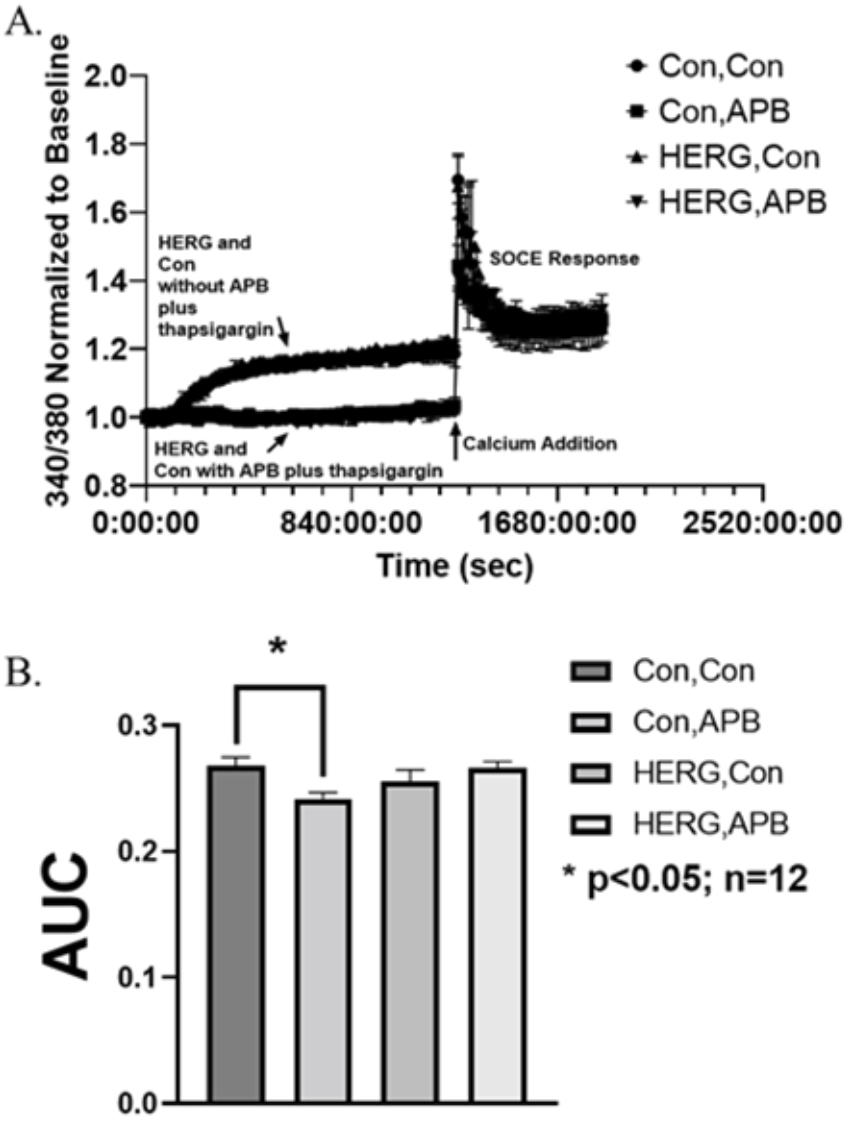

Figure 3. Store operated calcium entry (SOCE) is not affected by HERG expression in myotubes. SOCE is the response to calcium treatment after thapsigargin depletion of SER calcium and is blocked by 2-APB. A. SOCE does contribute to the intracellular calcium response in myotubes. B. AUC analyses shows that the SOCE blocker 2-APB lowers intracellular calcium in control cells, but not in HERG treated cells, suggesting HERG does not modulate SOCE.

Hockerman Report 2021). However, we discovered that when we removed sodium from the cell recording bath, these currents were eliminated (Figure 4), suggesting that HERG modulates sodium channels. This conclusion is confirmed by data revealing that HERG increases sodium current through Nav channel modulation (Figure 5). The obvious question is: How does HERG modulate the sodium channel? What are the consequences of this increased current conductance? Interestingly, it has been reported that muscle disuse increases sodium current density in rat *Soleus* (slow twitch) muscle fibers, but not in the fast twitch EDL muscle fibers (Desaphy et al., 2001). We have indeed reported more abundant detection of HERG in atrophying rat *Soleus* than EDL muscle (Anderson et al., 2019). Further, HERG-modulation of Nav channel expression was predicted by our RNAseq data (see also Major Task 3).

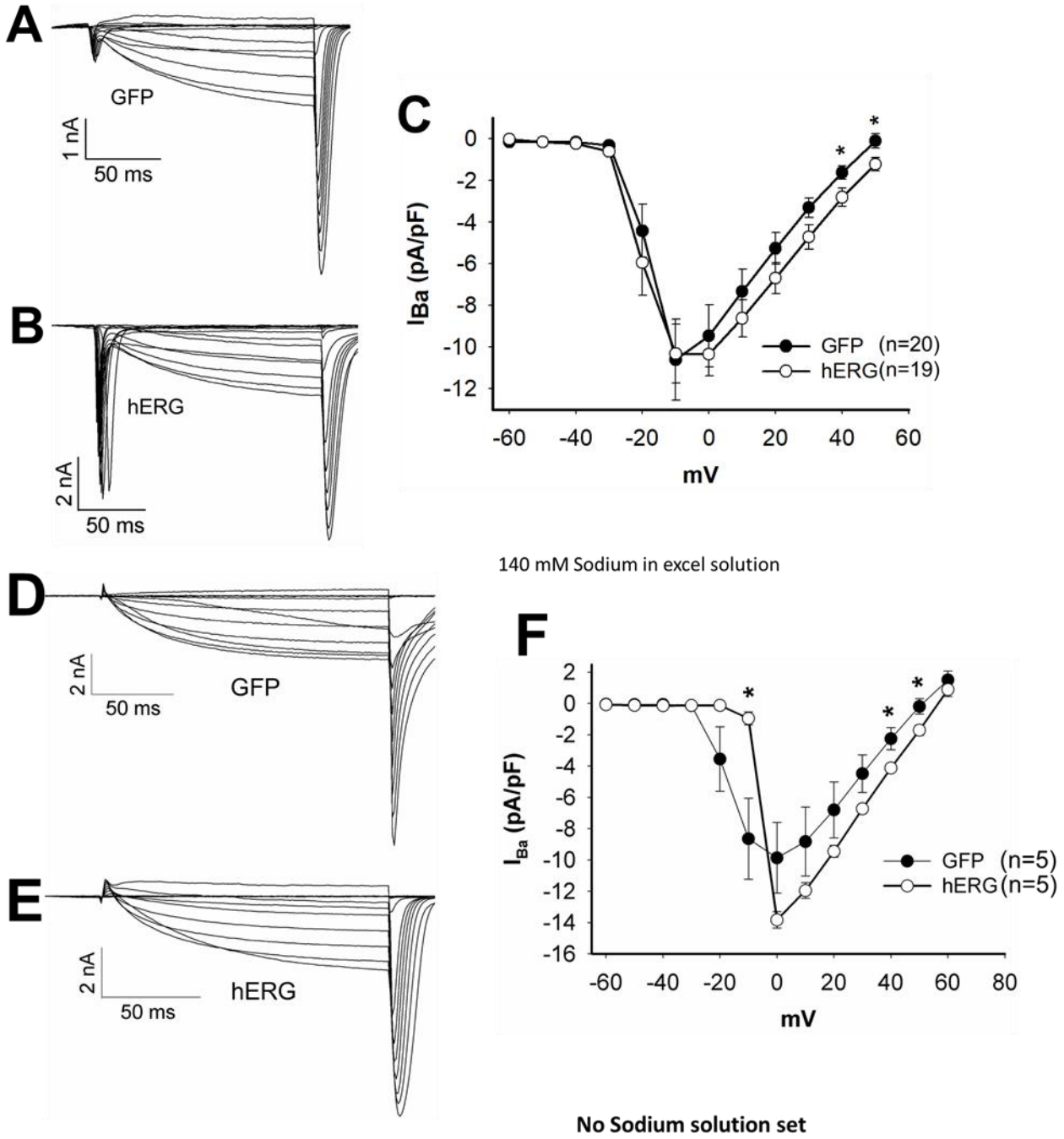

Figure 4. **HERG Expression doesn't change peak Ca_v channel current density in C2C12 myotubes.** Example ensemble of current traces elicited by stepping from -80mV to 50 mV in 10 mV increments for 150 msec, from a holding potential of -60 mV in a GFP transduced myotube **A)** or a HERG transduced myotube **B).** Currents in **A)** and **B)** were recorded in a bath solution containing 140 mM Na^+ and 10 mM Ba^{2+}. **C)** Compiled IV curves for peak Ba^{2+} (slow) currents measured in GFP or HERG expressing myotubes. The current density differed only at the two most positive voltages (40 and 50 mV) *, $P < 0.05$. Example ensembles of currents recorded as described above in myotubes transduced with GFP **D)** or HERG **E)** recorded in bath solution containing 0 Na^+ and 10 mM Ba^{2+}. Note that the fast Na^+ currents observed at the beginning of the traces in **A)** and **B)** are absent, leaving only the more slowly developing Ba^{2+} current. **F)** Compiled IV curves for peak Ba^{2+} currents measured myotubes transduced with GFP or HERG. and recorded in sodium-free bath solution. Ba^{2+} current density was increased at 40 and 50 mV by HERG expression. The difference at -10 mV is likely a voltage-clamp artifact.

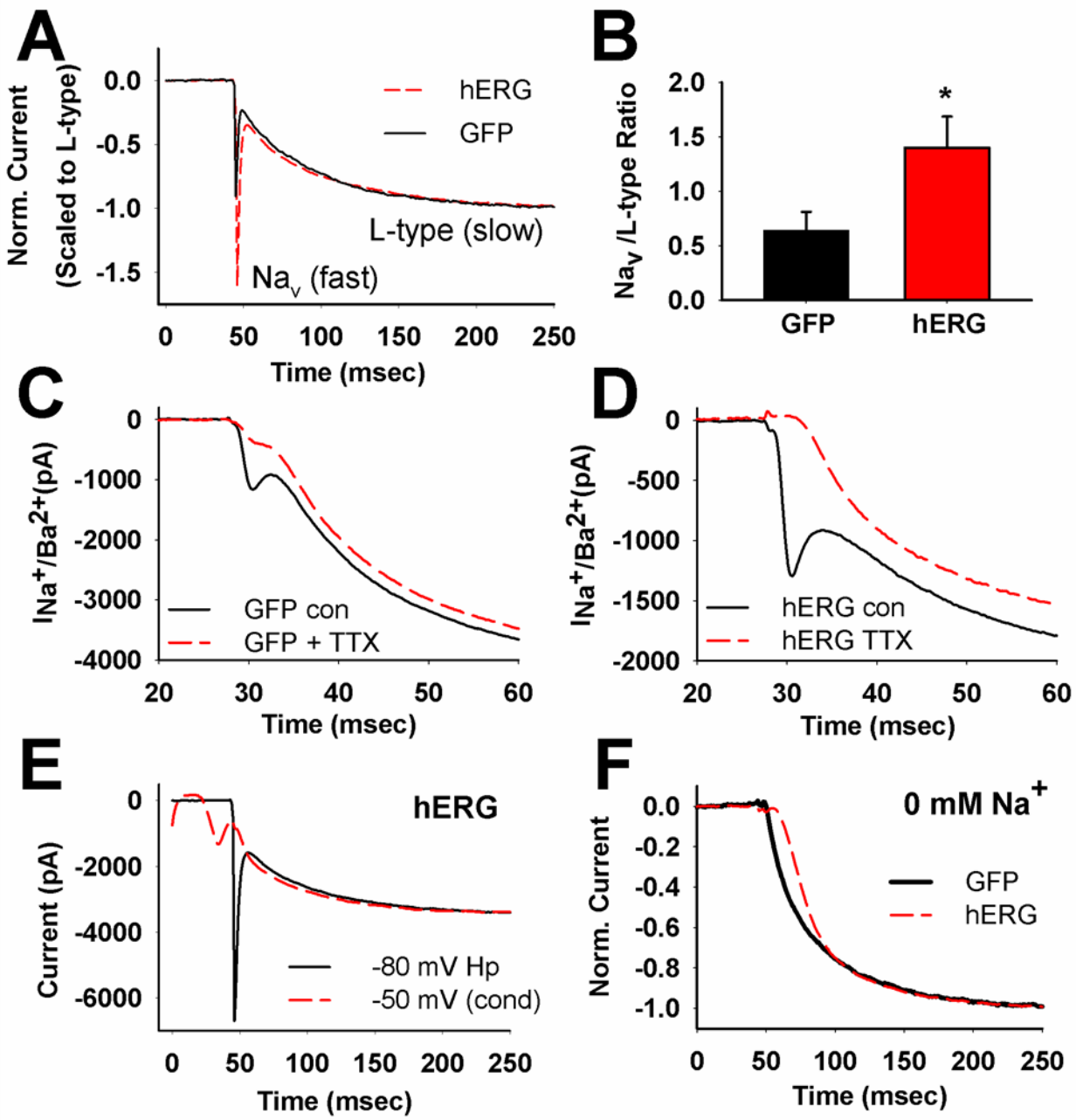

Figure 5. **HERG Expression increases Na_v current in C2C12 myotubes A)** Example current traces recorded in bath solution containing both 140 mM Na^+ and 10 mM Ba^{2+}. Traces were elicited by stepping to 10 mV from -80 mV for 250 msec. Note the fast Na_v current, and the slow Ca_v (L-type) Ba^{2+} current. **B)** Peak Na^+ currents were normalized to peak Ba^{2+} current in myotubes transduced with GFP or HERG. HERG expression increased the peak Na^+ current relative to the peak Ba^{2+} current. Fast Na^+ currents were blocked by 1 µM tetrodotoxin in both GFP **C)** and HERG **D)** transduced myotubes. **E)** Fast Na^+ currents were inhibited by a 20 msec conditioning pulse to -50 mV. Both traces were recorded from the same myotube expressing HERG. **F)** Removing Na^+ from the bath solution eliminates the fast Na^+ current. Traces were recorded as described for **A)** except that the bath solution contained no Na^+.

Subtask 1.B.2 (Pond). The HERG-modulated increase in calcium is not a consequence of increased expression of Cav 1.X L-type calcium channel genes or Cav1.X L-type channel protein abundances (Pond & Hockerman Report 2019).

Subtask 1.C (Hockerman). The Hockerman laboratory demonstrated that, although a small increase in IP1concentration (indicating IP3 signal cascade activation) was seen in C2C12 myotubes in response to 100 uM Carbachol (i.e., which stimulates both muscarinic and nicotinic receptors; Powell et al., 2001), this response was not different between control and HERG-expressing myotubes (Pond & Hockerman Report 2021). However, using Fura 2 experiments and single cell calcium imaging, we have now shown that IP3 signaling is indeed stimulated by HERG expression in C2C12 myotubes because bethanechol (which activates IP3 signaling ONLY through muscarinic receptor activation) induces an increase in $[Ca^{++}]i$ which is greater in HERG-expressing myotubes and is blocked by the specific IP3 receptor (IP3R) antagonist xestospongin C (Pond & Hockerman Report 2021). Interestingly, the significant increase in $[Ca^{++}]i$ that occurs in bethanechol treated HERG-expressing myotubes is not blocked by the phospholipase C (PLC) inhibitor U73211 (Pond & Hockerman Report 2021). Thus, we conclude that the low level of IP3 signaling stimulation that occurs in HERG-expressing myotubes in response to muscarinic agonists is not a consequence of enhanced PLC activity, but must be a result of IP3 receptor modulation by HERG. Indeed, the increased IP3 receptor response to bethanechol may be a consequence of reduced calcium buffering in the SR resulting from decreased CaSeq1 levels; that is, with decreased calcium binding to CaSeq1, there would be more free calcium to exit the SR via IP3R when those receptors are activated. Indeed, the IP3 cascade is not as prevalent in skeletal muscle as in other tissues, so this finding may have more relevance to other tissues. Results of Major Task1 have resulted in numerous published abstracts and a journal paper (see Appendix).

Major Task 2 (Pond). During the first year of the project, we completed this task. We validated our *in vitro* model of skeletal muscle atrophy and discovered that expression of human *ERG1A* in C2C12 myotubes increases basal intracellular calcium concentration 51.7% ($p<0.0001$; n=177). Further, it increases the combined activity of the calcium activated cysteine proteases, calpain 1 and 2, by 31.9% ($p<0.08$; n=24); these enzymes are known to contribute to degradation of myofilaments. Obviously, the increased calcium levels are a contributor to the increased calpain activity; however, the change in calpain activity may also be attributable to increased calpain protein abundance and/or a decrease in levels of the native calpain inhibitor, calpastatin. To explore the enhanced calpain activity further, we evaluated expression of calpain and calpastatin genes and observed no significant differences. Additionally, there was no change in calpain 1 protein abundance, however, calpain 2 protein abundance decreased 40.7% ($p<0.05$; n=6). Obviously, these changes do not contribute to an increase in calpain activity; however, we detected a 31.7% decrease ($p<0.05$; n=6) in calpastatin which would contribute to enhanced calpain activity. These findings have resulted in numerous published abstracts and the manuscript published in the journal *Skeletal Muscle* (see Appendix).

Major Task 3 (Pond). Originally, we proposed exploring the effect of HERG on gene expression using PCR arrays. The DOD reviewers kindly suggested we perform Next Generation Sequencing instead. We prepared the samples and the Purdue Genomics facility completed the RNAseq assay, data collection, and statistical analyses (see Appendix). With the guidance of the statisticians of the Purdue Bioinformatics Core, we began the task of interpreting this massive amount of data. As predicted by the RNAseq, we have determined that genes encoding CaSeq1 and Nav channel members are modulated by HERG (see Major Task 1, Subtasks 1.A [Figure 2] and 1.B.1 [Figures 4 & 5]). Further, the NGS data revealed a decrease in expression of the gene encoding IRBIT, a protein shown to modulate IP3 receptors and $[Ca^{2+}]i$. We asked: Could IRBIT be involved in the greater release of Ca^{2+} through IP_3 receptors in HERG-expressing versus control GFP tubes? The Pond lab performed an immunoblot probing control and HERG-expressing cell lysates for IRBIT and did not detect this protein in the C2C12 myotubes (Pond & Hockerman Report 2021).

Many other proteins could potentially regulate IP3 receptor response. This may be better addressed by immunoprecipitation of HERG from C2C12 myotubes or perhaps other tissues.

Some of the potentially interesting HERG1A-modulated genes to explore at this time encompass players in the retinol pathway and in modulation of reactive oxygen species expression (Pond & Hockerman Report 2021). Indeed, the retinol pathway plays a role in both musculoskeletal health and insulin production: 1) mRNAs encoding proteins critical for the import of retinol (STRA6, -85%, $P = 0.029$), its conversion to retinal (SRP35, -80%, $P = 0.01$), and then to RA (RALDH3, -95%, $P = 0.005$) are markedly reduced; and 2) mRNA levels of secreted factors that exert an autocrine/paracrine, anabolic effect on skeletal muscle (12-14): IGF-1 (-70%; $P = 0.033$), HB-EGF (-75%; $P = 0.020$), and decorin (- 70%; $P = 0.031$) are affected. Further, reactive oxygen species are known to contribute to muscle atrophy. When transduced with an ERG1A-encoded adenovirus (and analyzed against cells transduced with an appropriate control adenovirus), C2C12 myotubes exhibit significant *increases in expression of genes*: **1)** encoding enzymes which produce ROS (i.e., specifically, superoxide dismutase, amine oxidase, aldo-keto reductase family1, ubiquinol-cytochrome C reductase binding protein [which increases mitochondrial production of ROS], etc.); and **2)** related to ROS-induced Ca^{2+} modulation (which may occur through endocannabinoid/TRP signaling). We started the quantitative PCR work and confirmed (report 2021) that HERG expression causes a significant 2.4-fold decrease in IGF1 expression ($p<0.05$; $n=6$) and a 5.7-fold decrease in expression of the decorin gene ($p=0.15$; $n=6$). Power analysis of the decorin gene data suggested that an increase in the number of samples to $n=10$ would yield a p value below 0.05. We were working to determine the efficiency for primers designed to recognize HB-EGF, STRA6, SRP35, and RA, however, the person performing these experiments accepted a nice position shortly after becoming quite proficient with rtPCR technique. We have confirmed some of the numerous HERG-mediated changes in gene expression predicted by the NGS and will submit this data for publication.

SUMMARY: HERG has been detected in atrophying skeletal muscle and has been shown to induce proteolysis when expressed in non-atrophying muscle (Wang et al., 2006). Our current work shows that the HERG potassium channel increases intracellular calcium concentration ($[Ca^{++}]i$) and calpain activity in C2C12 myotubes (Whitmore et al., 2020). Indeed, calpain mediated proteolysis is known to contribute to atrophy in muscle. Most interestingly, our novel work shows that HERG increases $[Ca^{++}]i$ through modulation of RYR receptors (and CaSeq1), known to release calcium from the sarcoplasmic reticulum (SR). We also show here that HERG enhances IP3 signaling, a cascade responsible for increases in intracellular calcium by release from the endoplasmic reticulum (ER) through IP3 receptors. Specifically, our data suggest that HERG specifically modulates IP3 receptors, but not phospholipase C activity. Unexpectedly, here we also show that HERG enhances Nav sodium channel activity, which has been shown to occur in atrophic slow twitch muscle fibers. Indeed, we have shown that HERG protein increases more abundantly in slow muscle fibers than in fast muscle fibers (Anderson et. al., 2019). Taken together, the data demonstrate that further investigation of the HERG channel function in muscle is merited. Indeed, it could lead to discovery of a small regulating, skeletal muscle specific molecule(s) which could be targeted for treatment of skeletal muscle atrophy.

FUTURE ENDEAVORS: This project has opened several potential research opportunities. There is now an obvious need for exploration of: 1) How HERG modulates abundances of the calpain 2 and calpastatin proteins; 2) How HERG modulates gene expression, specifically of genes related to RYR1 signaling (e.g., CaSeq1); 3) How does HERG interact with RYR1 to modulate calcium release; 3) How does HERG modulate IP3 receptor activation; Is there a small ligand involved?; 4) How does HERG modulate skeletal muscle sodium channel (Nav) activity; What are the consequences of this action?; and 5) Does HERG modulate the retinol pathway and expression of reactive oxygen species? Further, now that we have a better understanding of how HERG1A affects calcium signaling in skeletal muscle, does (and if so how) this

information has implications for the HERG1A/1B heteromultimeric channel in the heart, where calcium dysregulation can have dire consequences.

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What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

PI. Dr. Pond has presented data from this project at 5 conferences over the funding period, both in person and virtually, missing two conferences which were cancelled in 2020 as a result of the COVID-19 pandemic. She has published 8 abstracts (including student abstracts) and a peer-reviewed journal article (in **Skeletal Muscle**) from this project (see Appendix). One more manuscript concerning the source of the intracellular

calcium increase induced by HERG is in preparation and a third describing the RNAseq results is currently outlined for write up.

Co-PI. Dr. Hockerman has supervised his graduate student, Ms. Rantz, and worked on the electrophysiology to measure both L-type calcium and Nav sodium currents. Dr. Hockerman is an author on 8 abstracts and on the paper published in **Skeletal Muscle** (see Appendix). He will be co-author on the two papers in preparation.

Graduate Students.

Clayton Whitmore (Pond laboratory) worked on the project from its inception and defended his Masters thesis in October 2018: *Investigation of ERG1A potassium channel modulation of calpain activity in C2C12 myotubes.* He presented his work at 3 research forums, co-authored 9 abstracts, and is first author of the paper published in **Skeletal Muscle**: *The ERG1a potassium channel increases basal intracellular calcium concentration and calpain activity in skeletal muscle cells* (see Appendix). He currently works as a highly skilled Research Assistant at Vanderbilt University in Nashville, TN.

Emily Rantz (Hockerman laboratory) is a PhD student who has worked on the project since 2019. She performed IP1 assays and single-cell Ca^{2+} imaging to measure cellular calcium levels in response to HERG expression and various treatments to block specific pathway steps. She has learned skeletal muscle biology and comparative pancreatic and muscular physiology in addition to learning how to develop and perform IP1 assays and single cell calcium imaging. She has worked on this project as part of her dissertation titled *Investigating the role of RYR2 in Ca^{2+} dynamics, insulin secretion, and electrophysiological properties in pancreatic beta-cells* and will graduate August 2022.

Undergraduate Students.

Luke Anderson worked on the project since its inception until May 2020 when he graduated with a Baccalaureate degree. Luke worked on culturing the cells and validated the *in vitro* model. He performed enzyme assays and immunohistochemistry. Luke has presented his work at 5 research forums and earned authorship on 12 abstracts and the paper supported by this grant published in *Skeletal Muscle*. (See Appendix.) He also had a first author paper (from an Honors Project) published in a peer reviewed jouranl: Anderson LB, Latour CD, Khader O, Massey BH, Cobb B, **Pond AL**. 2019. *Ether-a-go-go related gene-1a* potassium channel abundance varies within specific skeletal muscle fiber type. *European Journal of Translational Myology* 29(3):8402.

Omar Khader has worked on the project since 2018 and learned basic laboratory and safety skills in addition to performing the fura2 assays to assay intracellular calcium concentration. Omar has presented his work at 3 research forums and earned authorship on 7 abstracts and has earned authorship on the paper (in preparation), describing the how the HERG channel induces an increase in intracellular calcium concentration. (See Appendix.)

Ashley Bryant joined the lab in summer 2020. She learned basic laboratory and safety skills and aseptic technique which she used with cell culture. Ashley learned real time PCR and used this technique to validate a few of the results observed with NGS (Task 3). Ashley graduated in May 2021 and left the lab earlier than expected to take a job performing rtPCR with a pharmaceutical company in Hawaii.

Anna-Jonesboro High School Senior.

Bryce Massey worked in the laboratory and learned basic lab skills and safety procedures as well as basic muscle immunohistochemistry. He improved the flow of work by taking care of lab basic needs. He is currently a sophomore at Brown University.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Results from the project have been published in the peer reviewed journal *Skeletal Muscle* (See Appendix). Eight different abstracts have been presented at 4 separate conferences by Dr. Pond or her students. Dr. Pond has presented the data at 3 national and 2 international conferences over the funding period, including some of which were virtual. Two conferences (one national and one international) were cancelled in 2020 as a result of the COVID-19 pandemic, however, the abstracts were still published. Dr. Pond has also judged 3 regional middle and high school science fairs where she discussed simple aspects of the project with students. Students have presented the work at a total of 3 local and 1 national forums.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Nothing to report.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

We have reported that HERG has an effect on calcium levels in skeletal muscle cells. We have now shown that HERG modulates intracellular calcium concentration by two mechanisms: 1) modulation of RYR1 signaling; and 2) modulation of IP3 signaling. We have also reported the novel finding that HERG modulates Nav sodium current. Indeed, it has been shown that sodium currents vary with muscle condition. We have published some of our data and are currently developing 2 publications: 1) describing the role of HERG in modulation of intracellular calcium concentration; and 2) one describing the effect of HERG on gene expression (as elucidated by RNAseq). This work opens a new area of research for researchers interested in calcium signaling in skeletal muscle and any tissue in which HERG is expressed. Indeed, HERG has been detected in numerous tissues including heart, brain, and cancer cells (Wang et al., 2006).

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

This work opens a new area of research for researchers interested in calcium signaling not only in skeletal muscle, but also in numerous tissues, including heart. Calcium dysregulation is a serious concern in cardiac

tissue. Indeed, HERG1A has also been reported in malignant cells where it appears to modulate cellular growth.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

The work on this project has included many young people. It is the hope that these people enjoyed their exposure to science and will continue to study the sciences.

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

There have been no changes made to the approach or plan as originally revised per reviewer request at the outset.

Actual or anticipated problems or delays and actions or plans to resolve them

Need for Two No Cost Extensions:

We concluded the initial year and a half of the grant, one 1-year no cost extension, and a second 1-year no cost extension. We completed Major Task 2 in the first year as proposed and also completed subtask 1.B.2 of Task 1 during this time, a bit ahead of schedule. We completed aspects of Major Tasks 1 and 3 during the first no cost extension (NCE), however, did not 100% complete them until the second NCE. We have one paper and numerous abstracts published (see Appendix) and are now focused on writing a paper describing our results regarding HERG modulation of intracellular calcium levels. Indeed, HERG modulation of RYR1 by regulation of CaSeq1 expression is most exciting. We will submit this manuscript to a prestigious, peer-reviewed journal. Once this paper is submitted, we will focus on writing a paper summarizing our RNAseq results. This paper will reference results in the paper upon which we are now focused. Overall, our labs have experienced numerous events which have delayed progress. The Hockerman lab had a student injury in year 1 which required subsequent surgery and extensive rehabilitation. In year two the student experienced a significant stressful family situation and both of these life events have interfered with timely execution of Major Task 1. In keeping with the rest of the world, we have experienced extensive delays resulting from the COVID-19 Pandemic, which resulted in temporary restructuring of both Purdue and Southern Illinois University teaching, research, and business practices. Both the Pond and Hockerman laboratories were administratively closed from mid-March to mid-June 2020. Once re-opened, the laboratories had to restructure how they functioned to accommodate social distancing and major delays in order deliveries. The labs had to renew and test cell cultures and clean equipment, etc.

Further delays for completion of Major Tasks 1 and 3 include 3 hospitalizations and subsequent deaths in the immediate family of Dr. Pond:

Stepfather (who raised her), Roger G. Simpkins, died August 25, 2020: https://www.springhillfh.com/obituaries/Roger-Simpkins-3/#!/Obituary;

Stepmother, Peggy Jane Pond, died April 4, 2021, (leaving behind Dr. Pond's father, who needed/needs support): https://www.dignitymemorial.com/obituaries/old-hickory-tn/peggy-pond-1013731;

Partner, Dr. Darwin Shane Koch, died at home in hospice care on May 14, 2021: https://www.meredithfh.com/obituaries/Darwin-Shane-Koch?obId=21107122

All hospitalizations and funerals were negatively impacted by the COVID-19 pandemic, extending the time necessary to bring closure to these sad life events.

With improvement of the pandemic situation, both laboratories have recently returned to a full capacity workload, albeit with some minor restrictions remaining. Indeed, we have completed the grant aims and whole-heartedly thank the DOD for the gracious No Cost Extension opportunities.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

The project does not involve human subjects.

Significant changes in use or care of vertebrate animals

The project does not involve vertebrate animals.

Significant changes in use of biohazards and/or select agents

The project does not involve use of biohazards and/or select agents.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Publications, conference papers, and presentations
Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Whitmore C, Pratt E, Anderson LB, Bradley K, Latour SM, Hashmi MN, Urazaev AK, Weilbacher R, Davie JK, Wang W-H, Hockerman GH, **Pond AL**. The ERG1a potassium channel increases basal intracellular calcium concentration and calpain activity in skeletal muscle cells. 2020. *Skeletal Muscle*. 10:1-15. doi.org/10.1186/s13395-019-0220-3.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

All funding sources were acknowledged for each publication below.

2019

***Pond AL**, Whitmore C, Pratt EE, Anderson LB, Rantz E, Salyer A, Carraro U, Wang W-H, Weilbaecher R, Davie JK, Hockerman GH. 2019. *The ERG1a K^+ channel increases calpain activity in C2C12 myotubes and mouse skeletal muscle.* Oral presentation given at Padua Muscle Days conference in Padua, Italy, March 28, 2019.

***Pond AL**, Whitmore C, Pratt EE, Anderson LB, Rantz E, Salyer A, Carraro U, Zampieri S, Weilbacher R, Davie JK, Hockerman GH. 2019. *The ERG1a K^+ channel increases intracellular calcium and calpain activity in C_2C_{12} myotubes.* Poster presentation given at Experimental Biology Conference in Orlando, FL, April 8, 2019.

*Clayton Whitmore. 2019. *Investigation of ERG1A potassium channel modulation of calpain activity in C2C12 myotubes.* Thesis presented to the Faculty of Southern Illinois University in partial fulfillment of the requirements for the Masters degree.

Anderson LB, Latour CD, Khader, O, Pond AL. 2019. *ERG1a abundance varies by skeletal muscle in mice.* Poster presentation given at SIU Undergraduate Research Forum, April 8, SIU. Carbondale.

2020

Pond AL, C Whitmore, JK Davie, S Choudhari, J Thimmapuram, GH Hockerman. Viral transduction of C2C12 myotubes with the HERG potassium channel induces expression of genes related to multiple signaling pathways. 2020. To be presented at the Padua Muscle Days conference in Padua, Italy, March 2020, but canceled as a result of the COVID-19 Pandemic.

Pond AL, Zampieri S, Sandri M, Cheatwood J, Kohli P, Balaraman R, Anderson, LB, Latour CD, Hockerman GH, Kern H, Sartori R, Merigliano S, Da Dalt G, Davie JK, Carraro U, Pond AL. The ERG1\ potassium channel is abundant in cachectic human skeletal muscle. 2020. FASEB J. Presented at the 2020 Experimental Biology Conference in San Diego, CA, April 4-7; but canceled as a result of the Pandemic.

2021

Pond AL, Davie JK, Kohli P, Zampieri S. Update on the ERG1A potassium channel: Potential for many cellular roles in aging and cancer. Presented at the 2021 Spring Padua Muscle Days, May 26-29; University of Padua, Italy. Presented by ZOOM on May 29, 2021 as a result of COVID-19 pandemic.

Pond AL, Whitmore C, Thimmapuram J, Hockerman GH. The ERG1A potassium channel induces expression of genes related to skeletal muscle atrophy in C2C12 myotubes**.** 2020. FASEB J 35(S1):1. https://doi.org/10.1096/fasebj.2021.35.S1.04316. Presented Virtually at the 2020 Experimental Biology Conference on April 27, 2021.

Khader O, Hockerman GH, Pond AL. Investigation of the mechanism by which the ERG1 potassium channel increases intracellular calcium concentration. Presented virtually at the Southern Illinois University Undergraduate Research Forum on April 15, 2021.

LaVigne, Emily. 2021. Investigating the role of RYR2 in Ca^{2+} dynamics, insulin secretion, and electrophysiological properties in pancreatic beta-cells. Dissertation presented to the Faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report.

Website(s) or other Internet site(s)
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

Technologies or techniques
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report.

Inventions, patent applications, and/or licenses
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

Other Products
Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life.

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Amber L. Pond
Project Role:	PI – No Change
Researcher Identifier:	0000-0002-2836-889X (ORCID ID)
Nearest person month worked:	8.4 total for funding period
Contribution to the Project:	Generated and collated data, supervised students, wrote abstracts and presentations, co-wrote manuscript, currently co-developing one manuscript and have outlined another.
Funding Support:	Southern Illinois University

Name:	Dr. Gregory Hockerman
Project Role:	Co-PI – No Change
Researcher Identifier:	0000-0001-6147-8604
Nearest person month worked:	1.9

Contribution to the Project:	Generated and collated data, supervised students, edited abstracts and presentations, co-wrote manuscript, currently co-developing one manuscript.
Funding Support:	DOD CDMRP Discovery Award W81XWH-18-1-0052, Log Number PR170326 (1.9 person months)

Name:	Clayton Whitmore
Project Role:	Graduate Student
Researcher Identifier:	None
Nearest person month worked:	3
Contribution to the Project:	Mr. Whitmore worked in the lab, performing rtPCR, immunoblots, and calpain assays.
Funding Support:	Southern Illinois University

Name:	Emily Rantz
Project Role:	Graduate Student
Researcher Identifier:	None
Nearest person month worked:	13.14 DOD CDMRP Discovery Award W81XWH-18-1-0052, Log Number PR170326
Contribution to the Project:	Ms. Rantz works in the lab, performing single cell calcium assays, electrophysiology, and IPOne assays.
Funding Support:	Current CDMRP project (13.14 person months) and R21 from NINDS

Name:	Omar Khader
Project Role:	Undergraduate Student
Researcher Identifier:	None
Nearest person month worked:	4.7
Contribution to the Project:	Mr. Khader helps maintain the lab and cell culture facilities. He cultures cells and is working with the fura calcium assays.
Funding Support:	Mr. Khader was funded by this DOD CDMRP Discovery Award and by a REACH award from SIU.

Name:	Ashley Bryant
Project Role:	Undergraduate Student
Researcher Identifier:	None
Nearest person month worked:	3.9
Contribution to the Project:	Ms. Bryant graduated and left the lab at the end of May 2021. Her duties included: maintenance of the lab and performance of quantitative PCR.
Funding Support:	Ms. Bryant was funded by this DOD CDMRP Discovery Award.

Name:	Bryce Massey
Project Role:	Recent High School Graduate
Researcher Identifier:	None
Nearest person month worked:	0.25

Contribution to the Project:	Mr. Massey left the lab at the end of summer 2019 and matriculated at Brown University. His duties included: maintenance of the lab.
Funding Support:	Mr. Massey was funded by this DOD CDMRP Discovery Award.

Name:	Luke Anderson
Project Role:	Recent SIU Graduate: BS
Researcher Identifier:	None
Nearest person month worked:	5.9
Contribution to the Project:	Mr. Anderson graduated in 2020 with a BS degree from SIU. His duties: lab maintenance, immunoblots, cell culture, and enzyme assays.
Funding Support:	Mr. Anderson was funded by this DOD CDMRP Discovery Award.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Purdue University
West Lafayette, IN, USA

Purdue University houses Dr. Greg Hockerman, who is the Co-PI on this grant. To him it supplies an office and a lab, a computer, and some students.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

Not Applicable.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

A. Bibliography of all publications and meeting abstracts.

1. Publications in peer reviewed journal.

Whitmore C, Pratt E, Anderson LB, Bradley K, Latour SM, Hashmi MN, Urazaev AK, Weilbacher R, Davie JK, Wang W-H, Hockerman GH, Pond AL. The ERG1a potassium channel increases basal intracellular calcium concentration and calpain activity in skeletal muscle cells. 2020. *Skeletal Muscle*. 10:1-15. doi.org/10.1186/s13395-019-0220-3.

2. Meeting abstracts.

Anderson LB, Latour CD, Khader O, and Pond AL. ERG1a abundance varies by skeletal muscle fiber in mice. Presented at the SIU Undergraduate Research Forum, Carbondale, IL, April 8, 2019.

Khader O, Anderson LB, Hockerman GH, Pond AL. ERG1A increases intracellular calcium concentration by enhancing flux from internal calcium stores. Accepted for presentation at the SIU Undergraduate Research Forum, Carbondale, IL, April 6, 2020. The forum was canceled because of the COVID-19 pandemic.

Khader O, Hockerman GH, Pond AL. Investigation of the mechanism by which the ERG1 potassium channel increases intracellular calcium concentration. Presented virtually at the Southern Illinois University Undergraduate Research Forum on April 15, 2021.

Khader O, Latour SM, Graham VM, and Pond AL. The effect of *Erg1a* expression on caspase enzyme activity in C_2C_{12} myotubes. Presented at the SIU Undergraduate Research Forum, Carbondale, IL, April 8, 2019.

Pond AL, Davie JK, Kohli P, Zampieri S. Update on the ERG1A potassium channel: Potential for many cellular roles in aging and cancer. Presented at the 2021 Spring Padua Muscle Days, May 26-29; University of Padua, Italy. Presented by ZOOM on May 29, 2021 as a result of COVID-19 pandemic.

Pond AL, Whitmore C, Davie JK, Choudhari S, Thimmapuram J, Hockerman GH. Viral transduction of C2C12 myotubes with the HERG potassium channel induces expression of genes related to multiple signaling pathways. Accepted for oral presentation at 2020 Spring Padua Muscle Days, March 18-21; University of Padua, Italy. Presented by ZOOM on November 20, 2020 as a result of COVID-19 pandemic. *****2020PMD: Translational Mobility Medicine – Collection of Abstracts EJTM 2020; 30 (1):20-21.

Pond AL, Whitmore C, Pratt EE, Anderson LB, Rantz E, Salyer A, Carraro U, Wang W-H, Weilbaecher R, Davie JK, and Hockerman GH. The ERG1a K^+ channel increases calpain activity in C2C12 myotubes and mouse skeletal muscle. Presented at 2019 Spring Padua Muscle Days, March 27-29; University of Padua, Italy.

Pond AL, Whitmore C, Pratt EE, Anderson LB, Rantz E, Salyer A, Carraro U, Zampieri S, Weilbaecher R, Davie JK, and Hockerman GH. The ERG1a K^+ Channel increases intracellular calcium concentration and calpain activity in C_2C_{12} myotubes. Presented at the 2019 Experimental Biology Conference in Orlando, FL, April 6-10.

Pond AL, Whitmore C, Thimmapuram J, Hockerman GH. The ERG1A potassium channel induces expression of genes related to skeletal muscle atrophy in C2C12 myotubes**.** 2021. FASEB J 35(S1):1. https://doi.org/10.1096/fasebj.2021.35.S1.04316. Presented Virtually at the 2021 Experimental Biology Conference on April 27, 2021.

Pond AL, Zampieri S, Sandri M, Cheatwood J, Kohli P, Balaraman R, Anderson, LB, Latour CD, Hockerman GH, Kern H, Sartori R, Merigliano S, Da Dalt G, Davie JK, Carraro U, Pond AL. The ERG1 potassium channel is abundant in cachectic human skeletal muscle. 2020. FASEB J 34(S1):1. https://doi.org/10.1096/fasebj.2020.34.s1.05. Presented at the 2020 Experimental Biology Conference in San Diego, CA, April 4-7; but canceled as a result of the Pandemic.

3.Graduate Student Theses and Dissertations.

Clayton Whitmore. 2019. *Investigation of ERG1A potassium channel modulation of calpain activity in C2C12 myotubes.* Thesis presented to the Faculty of Southern Illinois University in partial fulfillment of the requirements for the Masters degree.

LaVigne, Emily. 2021. Investigating the role of RYR2 in Ca^{2+} dynamics, insulin secretion, and electrophysiological properties in pancreatic beta-cells. Dissertation presented to the Faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

B. All personnel receiving pay from the research effort.

Gregory H. Hockerman – Co-Principal Investigator
Luke Brian Anderson – former undergraduate student
Omar Khader - former undergraduate student
Ashley Bryant - former undergraduate student
Bryce Massey – former high school student

C. Papers and Abstracts in Entirety.

Published Research Paper:

Whitmore C, Pratt E, Anderson LB, Bradley K, Latour SM, Hashmi MN, Urazaev AK, Weilbacher R, Davie JK, Wang W-H, Hockerman GH, **Pond AL**. The ERG1a potassium channel increases basal intracellular calcium concentration and calpain activity in skeletal muscle cells. 2020. *Skeletal Muscle*. 10:1-15. doi.org/10.1186/s13395-019-0220-3.

Published Research Paper

Whitmore *et al. Skeletal Muscle* (2020) 10:1
https://doi.org/10.1186/s13395-019-0220-3

Skeletal Muscle

RESEARCH Open Access

The ERG1a potassium channel increases basal intracellular calcium concentration and calpain activity in skeletal muscle cells

Clayton Whitmore[1], Evan P.S. Pratt[2], Luke Anderson[1], Kevin Bradley[1], Sawyer M. Latour[3], Mariam N. Hashmi[1], Albert K. Urazaev[4], Rod Weilbaecher[5], Judith K. Davie[5], Wen-Horng Wang[6], Gregory H. Hockerman[2] and Amber L. Pond[1,7*]

ABSTRACT

Background: Skeletal muscle atrophy is the net loss of muscle mass that results from an imbalance in protein synthesis and protein degradation. It occurs in response to several stimuli including disease, injury, starvation, and normal aging. Currently, there is no truly effective pharmacological therapy for atrophy; therefore, exploration of the mechanisms contributing to atrophy is essential because it will eventually lead to discovery of an effective therapeutic target. The *ether-a-go-go related gene* (*ERG1A*) K^+ channel has been shown to contribute to atrophy by upregulating ubiquitin proteasome proteolysis in cachectic and unweighted mice and has also been implicated in calcium modulation in cancer cells.

Methods: We transduced C_2C_{12} myotubes with either a human *ERG1A* encoded adenovirus or an appropriate control virus. We used fura-2 calcium indicator to measure intracellular calcium concentration and Calpain-Glo assay kits (ProMega) to measure calpain activity. Quantitative PCR was used to monitor gene expression and immunoblot evaluated protein abundances in cell lysates. Data were analyzed using either a Student's *t* test or two-way ANOVAs and SAS software as indicated.

Results: Expression of human *ERG1A* in C_2C_{12} myotubes increased basal intracellular calcium concentration 51.7% ($p < 0.0001$; $n = 177$). Further, it increased the combined activity of the calcium-activated cysteine proteases, calpain 1 and 2, by 31.9% ($p < 0.08$; $n = 24$); these are known to contribute to degradation of myofilaments. The increased calcium levels are likely a contributor to the increased calpain activity; however, the change in calpain activity may also be attributable to increased calpain protein abundance and/or a decrease in levels of the native calpain inhibitor, calpastatin. To explore the enhanced calpain activity further, we evaluated expression of calpain and calpastatin genes and observed no significant differences. There was no change in calpain 1 protein abundance; however, calpain 2 protein abundance decreased 40.7% ($p < 0.05$; $n = 6$). These changes do not contribute to an increase in calpain activity; however, we detected a 31.7% decrease ($p < 0.05$; $n = 6$) in calpastatin which could contribute to enhanced calpain activity.

Conclusions: Human *ERG1A* expression increases both intracellular calcium concentration and combined calpain 1 and 2 activity. The increased calpain activity is likely a result of the increased calcium levels and decreased calpastatin abundance.

Keywords: ERG1A, Skeletal muscle atrophy, Calpains, Calpastatin, Intracellular calcium

* Correspondence: apond@siumed.edu
[1]Anatomy Department, Southern Illinois University School of Medicine, Carbondale, IL 62902, USA
[7]Southern Illinois University, 1135 Lincoln Drive, Carbondale, IL 62902, USA Full list of author information is available at the end of the article

BACKGROUND

Skeletal muscle comprises approximately 40% of total human body weight and contains 50–75% of all bodily proteins. Skeletal muscle is needed for the production of mechanical energy, body posture, modulation of body temperature, and for generating force and movement. Thus, a certain amount of skeletal muscle tissue is necessary for well-being and a reduction in this tissue could compromise health [1]. Skeletal muscle mass is maintained by a continuous, fluctuating balance between protein degradation and protein synthesis; however, when the rate of degradation increases or the rate of protein synthesis decreases, muscle mass can be lost in a process known as atrophy. Skeletal muscle atrophy is defined as a 5% or greater decrease in muscle mass and strength and can be induced by certain stimuli: muscle disuse, denervation, starvation, disease (e.g., diabetes and cancer), loss of neural input, and even normal aging [2, 3]. Treatments for skeletal muscle atrophy currently under study include administration of pharmaceuticals such as growth factors [4], beta-agonists [5], inhibitors of proteolysis [6, 7], stimulators of protein synthesis [8], and myostatin inhibitors [9–11]; however, these are not adequately effective. Thus, further investigation into the mechanisms resulting in atrophy is needed to reveal new and improved targets for therapy.

The protein degradation that contributes to atrophy occurs mainly through four proteolytic pathways: the ubiquitin proteasome pathway (UPP), cathepsins (the autophagy-lysosome system), caspases (the apoptosis protease system), and calpain enzymes. Calpains are a family of calcium activated cysteine proteases that cleave specific proteins to release large fragments [7]. In skeletal muscle, calpain activity disassembles the sarcomere, releasing actin and myosin to become accessible for ubiquination and subsequent degradation by the proteasome (i.e., the UPP) [12–14]. Indeed, calpains have been shown in vitro to act upon anchoring proteins (e.g., titin, nebulin, and desmin) which attach the sarcomere's myofilaments to the sarcomeric Z-disc [13]. The cleavage of these proteins subsequently releases α-actinin and thus results in the release of the actin thin filament from the myofibril [13, 14]. Calpains have also been shown to degrade tropomyosin and troponin proteins [13] and, combined with the cleavage of titin, this degradation allows for the removal of the thick filaments from the myofibrils. Calpain activity has also been shown to affect the Akt pathway which modulates the balance of protein synthesis and degradation [14].

The ERG1a (*ether-a-go-go related gene*) gene encodes a potassium channel known to conduct cardiac I_{Kr} current and be partially responsible for the repolarization of the heart action potential [15–17]. ERG1 is detected in numerous mammalian tissues including brain and heart, but had not been reported in skeletal muscle until we demonstrated that ERG1a protein abundance increases in the skeletal muscle of mice in response to hind limb suspension and tumor expression [18]. We further showed that, when ectopically expressed in the skeletal muscle of weight bearing mice, ERG1a increases the abundance of the UPP E3 ligase, MuRF1, and overall UPP activity [18]. These data suggest that ERG1a participates in the process of skeletal muscle atrophy at least partially through modulation of the UPP [15]. We hypothesized that ERG1a could affect other proteolytic pathways. Indeed, human ERG1A (HERG1A) has been shown to increase the basal intracellular calcium concentration ($[Ca^{2+}]i$) of SKBr3 breast cancer cells [19] and is detected in the t-tubules of cardiac tissue [17, 20] where it has the potential to affect the calcium release mechanism. Thus, we hypothesized that HERG1A would increase intracellular concentration in C_2C_{12} myotubes and consequently enhance calpain activity. Here, we describe studies designed to explore this hypothesis and demonstrate that indeed, ERG1A enhances both intracellular calcium concentration and calpain activity.

METHODS AND MATERIALS

Antibodies

The following antibodies were used: Calpain-1 polyclonal antibody 3189-30 T (BioVision, Milpitas, CA); Calpain-2 polyclonal antibody 3372-30 T (BioVision, Milpitas, CA); Calpain-3 polyclonal antibody A11995 (ABclonal, Woburn, MA); Calpastatin polyclonal antibody A7634 (ABclonal, Woburn, MA); MF-20 myosin antibody (Developmental Studies Hybridoma Bank, Iowa City, IA); laminin antibody NBP2–44751 from rat (Novus, Centennial, CO); erg1 antibody P9497 (Sigma, St. Louis, MO); and GAPDH polyclonal antibody ABS16 (Sigma, St. Louis, MO).

Cell culture

C_2C_{12} myoblasts were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator with 10% CO_2 at 37 °C. To differentiate myoblasts into myotubes, cells were grown in DMEM supplemented with 10% FBS to ~85% confluence. The FBS medium was then replaced with DMEM medium supplemented with 2% heat-inactivated horse serum. Cells were incubated for 4 days to allow for terminal differentiation.

Viral transduction

Terminally differentiated C_2C_{12} myotubes were treated with 200 MOI virus to produce HERG1A protein after 48 h. Specifically, for experimentation one set of cells was treated with control GFP encoded adeno-virus (VQAd EMPTY-eGFP; ViraQuest, New Liberty, IA) while the other received the same GFP encoded adeno-viral particles also encoding the human ERG1A K^+ channel (VQAd

CMV Herg-GFP; ViraQuest). The cells were then incubated for 48 h and monitored via fluorescence to verify that the transduction was successful.

Animals

All procedures were approved by the Southern Illinois University Carbondale (SIUC) Animal Care and Use Committee. A total of 80 ND4-Swiss Webster 7–8- week-old male mice (Harlan-Sprague; Indianapolis, IN) were used. Animals were housed in SIUC vivarium facil- ities on a 12 h light/dark cycle, monitored by lab animal veterinarians, and provided food and water ad libitum.

Western blot

Membrane proteins were extracted from C_2C_{12} myoblasts and myotubes for Fig. 1a and from C_2C_{12} myotubes at 48 h after viral transduction for Figs. 1, 5, 6, 7, and 8c, b, b. Membrane proteins were extracted from C_2C_{12} cells using Tris buffer (10 mM, pH 7.4) containing 1 mM EDTA, 2% Triton X-100, and protease inhibitors (0.5 mM pefabloc, 0.5 mM PMSF, 1 mM benzamidine, 1 mM pepstatin, and 1 mM 1,10-phenanthroline). Samples were triturated using a tuberculin syringe and 23G needle and allowed to incubate on ice at 4 °C for 30 min and then centrifuged for 2 min at 15,000 rpm. Cellular proteins for Fig. 2b were extracted from C2C12 myotubes at 24, 48, and 72 h after transduction using Tris buffer (10 mM, pH 7.4) containing 1 mM EDTA, and protease inhibitors (0.5 mM pefabloc, 0.5 mM PMSF, 1 mM benzamidine, 1 mM pepstatin, and 1 mM 1,10-phenanthroline). The samples were then centrifuged for 2 min at 15, 000 rpm. All supernatants were collected and the protein content was determined using a DC protein assay kit (BioRad, Hercules, CA) and manufacturer's instructions. Samples were electrophoresed through a 4% poly-acrylamide stacking gel followed by a 7.5% poly-acrylamide separating gel and finally transferred to PVDF membrane (BioRad, Hercules, CA). Membranes were immunoblotted using one or more of the antibodies listed above and developed with Immun-Star AP chemiluminescent substrate (BioRad, Hercules, CA). Optical densities of the protein bands were determined using ImageJ software (NIH).

Fusion index

Myoblasts were grown on glass coverslips coated with rat tail collagen and then treated with either the HERG-encoded or the control virus and allowed to terminally differentiate. These were then immunostained for myosin using the DSHB antibody recognizing myosin and a mouse on mouse (M.O.M.) Kit (Vector Labs, Inc.; Burlingame, CA) per manufacturer's instructions. The coverslips were then mounted to slides with a mounting substance containing DAPI, and images were acquired using a Leica DM4500 microscope with a Leica DFC 340FX camera. The nuclei of myosin-positive cells were counted in three fields from ten slides (five treated with HERG-encoded virus and five treated with control virus).

Resting intracellular Ca^{2+} assay

C2C12 myoblasts were cultured in DMEM supplemented with 10% FBS and 1% P/S and plated at a density of 5×10^4 cells/well in a black-walled 96-well plates (Corning Life Sciences). Once myoblasts reached 80–90% confluency, culturing media was exchanged for differentiation media (DMEM supplemented with 2% horse serum and 1% P/S) to promote differentiation and fusion of myoblasts into myotubes. Myoblasts were differentiated for 3–4 days (2–3 days prior to a decrease in myotube viability within a 96-well plate), and the differentiation media was exchanged daily. Using a multiplicity of infection of 100 (based on the initial number of myoblasts plated), myotubes were transduced with adenovirus encoding EGFP control or HERG. Myotubes were grown for two additional days, and the differentiation media was refreshed daily. Prior to Ca^{2+} measurements, the media was removed and myotubes were washed twice with 200 μL PBS. Then, 5 μM Fura2-AM (Molecular Probes, Eugene, OR) was diluted in Krebs-Ringer HEPES buffer (KRBH), and each well of myotubes was incubated in 100 μL of this solution for 1 h at RT. KRBH contained 134 mM NaCl, 3.5 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM $NaHCO_3$, and 10 mM HEPES and was supplemented with 0.05% fatty-acid free BSA (pH 7.4). After this period, the Fura2-AM was removed, and myotubes were washed twice with KRBH. Lastly, myotubes were equilibrated in KRBH for 30 min at RT. Fura2 fluorescence was monitored every 0.7 s for a total of 15 s using a Synergy 4 Multimode Microplate Reader (BioTek Instruments, Winooski, VT). Fura2 was excited using a 340/20 nm band-pass excitation filter or 380/20 nm band-pass excitation filter, and emission was collected in both cases using a 508/20 nm band-pass emission filter. The 340/380 nm ratio at each time point was calculated by dividing the Fura2 signal collected at 340 nm by 380 m, and these data points were averaged to yield a resting 340/380 nm ratio, or resting Ca^{2+} level, for each well of myotubes. Seven independent calcium measure- ments were performed, with each experiment containing between six and 16 replicates, and the average 340/380 nm ratio ± SE was calculated among all wells for GFP- and HERG-transduced myotubes.

Quantitative real time PCR

Total RNA was extracted from C_2C_{12} myotubes using Trizol reagent (Life Technologies; Carlsbad, CA) according to manufacturer's instructions followed by chloroform

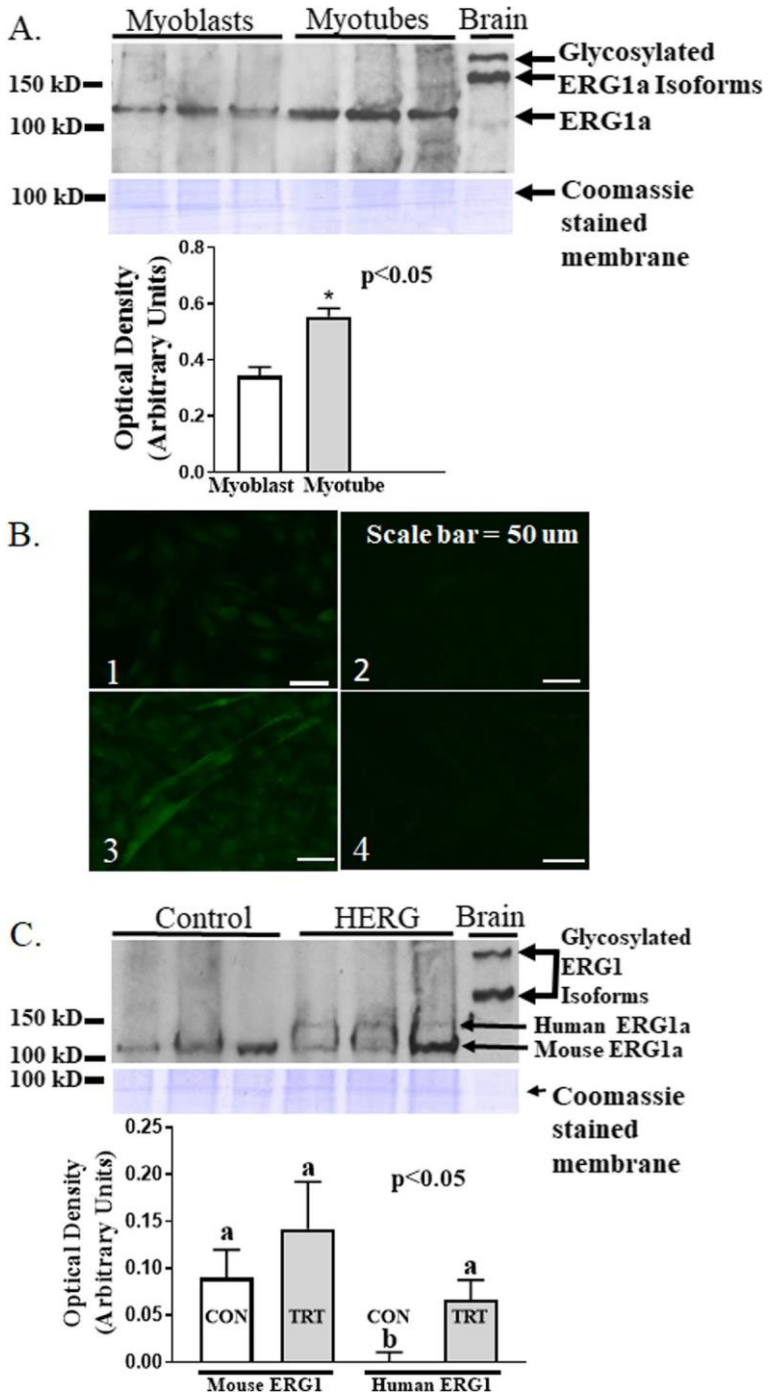

Fig. 1 Transduction of C_2C_{12} myotubes with a HERG1A-encoded adenovirus results in elevated HERG1A protein. a Immunoblot of equal protein content (50 μg) from lysates of non-transduced cells reveals that native ERG1 protein is 40.7% ($p < 0.01$; $n = 6$; Student's t test) more abundant in myotubes than in myoblasts. Coomassie stained membrane confirms that equal amounts of cell lysate protein were loaded into each lane. b Immunohistochemistry labeling ERG1 protein with Alexfluor 488 (green) secondary antibody confirms that native ERG1 protein is more abundant in myotubes than in myoblasts. Representative images of immune-stained cells: (1) myoblasts immunostained with ERG1 primary antibody; (2) myoblasts immunostained without ERG1 primary antibody as control; (3) myotubes immunostained with ERG1 primary antibody; (4) myotubes immunostained without ERG1 primary antibody as control. Scale bar = 50 μm. c Transduction of C_2C_{12} myotubes with a HERG1A-encoded adenovirus results in synthesis of HERG1A protein as demonstrated by immunoblot ($p < 0.05$; $n = 6$; two-way ANOVA). Coomassie stained membrane (blue) reveals that equal amounts of cell lysate protein were loaded into each lane

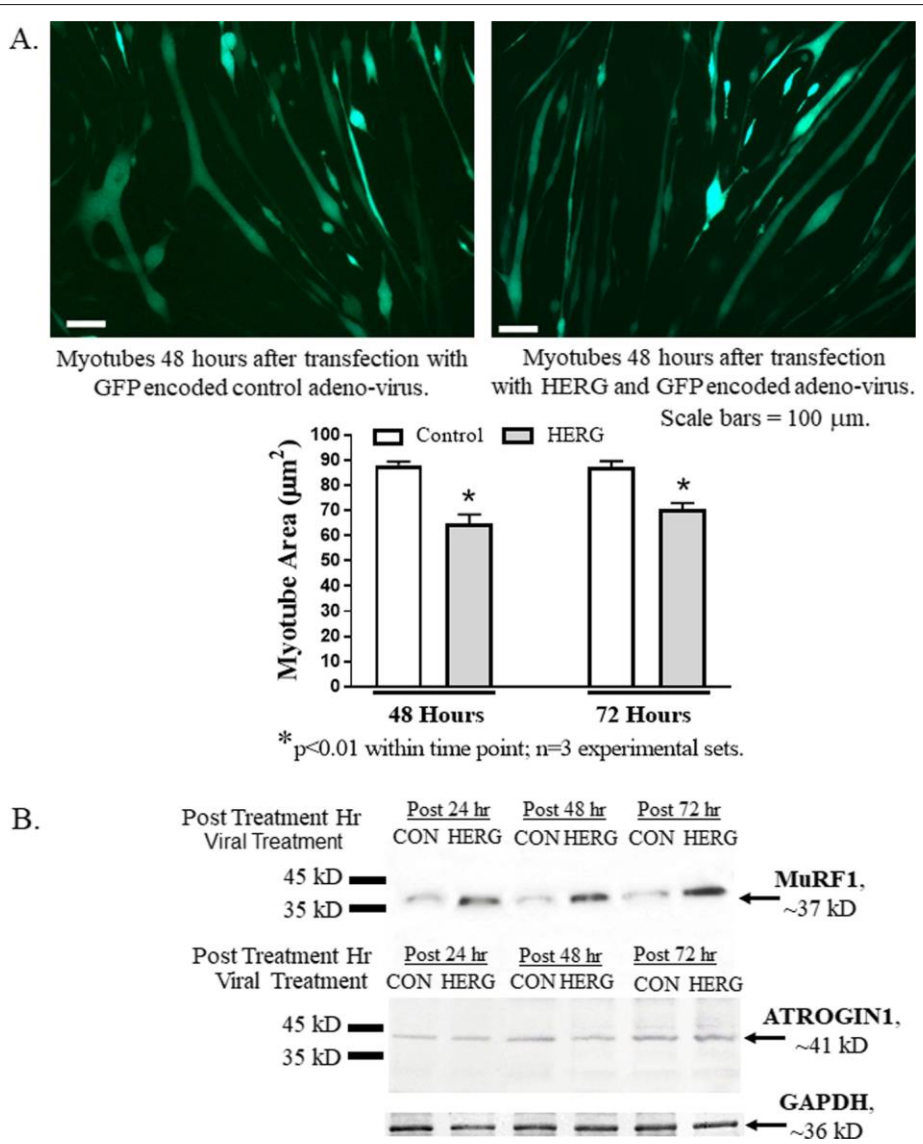

Fig. 2 Transduction of myotubes with HERG1A-encoded adenovirus is a valid in vitro skeletal muscle atrophy model. a The area of myotubes treated with HERG1A-encoded adenovirus is a significant 26.4% smaller ($p < 0.01$; $n = 3$ experimental sets) than that of control myotubes at 48 h after transduction and a significant 19.3% smaller ($p < 0.01$; $n = 3$ experimental sets) at 72 h after transduction. Scale bar = 100 μm. Bars of the graph represent the mean myotube area (μm²) while the error bars represent the standard error of the mean. b Immunoblot shows that transduction of C_2C_{12} myotubes with a HERG1A-encoded adenovirus yields an early increase in MuRF1 E3 ligase protein abundance while it does not increase abundance of ATROGIN1 protein. Immunoblots are representative of three experiments

solubilization and ethanol precipitation. Contaminating DNA was degraded via DNase (RQ1 RNase-Free DNase; ProMega, Madison WI). The total RNA was then reverse transcribed using a GOScript™ Reverse Transcription System Kit (Promega) per manufacturer's instructions. Quantitative PCR was then performed using PowerUp SYBR green master mix (Applied Biosystems, Foster City, CA) and primers for the gene of interest along with primers for the 18S ribosomal subunit "housekeeping gene" (Table 1). An Applied Biosystems 7300 real-time PCR system was used to detect SYBR green fluorescence as a measure of amplicon. Changes in gene expression were determined using the Livak method to normalize the gene of interest to the "housekeeping gene."

Tissue sections and immunohistochemistry

For Fig. 4, mouse *Gastrocnemius* muscles were embedded in OCT, cryo-sectioned (20 μm), and stained for β-galactosidase (lacZ) activity as described earlier [18]. Sections for immunohistochemistry were fixed in cold methanol at − 20 °C for 10 min. These were then rinsed with PBS at room temperature (RT) and incubated in

Table 1 Sequences of primers used for quantitative PCR

Primer name (mouse)	Primer sequence 5′–3′	Size (bp)[a]	Tm (°C)	GC (%)	Amplicon size (bp)[a]
Merg1a forward	cctcgacaccatcatccgca	20	59.6	55.0	145
Merg1a reverse	aggaaatcgcaggtgcaggg	20	60.3	60.0	
18S subunit forward	cgccgctagaggtgaaattct	21	57.2	52.4	101
18S subunit reverse	agaacgaaagtcggaggttc	20	57.0	52.4	
Calpain 1 forward	gctaccgtttgtctagcgtc	20	58.73	55.0	98
Calpain 1 reverse	taactcctctgtcatcctctggt	23	59.99	47.83	
Calpain 2 forward	ttttgtgcggtgtttggtcc	20	59.83	50.0	107
Calpain 2 reverse	aactcagccacgaagcaagg	20	60.89	55.0	
Calpain 3 forward	ttcacaggaggggtgacaga	20	60.11	55.0	122
Calpain 3 reverse	ttcgtgccatcgtcaatggag	21	61.01	52.38	
Calpastatin forward	gccttggatgacctgataga	20	53.8	50.0	115
Calpastatin reverse	gtgcctcaaggtaggtagaa	20	53.7	50.0	

[a]*bp* base pair

3% H_2O_2 for 1 h. These were then rinsed thoroughly in PBS and incubated with blocking reagent I (10% normal goat serum [NGS], 0.1% bovine serum albumin [BSA; Sigma, St. Louis, MO], and 0.1% Tween-20 in PBS) for 1 h at RT. The slides were then incubated for one hour with the laminin antibody (2 μg/mL in blocking reagent II−5% NGS and 0.2% TritonX100 in PBS) or in blocking reagent II only as a control for primary antibody binding. After a thorough rinsing with PBS, the slides were incubated overnight in the erg1 antibody (1:10 in blocking reagent 2) or in blocking reagent 2 alone on the control sections. The next day, the sections were rinsed thoroughly in PBS containing 0.1% Tween-20. All sections were then incubated for 1 h at RT in Alexafluor 568 goat anti-rat IgG (1:1000 in blocking reagent II) to bind the laminin primary antibody from rat. The slides were then again rinsed with PBS and incubated for one hour at RT in the goat anti-rabbit secondary antibody from the Alexafluor 488 Tyramide Super Boost Kit (Invitrogen, Carlsbad, CA). The tyramide reaction was carried out per manufacturer's instructions to identify ERG1 protein with green fluorescence. Finally, the sections were rinsed thoroughly with PBS and mounted with Fluoromount G with DAPI (EMS; Hatfield, PA). Two sections from each muscle mid-section were analyzed.

Imaging

Images were acquired using a Leica DM4500 microscope with a Leica DFC 340FX camera. Acquisition parameters were maintained identically across samples to allow for comparison of immunofluorescence levels when these comparisons were made. For assay of laminin protein fluorescence, two fields were imaged per slide (one slide per mouse) and the single point brightness was measured for 50 random consecutive points within the sarcolemma of each complete fiber within each field using ImageJ [21] and methods adapted from those published previously [22]. Brightness values were recorded as integers ranging from 0 (no signal) to 256 (white). The average brightness value (±standard error of the mean, SEM) for each section was determined and analyzed by two-way ANOVA using the General Linear Model Procedure of SAS 9.4 (SAS Institute Inc., Cary, NC).

Plasmids

The mouse *Erg1a* (*Merg1a*) clone in pBK/CMV plasmid [23] was a generous gift from Dr. Barry London (Cardiovascular Institute, University of Pittsburgh, PA). The phRL synthetic *Renilla* luciferase reporter vector was purchased from ProMega (Madison, WI).

Electro-transfer

Mouse anesthesia was induced with 4% isoflurane in a vented chamber and maintained by administration of 2.5% isoflurane in oxygen using a properly ventilated nose cone with anesthesia machine and scrubber. Once the animals were well anesthetized, the hind limbs were shaved and the *Gastrocnemius* muscles were injected with expression plasmids in 50 μL sterile saline and then stimulated with 8 pulses at 200 V/cm for 20 ms at 1 Hz with an ECM 830 ElectroSquare Porator (BTX; Hawthorne, NY). This method has been shown to result in ERG1a protein synthesis in skeletal muscle [15, 18].

Animal study design

Study 1

The *Merg1a* plasmid (30 μg) and a plasmid encoding *Renilla* reporter (5 μg) were injected into the left *Gastrocnemius* muscles of mice (n = 40). An empty control plasmid (30 μg) and the *Renilla* reporter plasmid (5 μg) were injected into the *Gastrocnemius* muscles of the right legs. All legs were electro-transferred to improve

plasmid uptake and expression. Each day, at days 0–7, five mice were humanely killed and the *Gastrocnemius* muscles were harvested and frozen immediately in liquid nitrogen. These were then stored at − 80 °C. All muscles were later thawed, homogenized, and assayed for (1) protein content, (2) *Renilla* activity to determine transfection efficiency, and (3) calpain activity.

Study 2

The *Gastrocnemius* muscles of a second set of animals, consisting of five animals per day for days 0–5 and 7 (n = 35), were injected and electro-transferred as described above. After the appropriate amount of time, the animals were humanely sacrificed, the muscles were harvested, and total RNA was extracted for rtPCR assay.

Study 3

The *Merg1a* plasmid (30 μg) and a plasmid encoding a β-galactosidase (LacZ) reporter (5 μg) were injected into the left *Gastrocnemius* muscles of mice (n = 5). An appropriate empty control plasmid (30 μg) and the LacZ reporter plasmid (5 μg) were injected into the *Gastrocnemius* muscles of the right legs. All legs were electro-transferred to improve plasmid uptake and expression. At day 5, the five mice were humanely killed and the *Gastrocnemius* muscles were harvested and frozen immediately in liquid nitrogen. These were then stored at −80 °C. All muscles were later thawed and painstakingly serially sectioned. Serial sections were then stained for either lacZ or dually immunostained for MERG1 and laminin proteins as described above.

Protein assay

The BCA D/C Protein Assay Reagents (BioRad; Carls Bad, CA) were used to assay both samples and standards (0, 0.25, 0.5, 1.0, 1.25, 1.5, 2.0 mg/mL bovine serum albumin in Passive Lysis Buffer [ProMega; Madison, WI]) for protein content, using a Synergy H1 Hybrid Reader (BioTek; Winooski, VT) to measure absorbance at 605 nm light wavelength. Sample absorbances were interpolated against the standard curve to determine the protein concentration of each sample.

Renilla activity

To control for differences in transfection efficiency in the animal muscle, a plasmid encoding the *Renilla* luciferase enzyme was electro-transferred into muscle along with the *Merg1a* plasmid (as described above). The *Renilla*-Glo™ Luciferase Assay System (ProMega) was used, according to manufacturer's instructions, to assay homogenates for *Renilla* enzyme activity. The reaction was allowed to proceed for the recommended 10 min and luminescence was measured using a Synergy H1 Hybrid Reader (BioTek; Winooski, VT). Luminescence was measured again 10 min later to ensure that the reaction had reached an end point after the first 10 min. The data are reported in relative light units (RLU).

Calpain assay

A Calpain-Glo Kit (ProMega; Madison, WI) was used to determine calpain activity in both myotubes and mouse muscle.

Myotubes

Myotubes were terminally differentiated and then transduced with either a HERG1A-encoded adeno-virus or the same (but non-HERG1A-encoded) virus as control (12 wells each). At 48 h post-transduction, wells were washed with two changes of 37 °C PBS and then PBS (200 μL) containing 0.2% Triton X-100 and 200 nM epoxomicin (BostonBiochem, Cambridge, MA, Cat. #I-110) was added to permeabilize the cells and to inhibit the proteasome, respectively. Six wells per viral treatment (HERG1A or control) received the buffer described (i.e., native activity); however, six wells per viral treatment received buffer supplemented with the calpain inhibitor MDL28170 (50 μM). These were allowed to sit at room temperature for 5 min to ensure the myotubes were permeabilized and the inhibitors had taken effect. Then 200 μL of Calpain-Glo reagent was added to all wells, mixed gently, and allowed to sit at room temperature. After 15 min, a 200 μL aliquot of the liquid was removed from each well and placed in a white-walled 96-well plate and luminescence was read using a Synergy H1 Hybrid Reader (BioTek Instruments, Winooski, VT). The remaining well contents were scraped from the back of the plate, triturated using a syringe and 26 gauge needle, and then centrifuged (13,000×g; 3 min) to remove any solid material. The supernatant was assayed for protein content using the BioRad DC Protein Assay kit. The protein data were used to normalize the calpain RLU activity.

Mouse muscle samples

The *Gastrocnemius* muscles were thawed, weighed, and homogenized in Passive Lysis Buffer (PLB; ProMega) at a concentration of 2.5 μL buffer/μg tissue. The sample homogenates were aliquoted and frozen at − 80 °C. Prior to assay, the homogenates were thawed and sample aliquots (40 μL) and positive control (purified porcine calpain) were added to wells of 96-well plates with assay buffer (40 μL) having either 2 mM calcium (to activate calcium dependent enzymes) or 2 mM calcium plus 50 mM MDL28170 (to inhibit calpain specifically while allowing other calcium activated enzymes to function). Each 96-well plate was read with a Synergy H1 Hybrid Reader (BioTek; Winooski, VT) and activity was measured in RLU. Calpain activity was determined by

subtracting the RLU of the wells treated with 2 mM calcium and MDL28170 from the RLU of the wells treated with 2 mM calcium only and normalizing this RLU to the RLU assayed with the *Renilla* kit to control for differences in transfection efficiencies. The result was then normalized to protein content (RLU/mg protein).

Statistics

In general, statistics were done using either a simple Student *t* test or an ANOVA (as indicated in results section and figure legends) and SAS (SAS Inc.; Carey, NC). Results were considered significant when $p < 0.05$ unless otherwise noted.

RESULTS

Transduction of C_2C_{12} myotubes with a HERG1A-encoded adenovirus results in elevated HERG1A protein

Immunoblot of equal protein aliquots from both non-virus treated C_2C_{12} myoblast and myotube lysates detects a 40.7% ($p < 0.01$; $n = 6$; Student's *t* test) greater abundance of the ERG1 protein in myotubes than in myoblasts (Fig. 1a). Immunohistochemistry work also demonstrates that there is more ERG1 protein in the C_2C_{12} myotubes than in the myoblasts, revealing a stronger signal in myotubes that is dispersed over the surface of the cell, while in myoblasts it reveals only a very faint fluorescent signal which appears to be mainly nuclear (Fig. 1b). We transfected myotubes with either virus-encoding HERG1A (and GFP) or with the same, but not HERG1A-encoded, virus as control. Immunoblot of the lysates shows that C_2C_{12} myotubes transfected with virus encoding HERG1A do synthesize the HERG1A protein, which appears as a single band of higher mass (likely a result of differential glycosylation) than the native mouse ERG1 and is absent from the myotubes treated with the control virus (Fig. 1c; $p < 0.05$; two-way ANVOA). Coomassie stained membrane confirms that equal amounts of protein were loaded into each well of the gel for immunoblot.

Transduction of C_2C_{12} myotubes with a HERG1a-encoded adenovirus results in decreased myotube area and increased MuRF1 E3 ligase abundance, but no change in myoblast fusion index

We transfected myotubes with either virus-encoding HERG1A (and GFP) or with the same, but not HERG1A-encoded, virus as control. Fluorescent imaging demonstrates that both viral particles infect myotubes (Fig. 2a). Further, when the average area (μm^2) of fluorescent myotubes from both sets is determined at both 48 and 72 h after transfection, we discover that, similarly to mouse skeletal muscle fibers electro-transferred with *Merg1a* plasmid [23], the myotubes transfected with HERG1A are significantly smaller than control myotubes. Specifically, the area of the HERG1A-expressing myotubes is decreased by 26.4% at 48 h post transfection ($p < 0.01$; $n = 6$; Student's *t* test) and by 19.3% at 72 h post transfection ($p < 0.01$; $n = 6$; Student's *t* test). Within each time point, the difference between the HERG1A-treated and control myotubes is statistically significant ($p < 0.01$); however, there is no significant difference in size between the myotubes treated with HERG1A-encoding virus at the two different time points (Fig. 2a). Also similarly to mouse skeletal muscle expressing *Merg1a* [23], myotubes transduced with HERG1A exhibit increased levels of the UPP E3 ligase, MuRF1, but not the E3 ligase ATROGIN1 (Fig. 2b). However, when we treated myoblasts with either the HERG-encoded or the control virus and allowed them to differentiate, we found that the HERG-expressing samples did not have a significantly different number of myotubes containing two or more nuclei than the cells treated with the control virus. That is, the fusion index (myosin-positive multi-nucleated cells:total myosin-positive cells evaluated) was 33.5 ± 5.0% (mean ± SEM) for the cells treated with the HERG-encoded virus while it was 31.6 ± 2.3% for the control-treated myoblasts ($p < 0.74$; $n = 14$; Student's *t* test). The data demonstrate that HERG1A treatment of myotubes results in atrophy (i.e., reduced myotube area) as it does in mouse skeletal muscle; however, it does not affect the degree to which the myoblasts fuse. We conclude that we have developed a valid in vitro model of skeletal muscle atrophy.

Transduction of myotubes with a HERG1A-encoded adenovirus yields a basal increase in both intracellular calcium levels and calpain activity

We transduced C_2C_{12} myotubes with either a GFP- and HERG1A-encoded adenovirus or an appropriate control GFP-only encoded adenovirus. At 48 h after viral treatment, we used a fura-2 calcium indicator assay and observed a significant 51.7% increase ($p < 0.0001$; $n = 90$ GFP and $n = 87$ HERG1A transduced wells; Student's *t* test) in basal intracellular calcium levels in HERG1A transduced myotubes relative to control (Fig. 3a). This demonstrates that HERG1A must either increase calcium influx and/or intracellular calcium release and/or decrease intracellular calcium re-uptake. Because HERG1A transduction results in increased basal intracellular calcium levels, we investigated the downstream effects of this increase. Specifically, using a Calpain-Glo assay kit (ProMega), we measured the combined activity of the calpain 1 and 2 enzymes in myotubes treated with either the control or the HERG1A-encoded virus. Some myotubes from both viral treatments were treated with either 50 μM MDL28170 to inhibit calpains or an equal volume of buffer vehicle. We observed that basically the same amount of enzyme activity (control myotubes = 160.8 ± 7.3 and HERG1A-expressing myotubes = 167.5 ± 5.34 RLU/mg protein; $n = 24$) was not blocked in each well treated with the MDL28170, indicating that a rather high level of non-calpain activity was assayed. Nonetheless, we find that in control cells, the calpain activity is

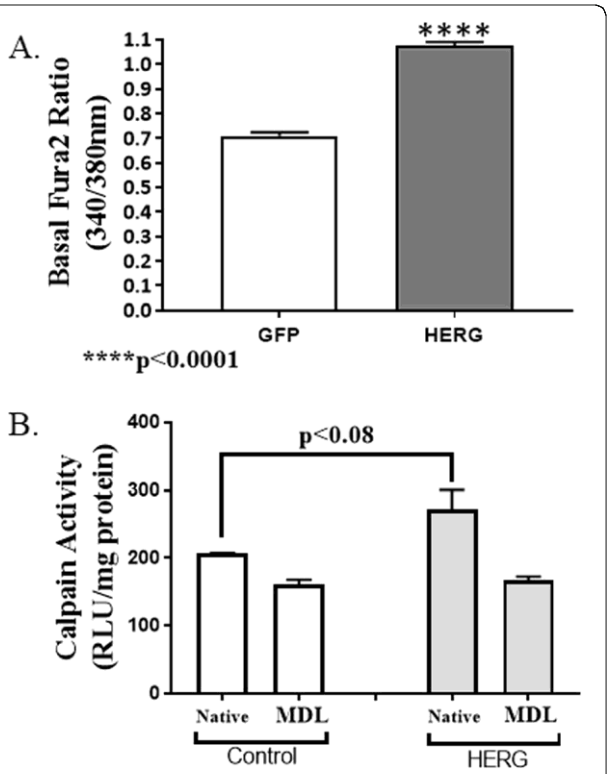

Fig. 3 Transduction of myotubes with HERG1A-encoded adenovirus increases basal intracellular calcium levels and basal calpain activity. a Fura-2 dye experiments reveal that expression of HERG1A in C_2C_{12} myotubes yields a 51.9% increase ($p < 0.0001$; $n = 90$ GFP and $n = 87$ HERG1A transduced wells) in basal intracellular calcium levels relative to myotubes transduced with a control virus. b Calpain assay reveals that transduction of C_2C_{12} myotubes with a HERG1A-encoded adenovirus increases combined native calpain 1 and 2 activity a significant 31.9% ($p < 0.08$; $n = 24$; two-way ANOVA) over control myotubes. All bars represent the mean while error bars represent the standard error of the mean

22.1% of the total native activity while it is 38.5% of the total in HERG1A-treated cells, demonstrating an increase in calpain activity in the HERG1A-treated cells. Because a two-way ANVOA reveals there is no real difference in the level of MDL28170 inhibited activity, we can compare the differences in assayed native activity (control versus HERG1A treated) and find that there is a 31.9% increase ($p < 0.08$) in activity in the HERG1A-expressing myotubes over the controls (Fig. 3b). Although the 0.08 probability is greater than the generally accepted statistical significance level of 0.05, we believe that the difference is nonetheless real.

Merg1a expression in mouse *Gastrocnemius* muscle increases calpain activity, but did not change the number of centrally located nuclei or laminin abundance

To test the effect of *Merg1a* expression on calpain activity in animals, we electro-transferred the left *Gastrocnemius* muscle of mice with an expression plasmid encoding *Merg1a* and the right leg muscle with an appropriate control plasmid ($n = 68$ mice). We then assayed total RNA extracted from the muscles for *Merg1a* expression ($n = 28$) and the muscle homogenates for calpain activity ($n = 40$). Quantitative PCR reveals that the electro-transfer did produce *Merg1a* expression which was significantly higher than day 0 at days 3–5 ($p < 0.05$; Student's *t* test was used to compare each day to day 0; Fig. 4a). *Merg1a* expression also yielded an increase in calpain activity, increasing nearly 4-fold (over day 0) by day 3 and 7.5-fold by day 4 ($p < 0.05$; Student's *t* test was used to compare each day to day 0; Fig. 4b). It returns to day 0 control levels by day 5. Thus, we show that MERG1a overexpression increases calpain activity and thus protein degradation. It is possible that the increase in intracellular calcium could lead to myofiber degeneration. Thus, we electro-transferred left mouse *Gastrocnemius* muscle with a *Merg1a*-encoded plasmid and a LacZ-encoded plasmid while expressing lacZ-encoded plasmid and a an appropriate control plasmid in the right *Gastrocnemius* muscle and performed studies to determine if over-production of this protein would bring about changes indicative of degeneration, specifically changes in the number of centrally located nuclei or in the abundance of basal laminin. Thus, we *painstakingly* stained muscle serial sections for lacZ (Fig. 4c) as a marker for MERG1 and dually immunostained matching serial sections for both MERG1 (green fluorescence, Fig. 4d) and laminin (red fluorescence, not shown) and used a DAPI containing immunomount to identify nuclei (Fig. 4d). There was no response in sections not stained with primary antibody (Fig. 4e). The lacZ stain (blue fibers in Fig. 4c) identifies where the MERG1 overexpression occurs. We find no evidence of any changes in the number of centrally located nuclei (Fig. 4d) nor in the amount of laminin fluorescence (Fig. 4f) in the fibers overexpressing MERG1 in any of the five mice examined nor have we seen any evidence of these occurrences in any of our past studies.

HERG1A expression in myotubes does not affect expression of calpains 1–3 or calpastatin although it does affect certain protein abundances

Calpain activity will augment with increased intracellular calcium; however, we cannot assume that the increased calcium is the only explanation for the increased calpain activity. Thus, we asked if expression and/or protein abundances of either calpains 1, 2, or 3 or calpastatin were affected by HERG1A expression. We used quantitative real-time PCR to discover that HERG1A expression does not produce a statistically significant change in calpain 1 mRNA levels for up to 84 h after viral treatment (Fig. 5a). As well, no change in gene expression was detected for calpains 2 or 3 (data not shown). Further, our results indicate that there is no significant change in calpain 1 protein abundance (Fig. 5b; $n = 6$; Student's *t* test). Calpain 2, when autolyzed and hence

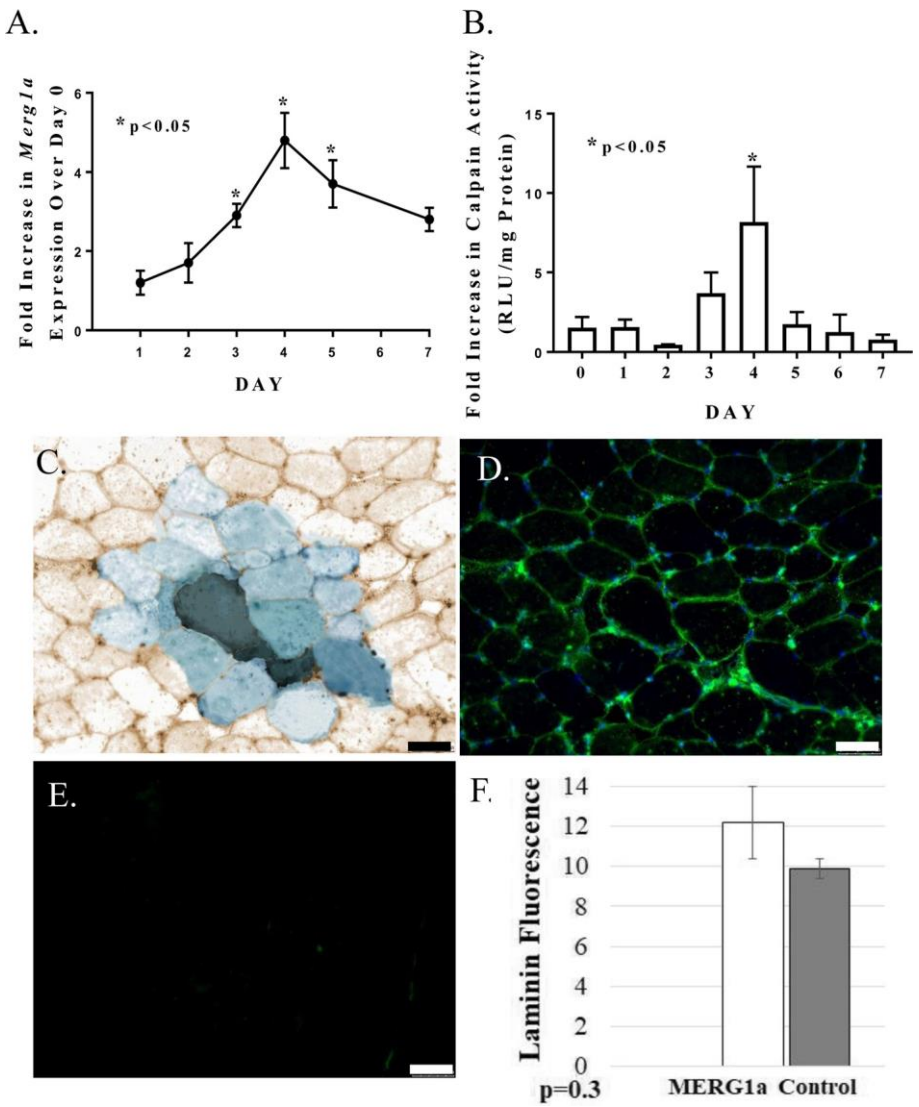

Fig. 4 Expression of mouse *erg1a* in mouse *Gastrocnemius* muscle increases *Merg1a* transcription and native calpain activity, but does not increase the number of centrally located nuclei or the abundance of laminin protein. a Quantitative PCR shows that electro-transfer of an expression plasmid encoding mouse *erg1a* (*Merg1a*) into mouse skeletal muscle produces *Merg1a* expression which is significantly higher than day 0 at days 3–5 ($p < 0.05$; $n = 28$). The enclosed circles of the line graph represent the mean while the error bars represent the standard error of the mean. b *Merg1a* transfection in mouse skeletal muscle increases calpain activity nearly 4-fold (over day 0) by day 3 and nearly 7.5-fold by day 4 ($p < 0.05$; $n = 40$). It returns to day 0 control levels by day 5 post transfection. Bars represent the mean calpain activity while error bars represent the standard error of the mean. c Positive assay for the β-galactosidase reporter (as an indicator of electro-transfer of plasmid encoding the Merg1a gene) produces a blue color. d Immunostain for MERG1 (green) of a serial section matched to the section in c demonstrates that there is indeed a greater amount of MERG1 in the fibers colored blue in c. There were no greater number of centrally located nuclei in the green fibers of any sections ($n = 5$ mice). e Representative of sections immunostained without primary antibody. f Over-expression of *Merg1a* does not produce a change in laminin abundance ($p = 0.3$; $n = 5$). Bars represent the mean single point laminin fluorescence while error bars represent the standard error of the mean. All scale bars = 50 μm

activated, appears as a doublet found at ~ 75 kD [24]. Interestingly, our results show that there is a 40.7% decrease ($p < 0.05$; $n = 6$; Student's *t* test) in total calpain 2 protein abundance in response to 48 h of HERG1A treatment (Fig. 6). Calpastatin expression was not significantly affected by the HERG1A channel for up to 84 h post-transduction (Fig. 7a); however, calpastatin protein abundance declined by a statistically significant 31.7% (Fig. 7b; $p < 0.05$; $n = 6$; Student's *t* test). Additionally, there is a decrease in two of the three noted calpain 3 autocatalytic products (25; Fig. 8): the 114 kD isoform is down 29.6% and the 60 kD isoform is down 29.2%, although the 30 kD isoform is not affected ($p < 0.03$; $n = 6$; Student's *t* test within protein isomer). When the optical

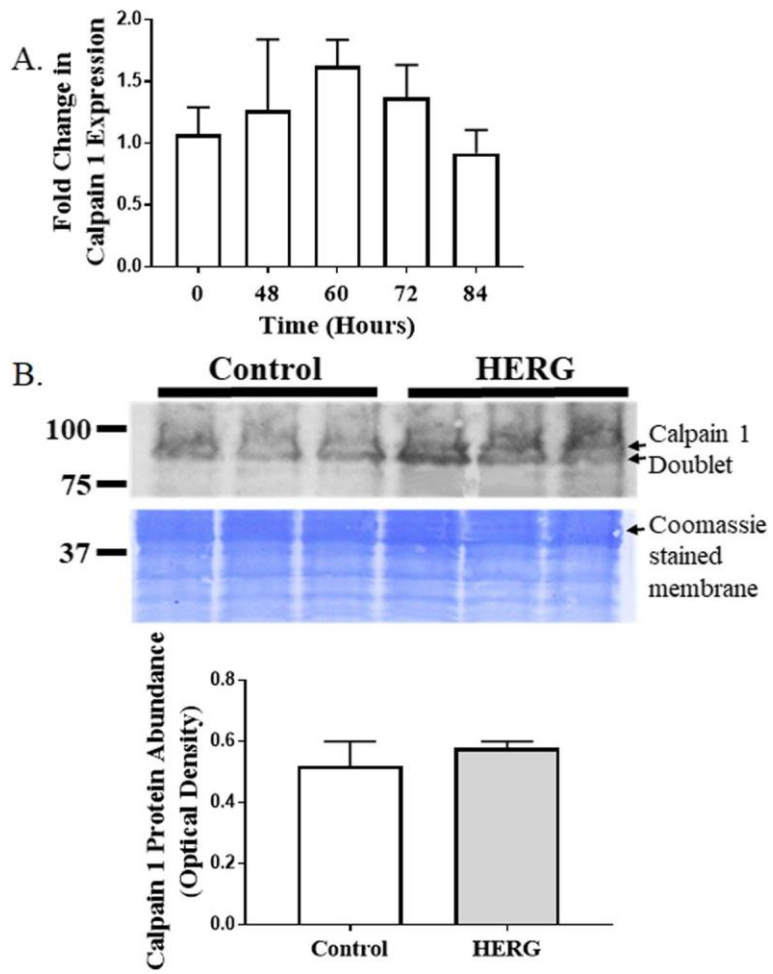

Fig. 5 Neither calpain 1 expression nor protein abundance changes after transduction of myotubes with HERG1A-encoded adenovirus. a Quantitative PCR reveals that there is no change in expression of calpain 1 for up to 84 h after transduction (n = 15). bImmunoblot demonstrates that there is no significant change in calpain 1 protein abundance at 48 h after viral transduction (n = 6). Bars represent the mean and the error bars represent the standard error of the mean. Coomassie staining of the blotted membrane shows that equal amounts of protein were loaded into each well of the gel

densities for all protein bands are summed, there is a total 21.0% decrease in calpain 3 protein abundance.

DISCUSSION

The ERG1a voltage-gated K^+ channel is responsible for late phase repolarization of the cardiac action potential and was reported to be absent from skeletal muscle [23, 25]; however, the Pond and Hannon labs demonstrated that this protein is detectable in the atrophying skeletal muscle of mice and in very low abundance in healthy rodent muscle with careful use of protease inhibitors and concentration of solubilized membrane proteins [18]. Subsequent studies showed that ERG1a expression leads to an increase in abundance of the MURF1 E3 ubiquitin ligase protein and enhances ubiquitin proteasome proteolysis, a pathway known to contribute to skeletal muscle atrophy [15, 18]. Here, using C_2C_{12} myotubes transduced with either control or HERG1A-encoded adenovirus, we show that HERG1A expression also increases basal $[Ca^{2+}]i$ and calpain activity. There are numerous potential sources of the calcium that contributes to the increased $[Ca^{2+}]i$. For example, it is possible that ERG1A is modulating Cav1.1 channels in the skeletal muscle sarcolemmal membrane, resulting in an influx potentially from both the external milieu and internal stores. Further, because ERG1A is located in the t-tubules of cardiac tissue [17, 20], it is possible that it is located in the t-tubules of skeletal muscle, where it could contribute to the release of calcium from internal stores by modulation of ryanodine receptors and/or IP3 receptors. Indeed, changes in regulation of sarcolemmal permeability could have severe consequences for skeletal muscle tissue, potentially producing diseases such as muscular dystrophies and Niemann-Pick disease [26, 27]. The source of the increased calcium is currently

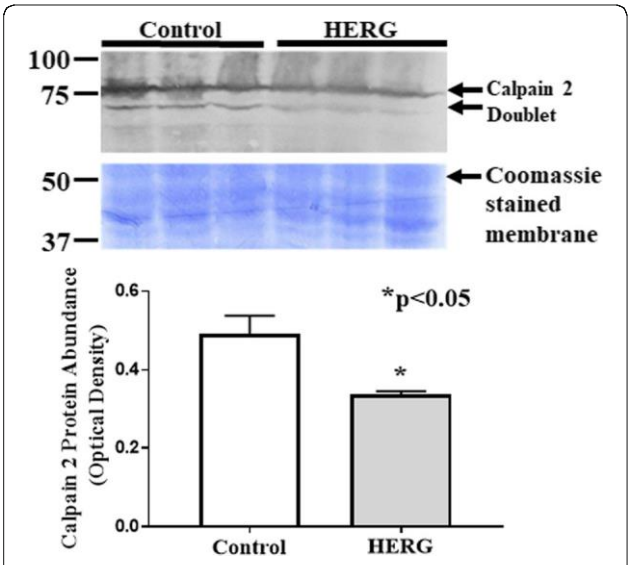

Fig. 6 Calpain 2 protein abundance decreases ($p < 0.05$; $n = 6$) 48 h after myotube transduction with HERG1A-encoded adenovirus. Bars represent the mean and error bars represent the standard error of the mean. Coomassie staining of the blotted membrane confirms that equal amounts of protein were loaded into each well of the gel

under investigation in our laboratories. However, because we find no change in the fusion index or an increase in either the number of centrally located nuclei or in the abundance of laminin fluorescence in the fibers overexpressing Merg1a, we believe that our data suggest that the channel (which we find to be in very low abundance in muscle normally) is simply upregulating protein degradation in our myotubes. It is also possible that the low levels of increased calcium are affecting signaling pathways, but that remains to be investigated.

The explanation for the increased calpain activity may seem obvious—the increase in $[Ca^{2+}]i$. However, we ectopically expressed mouse *erg1a* (*Merg1a*) in mouse *Gastrocnemius* muscle and homogenized the muscle, thereby disrupting the $[Ca^{2+}]i$ pool and equalizing the calcium concentration throughout the sample. We then assayed for calpain activity and discovered that even in the homogenate it is still higher in the *Merg1a*-expressing tissue. This study is evidence that increased $[Ca^{2+}]i$ may not be the only factor that contributes to the ERG1A-induced increase in calpain activity. Other pos- sible contributors include increased calpain 1 and/or 2 protein and/or decreased calpastatin protein.

Calpains 1 (μ-calpain) and 2 (m-calpain) are both classical calpains and are detected throughout the body, including skeletal muscle [28]. Indeed, calpain activity has been demonstrated to contribute to muscle atrophy [28]. For example, Shenkman and colleagues inhibited calpain activity in hind limb suspended mice by treatment with the calpain inhibitor PD150606 and demonstrated that blocking calpain activity reduced the activation of calpain 1 gene expression and attenuated skeletal muscle atrophy [29]. Here, we report that there is no detectable change in calpain 1 protein abundance in myotubes transduced with HERG1A while surprisingly we detect a decrease in calpain 2 protein abundance. These data demonstrate that the increased calpain activity is not a result of increased enzyme protein abundance. We suggest that the decreased calpain 2 protein abundance could result from either decreased calpain 2 synthesis and/or increased calpain 2 protein degradation. Quantitative PCR data demonstrate that there is no significant change in transcription of calpain 1 or 2 genes for up to 84 h post transduction. Interestingly, we observe a decrease in calpain 2 protein abundance without detecting a change in transcription of that gene. Thus, although mRNA production is not always directly correlated with protein abundance, we can speculate that the calpain 2 protein may be undergoing an increased level of degradation. Indeed, these proteins may be undergoing autolysis or it is possible that ubiquitin proteasome proteolysis of calpain 2 is enhanced. Indeed, we have shown that increased ERG1 expression increases UPP activity.

Calpastatin is a native calpain inhibitor which inhibits conventional calpains 1 and 2, but not calpain 3. Calpastatin requires calcium to bind calpains so that when the calcium concentrations rise, calpain activity is increased, but so is calpastatin binding [13, 30]. Indeed, a decrease in calpastatin protein would lower the inhibition of calpains and allow for increased calpain-mediated proteolysis. Certainly, the increased level of calpain activity assayed in the mouse muscle homogenates, in which the $[Ca^{2+}]i$ is disrupted, suggests that something other than $[Ca^{2+}]i$ must contribute to enhanced calpain activity.

Calpain 3 is a non-classical calpain which is detected mainly in skeletal muscle. It undergoes calcium-mediated autolysis that has been reported to be enhanced by ATP at lower calcium concentrations [31, 32]. Evidence has shown that the absence of calpain 3 leads to a reduction in protein turnover and results in accumulation of damaged and/or misfolded proteins which can lead to cellular stress and eventual muscle pathology [33, 34]. Indeed, the absence or reduction of this protein has been shown to lead to limb-girdle muscular dystrophy type 2A (LGMD2A) in humans [30–32, 34–37]. Studies suggest that calpain 3 takes part in remodeling of the sarcomere in response to cellular damage such as atrophy [34, 36, 37]. Interestingly, studies with calpain 3 knockout mice suggest that calpain 3 acts upstream of the UPP, although it is uncertain if calpain 3 directly cleaves proteins to make them accessible for ubiquitination [34]. Thus, calpain 3 appears to be protective against muscle loss and its protein abundance might be expected to be lower in an atrophic situation.

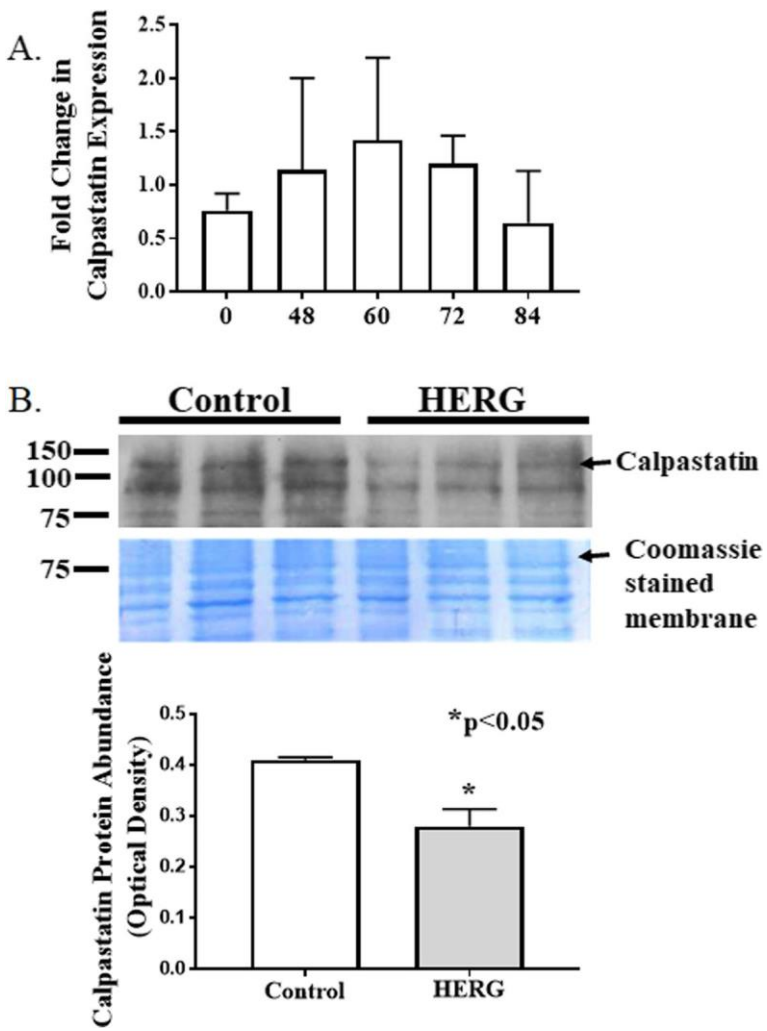

Fig. 7 Calpastatin expression does not change after transduction with HERG1A-encoded adenovirus although protein abundance decreases. a Quantitative PCR reveals that levels of calpastatin mRNA do not significantly change for up to 84 h after viral transduction with HERG1A encoded adenovirus. b, c. Immunoblot detects a significant 31.7% decrease in protein abundance ($p < 0.05$; $n = 6$) at 48 h after transduction. All bars represent the mean ± the standard error of the mean. Coomassie staining of the blotted membrane confirms that equal amounts of protein were loaded into each well of the gel

Indeed, we report that calpain 3 protein abundance decreases in response to HERG1A expression. The decrease may be related to a decreased ability to remodel the sarcomere during/after atrophy; however, this possibility would require much additional investigation.

In summary, we show that HERG1A increases calpain activity in myotubes, likely resulting from the increase in $[Ca^{2+}]i$. We detect no increases in abundances of calpains 1 or 2 proteins which would otherwise contribute to enhanced calpain activity. In fact, we report a decline in the abundance of calpain 2 protein. Thus, it would appear that the increased $[Ca^{2+}]i$ could be the main contributor to the enhanced calpain activity; however, there is a significant decline in calpastatin protein abundance which likely also contributes to the measured increase in calpain activity. This is not surprising considering that calpastatin binding is also enhanced by intracellular calcium. Calpain 3 activity was not measured here; however, the decline in calpain 3 protein is consistent with an atrophic environment. Interestingly, classical calpain activity has been shown to degrade sarcomeric anchor proteins (e.g., titin, nebulin) and this allows for release of contractile proteins (e.g., myosin and actin) into the cytosol where they can be accessed and degraded by the UPP [30, 38]. Here, we show that HERG1A modulates intracellular calcium and calpain activity. Because its interaction with calcium and calpains is upstream of the UPP, and it also modulates UPP activity [18], we hypothesize that ERG1A may indeed contribute to coordination of proteolytic systems which produce skeletal

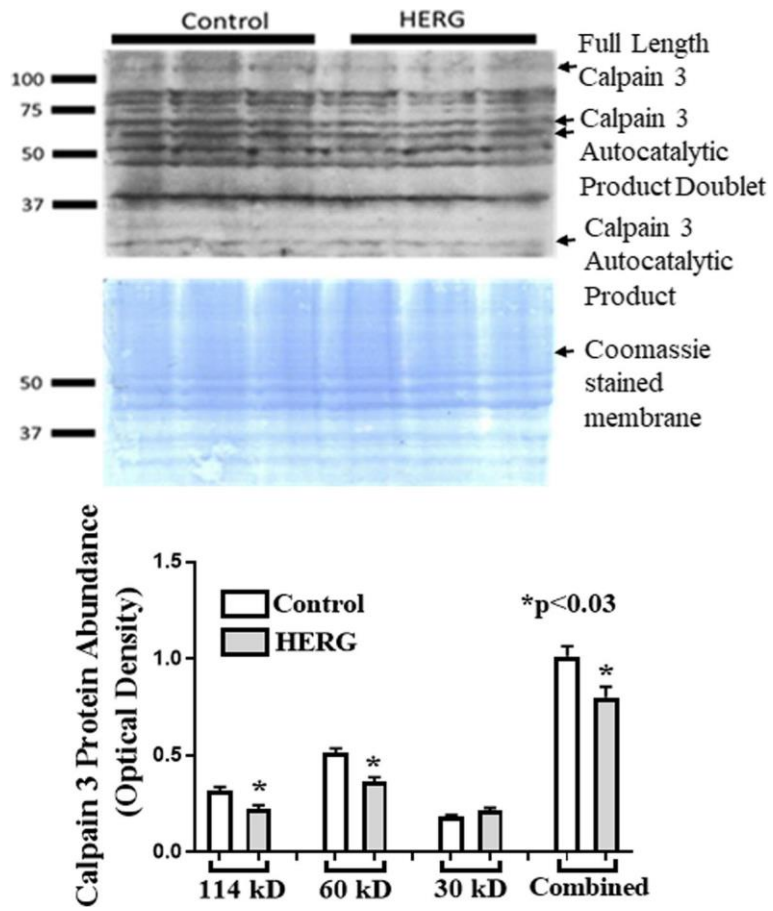

Fig. 8 Calpain 3 protein abundance decreased 21.0% in response to transduction of myotubes with HERG1A-encoded adenovirus. Immunoblot shows that calpain 3 degraded into numerous fragments as expected, including three notable autocatalytic products: 114 kD (down 29.6%), 60 kD (down 29.2%), and 30 kD which was not affected. Bars represent the mean ± the standard error of the mean. Coomassie staining of the blotted membrane shows that equal amounts of protein were loaded into each well of the gel

muscle atrophy, specifically calpain and UPP activities. Further study is needed to learn how ERG1A functions in skeletal muscle. Indeed, because of the role of the ERG1A/ERG1B heteromultimeric channel in cardiac action potential repolarization, ERG1A will likely never be a target for pharmacological treatment of atrophy; however, continuing study of this protein may reveal other possible targets to combat atrophy.

Abbreviations
DMEM: Dulbecco's modification of Eagle's medium; *ERG1A*: *Ether-a-gogo-related gene*; FBS: Fetal bovine serum; *HERG1A*: Human *ether-a-gogo-related gene*; *Merg1a*: Mouse *ether-a-gogo-related gene*; RLU: Relative light units; UPP: Ubiquitin proteasome pathway

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Authors' contributions
CW cultured and transduced myotubes, performed calpain assays, completed the PCR and immunoblotting work, and wrote the original draft of the manuscript. EP cultured and transduced myotubes and performed fura-2 assays to determine intracellular calcium concentrations. LA cultured and transduced myotubes and then imaged myotubes and determined their area. KB, SML, and MNH performed the electro-transfer on mice hind limbs. KB and SML performed the calpain assays on the electro-transferred muscles. AKU imaged myotubes and consulted on content and writing of manuscript. RW provided direction on calpain assays and consulted on content and writing of manuscript. JKD cultured, imaged, and evaluated myoblasts and myotubes and consulted on content and writing of manuscript. WHW cloned the *HERG1A* construct into the viral cassette, provided guidance for primer development, and consulted on content of manuscript. GHH provided over all guidance to EP for measurement of calcium concentration, acted as co-PI on the grant which funded the bulk of this work, and consulted on content and writing of manuscript. ALP worked in the laboratory to produce some of the data, provided over all guidance to the project, acted as co-PI on the grant which funded the bulk of this work, and consulted on content and writing of manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
All animal work and studies were approved the SIU IACUC.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Anatomy Department, Southern Illinois University School of Medicine, Carbondale, IL 62902, USA. [2]Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47906, USA. [3]Doisey College of Health Sciences, Saint Louis University, St. Louis, MO 63103, USA.
[4]School of Liberal Arts, Sciences and Education, Ivy Tech State college,
Lafayette, IN 47905, USA. [5]Biochemistry Department, Southern Illinois University School of Medicine, Carbondale, IL 62902, USA. [6]Gene Editing Core Facility, Purdue University, West Lafayette, IN 47906, USA. [7]Southern Illinois University, 1135 Lincoln Drive, Carbondale, IL 62902, USA.

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1.

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The ERG1a K^+ channel increases calpain activity in C2C12 myotubes and mouse skeletal muscle

Amber L. Pond (1), Clayton Whitmore (1), Evan E. Pratt (2), Luke Anderson (1), Emily Rantz (2), Amy Salyer (2), Ugo Carraro (3), Wen-Horng Wang (4), Rod Weilbaecher (5), Judy K. Davie (5), and Gregory H. Hockerman (2).
(1) Anatomy Dept., Southern Illinois Univ. School of Medicine, Carbondale, IL 62901; (2) Medicinal Chemistry and Molecular Pharmacology Dept., Purdue Univ. School of Pharmacy, West Lafayette, IN 47906; (3) IRCCS Fondazione Ospedale San Camillo, Venezia-Lido, Italy; (4) Gene Editing Core Facility, Purdue University, West Lafayette, IN; (5) Biochemistry Dept., Southern Illinois Univ. School of Medicine, Carbondale, IL.
E-mail: apond@siumed.edu

Increased intracellular calcium concentration ($[Ca^{2+}]i$; uM range) is necessary for excitation-contraction coupling in skeletal muscle; however, smaller increases in $[Ca^{2+}]i$ (nM range) can act as a second messenger (Fig. 1) to modulate other cellular processes in non-contracting muscle. In contrast, improper changes in $[Ca^{2+}]i$ can have detrimental effects on muscle and have been associated with pathologies, for example muscular dystrophy, amyotrophic lateral sclerosis, malignant hyperthermia, cancer cachexia, and atrophy.[1-4] Indeed, $[Ca^{2+}]i$ must be rigidly regulated in terms of time, space and amplitude for cellular processes to occur in a properly coordinated fashion.[1] In earlier reports, we have shown that the *ether-a-gogo related gene* (ERG) *1a* K^+ channel is detected in atrophying skeletal muscle and that *mouse erg1a* (*Merg1a*) expression in mouse muscle upregulates ubiquitin proteasome proteolysis.[5,6] Because ERG1a is detected in the t-tubules of cardiac muscle,[7] we hypothesized that it would affect intracellular calcium concentration and thus activity of calpains (calcium activated cysteine proteases known to contribute to protein loss in atrophy). We tested this hypothesis using the ratiometric Ca^{2+} indicator fura-2AM, and here show that viral transduction of human ERG1a (HERG1a) into C2C12 myotubes for 48 hours produces a 34% increase in basal intracellular calcium levels ($[Ca^{2+}]i$) relative to myotubes transduced with the appropriate control virus ($p<0.0001$; n=87 HERG1A; n=90 control). Further, we transduced myotubes with HERG1a and, using a CalpainGlo assay kit (ProMega; Madison, WI), determined that the combined calpain 1 and 2 activity increased by 25.7% relative to controls. The change in calpain activity is likely attributable to increased calcium levels; however, other explanations include increased calpain protein resulting from enhanced transcription and/or translation or decreased calpain protein degradation. Further, decreased abundance of the native inhibitor calpastatin could explain enhanced calpain activity. Thus, we performed quantitative PCR on myotubes transduced with control and HERG-encoded virus for 48 hours and detected no significant changes in expression of calpains 1, 2 and 3 nor of calpastatin. Immunoblot detected no change in calpain 1 protein abundance. However, immunoblot revealed a 40.7% decrease in abundance of calpain 2 protein and a 23.5% decrease in abundance of calpain 3. Obviously, these data do not explain the increase in calpain activity and, indeed, the increased degradation of the calpain proteins may be attributable to the increased calpain activity produced by

the increased calcium concentration. Further, calpastatin protein abundance also decreased by 37.7%. This lowered level of calpain inhibitor could indeed contribute to the increased calpain activity. Interestingly, we electro-transferred *Merg1a* encoded plasmid into mouse *Gastrocnemius* muscles and at day 4 post-electro-transfer detected a 7-fold increase in calpain activity over control in *homogenated* samples. Because the cellular structure had been disrupted in this experiment, the increase in calcium concentration cannot be the sole contributor to the increased calpain activity. Indeed, the decreased calpastatin protein levels may be important.

Key Words: skeletal muscle atrophy; ERG1 Potassium Channel; intracellular calcium; calpain; calpastatin

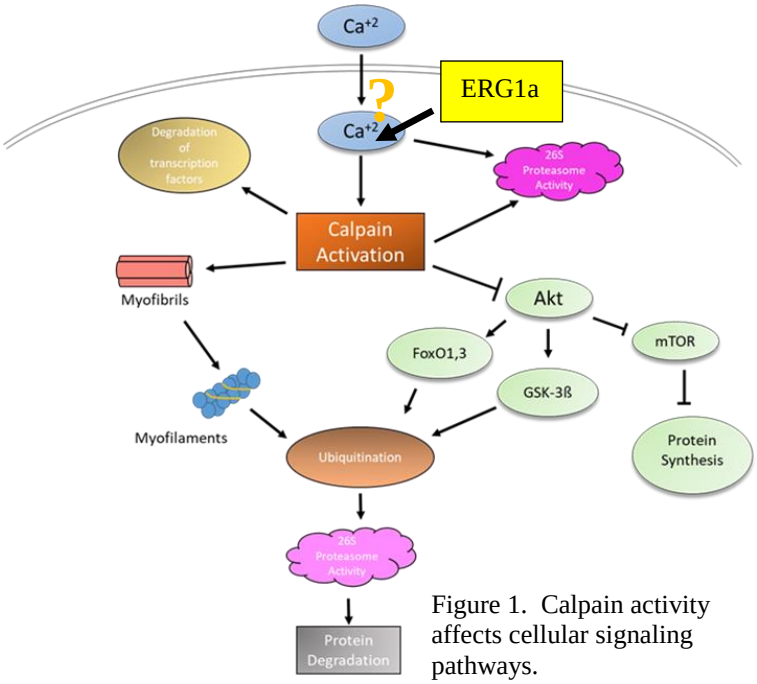

Figure 1. Calpain activity affects cellular signaling pathways.

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Pond AL, Whitmore C, Pratt EE, Anderson LB, Rantz E, Salyer A, Carraro U, Zampieri S, Weilbacher R, Davie JK, Hockerman GH. 2019. *The ERG1a K^+ channel increases intracellular calcium and calpain activity in C_2C_{12} myotubes.* Poster presentation given at Experimental Biology Conference in Orlando, FL, April 8, 2019.

The ERG1a K^+ Channel Increases Intracellular Calcium and Calpain Activity in C2C12 Myotubes

Amber L. Pond (1), Clayton Whitmore (1), Evan E. Pratt (2), Luke Anderson (1), Emily Rantz (2), Amy Salyer (2), Ugo Carraro (3), Sandra Zampieri (4), Rod Weilbaecher (5), Judy K. Davie (5), and Gregory H. Hockerman (2).

(1) Anatomy Dept., Southern Illinois Univ. School of Medicine, Carbondale, IL 62901; (2) Medicinal Chemistry and Molecular Pharmacology Dept., Purdue Univ. School of Pharmacy, West Lafayette, IN 47906; (3) IRCCS Fondazione Ospedale San Camillo, Venezia-Lido, Italy; (4) Biomedical Sciences Dept., University of Padova, Italy; (5) Biochemistry Dept., Southern Illinois Univ. School of Medicine, Carbondale, IL.

E-mail: apond@siumed.edu

In skeletal muscle increased intracellular calcium concentration ($[Ca^{2+}]i$; uM range) is necessary for excitation-contraction coupling; however, smaller increases in $[Ca^{2+}]i$ (nM range) can modulate other physiological processes in non-contracting muscle. Indeed, fluctuations in localized calcium concentration can serve as a second messenger. In contrast, inappropriate changes in $[Ca^{2+}]i$ can have detrimental effects on muscle tissue and are associated with numerous skeletal muscle pathologies, for example muscular dystrophy, amyotrophic lateral sclerosis, malignant hyperthermia, cancer cachexia, and atrophy. Therefore, $[Ca^{2+}]i$ must be tightly regulated in terms of time, space and amplitude for cellular processes to occur in a properly coordinated fashion. In earlier reports, we have shown that the ERG1a K^+ channel is upregulated in atrophying skeletal muscle and contributes to increased ubiquitin proteasome proteolysis. Here, using the ratiometric Ca^{2+} indicator fura-2AM, we show that ERG1a expression in C2C12 myotubes produces an increase in basal $[Ca^{2+}]i$ as well as a transient increase in $[Ca^{2+}]i$ as a consequence of depolarization. We explored this transient increase in $[Ca^{2+}]i$ using pharmacological agents. The data demonstrate that the ERG1a-induced increase is not sensitive to the L-type calcium channel blocker nifedipine, suggesting that it does not result from modulation of Cav1.1 channels. To further support this data, immunoblots reveal that there is no change in Cav1.1 channel abundance. However, the data do demonstrate that the increase in $[Ca^{2+}]i$ is sensitive to the SERCA blocking agent thapsigargin, suggesting that the source of the calcium is the sarcoplasmic reticulum stores. Additionally, the data reveal that ERG1a expression also increases basal calpain activity. In summary, to date the data show that ERG1a increases $[Ca^{2+}]i$ levels and suggest that this increase could occur, at least in part, as a result of release of calcium from sarcoplasmic reticulum stores. Additionally, the data reveal that ERG1a expression also increases calpain activity, suggesting that the increase in intracellular calcium results in increased calpain activity and possibly contributes to the increased proteolysis that occurs in atrophic skeletal muscle.

Key Words: skeletal muscle atrophy ERG1 Potassium Channel; intracellular calcium

3.

Anderson LB, CD Latour, O Khader, AL Pond. 2019. *ERG1a abundance varies by skeletal muscle in mice.* Poster presentation given at SIU Undergraduate Research Forum, April 8, SIU. Carbondale.

ERG1a Abundance Varies by Skeletal Muscle in Mice

Luke B. Anderson[1], Chase D. Latour[2], Omar Khader[1], and Amber L. Pond[1]

[1]Anatomy Department, SIU School of Medicine, Carbondale

[2]Mathematics Dept., Washington University, St. Louis, MO

Skeletal muscle atrophy is defined as a 5% or greater loss of skeletal muscle mass that results from an imbalance of protein degradation and synthesis resulting in net protein loss. It occurs naturally with aging as well as with diseases such as cancer and diabetes. It has been shown that the ERG1a potassium channel is upregulated in atrophying muscle and that atrophy occurs when ERG1a is expressed in healthy muscle; however, the mechanism by which ERG1a induces atrophy is not understood. More research is necessary to understand its role. For this, it is necessary to understand better the model we choose to study. Thus, we have explored ERG1a expression in mouse muscle fibers to learn if ERG1a expression is fiber type specific. First, we worked with the *Soleus* (*SOL*) and the *Extensor digitorum longus* (*EDL*) muscles each of which is nearly slow or fast type fiber homogeneous, respectively, based upon myosin heavy chain (MHC) composition. We cryo-sectioned these muscles (20 μm) and co-immunostained them for ERG1a and either fast or slow MHC. We found ERG1a to be more abundant in the *SOL*, suggesting that it is more abundant in slow-type fibers. However, because each of these muscles is indeed nearly homogeneous, we were not able to compare the ratio of ERG1a fluorescence in fast versus slow fibers within a single muscle. Thus, we co-immunostained sections from the mixed fiber *Gastrocnemius* muscles of mice and co-immunostained them for ERG1a and either fast or slow fiber type markers. Finally, we measured ERG1a fluorescence in each fiber type. The data suggest that there could be a difference in ERG1a expression between fiber types; however, additional data from on-going studies are needed to confirm this. Further research into this process could potentially lead to new therapies and treatments for those suffering from atrophy.

4.

Khader O, SM Latour, V Graham, AL Pond. 2019. *The Effect of Erg1a Expression on Caspase Enzyme Activity in C_2C_{12} Myotubes.* Poster presentation given at SIU Undergraduate Research Forum, April 8, SIU.

The Effect of *Erg1a* Expression on Caspase Enzyme Activity in C_2C_{12} Myotubes

Omar Khader[1], Sawyer M. Latour[2], Valerie[2], Amber L. Pond[1]
[1]Anatomy Department, SIU School of Medicine, Carbondale, IL
[2]Doisy College of Health Sciences, Saint Louis University, St. Louis, MO

Skeletal muscle atrophy occurs with injury, disease, starvation, and with natural aging, contributing to human morbidity and mortality. Muscle atrophy occurs when muscle protein degradation and synthesis are not appropriately balanced to maintain muscle mass. It has been suggested that caspase enzymes contribute to muscle loss by degrading muscle anchor proteins, allowing the contractile proteins to be degraded by the ubiquitin proteasome pathway (UPP). Because the ERG1a potassium channel increases UPP proteolysis in skeletal muscle, we hypothesized that ERG1a increases the activity of the caspase-3 enzyme known to cause the release of contractile proteins. We tested this hypothesis by expressing mouse *Erg1a* plasmid in left *Gastrocnemius* muscles of 40 mice and control plasmid in the right. We harvested *Gastrocnemius* muscle from 5 mice per day at days 0 through 7 after electro-transfer and assayed these muscles for combined caspase-3 and 7 activity. We determined the ratio of activity in the *Erg1a* treated left leg to that in the control treated right leg and compared this ratio at day 0 to those measured at each day 1-7. We also performed immunohistochemistry (IHC) to test for the presence of specific caspase-3 cleavage product. Data reveal that combined caspase-3,7 activity increased significantly ($p<0.05$) in ERG1a treated muscles: by 1.9-fold at day 3, 2.6-fold at day 4, and 2.9-fold at day 5; however, IHC demonstrated there was no increase in caspase-3 cleavage product. Thus, we conclude that the noted ERG1a-induced caspase activity must result from increased caspase-7 activity. It is hoped that our work will contribute to eventual development of improved atrophy therapies.

Abstracts 2020

1. Pond AL, Whitmore C, Davie JK, Choudhari S, Thimmapuram J, Hockerman GH. Viral transduction of C2C12 myotubes with the HERG potassium channel induces expression of genes related to Interferon gamma signaling pathways. Accepted for oral presentation at 2020 Spring Padua Muscle Days, March 18-21; University of Padua, Italy. Cancelled as a result of COVID-19 pandemic. *****2020PMD: Translational Mobility Medicine – Collection of Abstracts EJTM 2020; 30 (1):20-21.

The HERG potassium channel is detected as a heteromultimer of 2 alternative splice variants (1A and 1B) in heart and has been shown to be partially responsible for repolarization of the cardiac action potential.1 Both alternative splice variants have been reported in certain cancer cells, but their role in these cells is not clear.2 The HERG1A variant has been detected at low abundance in normal skeletal muscle, but is up-regulated in atrophying skeletal muscle, where it has been shown to increase protein degradation by modulation of both intracellular calcium levels and ubiquitin proteasome proteolysis (UPP).3,4,5 The pathways by which this modulation occurs is not clear. Therefore, we virally transduced C2C12 myotubes with either an adenovirus encoding HERG or an appropriate control virus (n=6). After 48 hours, we extracted total RNA from these cells and reverse transcribed them into cDNA, selecting for coding sequences (i.e., mRNA) by using poly(T) oligomers; the cDNA libraries were sequenced on Illumina's NovaSeq platform. Sequence quality was assessed using FastQC (v 0.11.7; https://www.bioinformatics.babraham.ac.uk/projects/fastqc) for all samples and quality trimming was done using FASTX-Toolkit (v 0.0.14; http://hannonlab.cshl.edu. fastx_toolkit/) to remove bases with Phred33 score of less than 30. Resulting reads of at least 50 bases were mapped against the reference genome using STAR.6 STAR derived mapping results and annotation file for reference genome were used as input for HTSeq7 (v 0.7.0) to obtain read counts. Counts from all replicates were merged together to produce a read count matrix for all samples and this count matrix was used for downstream differential gene expression analysis (DGEA). DGEA between treatment and control was carried out using 'R' (v 3.5.1; http://www.r-project.org/). The results show that HERG does result in numerous changes in gene expression. Limiting results to those with a $p<0.1$ that we find most interesting, we find that HERG potentially modulates expression of numerous genes connected with the UPP and with the cytokine interferon, which has been connected with muscle atrophy.8 Indeed, these results suggest HERG plays a role in protein degradation in skeletal muscle.

Keywords: HERG Expression, C2C12 Myotubes, gene upregulation, Interferon gamma, UPP

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Introduction. A heteromultimer of the ERG1a/1b potassium channel is known to contribute to repolarization of the cardiac action potential. A homomultimer of the ERG1a subunit has been detected in the atrophying skeletal muscle of mice experiencing muscle disuse and cancer cachexia and has been shown to contribute to muscle atrophy by enhancing ubiquitin proteolysis; however, to our knowledge, ERG1 has not been reported in human skeletal muscle. **Methods and Results.** Here, using immunohistochemistry we detect ERG1 immunofluorescence at low levels in *Rectus abdominis* muscle of young adult humans and show that it trends toward greater levels (10.6%) in the same muscle of healthy aged adults. Further, we detect ERG1 immunofluorescence at a statistically greater level (53.6%; $p<0.05$) in the *Rectus abdominis* muscle of older people having cancer cachexia than in age-matched adults. Additionally, we observe ERG1 immunofluorescence in skeletal muscle sarcolemma and detect that its fluorescent pattern is consistent with I-band localization. **Discussion.** The data suggest that ERG1 may be related to muscle loss in humans and may be located in t-tubules where it influences calcium handling.

3. Khader O, Anderson LB, Hockerman GH, **Pond AL**. ERG1A increases intracellular calcium concentration by enhancing flux from internal calcium stores. Accepted for presentation at the SIU Undergraduate Research Forum, Carbondale, IL, April 6, 2020. The forum was canceled because of the COVID-19 pandemic.

Skeletal muscle atrophy occurs with injury, disease and starvation, and with natural aging, contributing to human morbidity and mortality. Muscle atrophy can be caused by imbalances or defects in pathways modulating intracellular calcium level, which plays a crucial role in signaling and the excitation-contraction process in muscle fibers which cause movement. Because we have shown that the ERG1a potassium channel contributes to muscular atrophy, we hypothesized that it would also contribute to imbalances of intracellular calcium levels which are known to modulate protein degradation in atrophic muscle. In order to test this, we transduced cultured C2C12 myotubes with either ERG1-encoded adenovirus or an appropriate control adenovirus and determined that indeed intracellular calcium concentration is higher in ERG1-expressing myotubes than in control cells. To test for the source of the calcium concentration increase, we treated the control and ERG1-expressing myotubes with nifedipine, an L-type calcium channel blocking agent, and found no difference in intracellular calcium concentration, demonstrating that L-type channels do not contribute to this increase in calcium. Further, we treated the transduced myotubes with thapsigargin, a reagent which blocks SERCA and thus calcium reuptake into intracellular stores, and found that the intracellular calcium levels were not increased in the ERG1 expressing myotubes (relative to control cells), strongly suggesting that the source of the calcium increase is indeed intracellular stores. Further research is necessary to determine if the increase in intracellular calcium concentration is a result of modulation of IP3 or ryanodine receptors or potentially other ion channels. It is the hope of the researchers that investigation of this pathway will produce basic information which may lead to discovery of a more efficient therapy for skeletal muscle atrophy.

Abstracts 2021

1.Padua Muscle Days, March 2021.

Update on the ERG1A potassium channel: Potential for many cellular roles in aging and cancer

Amber Pond (1)*, Judy K. Davie (2), Punit Kohli (3), Sandra Zampieri (4,5)

(1) Dept. of Anatomy, Southern Illinois University School of Medicine, Carbondale, IL, USA; (2) Dept. of Biochemistry, Southern Illinois University School of Medicine, Carbondale, IL; (3) Dept. of Chemistry and Biochemistry Southern Illinois University School of Medicine, Carbondale, IL; (4) Department of Surgery, Oncology and Gastroenterology, and (5) Department of Biomedical Sciences University of Padova, Italy.
*Amber Pond: apond@siumed.edu

Abstract. The ether-a-go-go related gene 1 (ERG1) encodes alternative splice variants of a potassium channel alpha subunit which has been detected in numerous tissues and associated with multiple cellular functions.[1,2] Most copiously researched is the heteromultimeric ERG channel which is composed of the 1A and 1B subunits and known to be partially responsible for repolarization of the cardiac action potential.[1] Additionally, both the ERG1A and 1B subunits have been detected singly and together in cancer cells and associated with modulation of cell growth.[2] Although there were no prior reports of detection of ERG1 in normal skeletal muscle, we detected the ERG1A subunit in the atrophying skeletal muscle of hind limb unweighted and cachectic mice over ten years ago.[3] Since then, although it is not a hugely prominent protein in skeletal muscle, we have detected upregulation of the ERG1A alpha subunit in rodent skeletal muscle atrophying as a result of denervation (data not published), and to a lesser extent, aging.[4] Indeed, we have data revealing that ERG1A is mildly more abundant in the skeletal muscle of aged humans and significantly more so in the skeletal muscle of human cancer patients with low BMIs (data not published). We have shown that ectopic expression of ERG1A in normal rodent skeletal muscle results in decreased myofiber cross sectional area (CSA) and increased protein degradation, specifically calpain and ubiquitin proteasome activities (along with increased levels of the E3 ligase, MuRF1).[3,4] ERG1A protein is also detected at low abundance in C2C12 myoblasts and myotubes and over-expression of ERG1A in the myotubes results in decreased myotube area and increases in both MuRF1 protein and intracellular calcium concentration.[5] Interestingly, more recently we have found ERG1A is abundantly expressed in rhabdomyosarcoma cells, a skeletal muscle cancer cell line (data not published). The mechanisms by which ERG1A modulates pathways in atrophic muscle and malignant tissues are not clear and are under investigation. Research that is relevant to this discussion involves the β1 integrin chain, which is found in numerous integrin proteins, a group of transmembrane protein receptors involved in cell adhesion and reported to regulate tumorigenesis.[6] The β1 integrin has been shown to interact with ERG1 in numerous cancer types.[6] However, this interaction is not detected in cardiac cells because the cardiac K^+ channel subunit KCNE/Mink competes with β1 integrin for HERG, blocking the interaction in this tissue; this block of HERG-β1 integrin interaction has not been reported in cancer cells.[6] It is likely that a similar situation occurs in skeletal muscle, so that ERG1 expression does not normally result in tumorigenesis in this tissue; however, the ERG1A channel protein has many domains for interaction and modulation and thus the potential for many modulatory interactions.

Key Words: ether-a-gogo-related gene potassium channel; skeletal muscle atrophy; cancer

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The ERG1A potassium channel induces expression of genes related to skeletal muscle atrophy in C2C12 myotubes

[1]Amber L. Pond, [1]Clayton Whitmore, [2]Jyothi Thimmapuram, [3]Gregory H. Hockerman.
[1]Anatomy Dept., SIU School of Medicine, Carbondale, Illinois, USA
[2]Bioinformatics Core, Purdue University, West Lafayette, IN
[3]Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN

The ERG1A potassium channel alternative splice variant is detected at low abundance in normal skeletal muscle; however, it is up-regulated in atrophying skeletal muscle, where it has been shown to modulate both intracellular calcium levels and ubiquitin proteasome proteolysis (UPP). The pathways by which this modulation occurs are not known. Therefore, we transduced C2C12 myotubes with either an adenovirus encoding HERG or an appropriate control virus (n=6). At 48 hours after viral treatments, we extracted total RNA from these cells and reverse transcribed them into cDNA, selecting for coding sequences (i.e., mRNA) by using poly(T) oligomers. The cDNA libraries were sequenced on Illumina's NovaSeq platform and sequence quality was assessed using FastQC (v 0.11.7) for all samples. Quality trimming was performed with the FASTX-Toolkit (v 0.0.14) to remove bases with a Phred33 score of less than 30. The resulting reads of at least 50 bases were mapped against the reference genome using STAR. The mapping results and the annotation file for the reference genome were used as input for HTSeq[7] (v 0.7.0) to obtain read counts. Counts from all replicates were merged together to produce a read count matrix for all samples and this count matrix was used for downstream differential gene expression analysis (DGEA). DGEA between treatment and control was carried out using 'R' (v 3.5.1). The data demonstrate that HERG expression does produce numerous changes in the gene expression profile of C2C12 myotubes. Indeed, we find that HERG potentially modulates expression of numerous genes (see Table) connected with skeletal muscle atrophy, specifically ubiquitin proteasome proteolysis and with the cytokine interferon, which has been connected with muscle atrophy. The data suggest that HERG does play a role in modulation of protein degradation in skeletal muscle.

Gene Name	Fold Change	Adjusted p value
Ubiquitin specific peptidase 18	9.1	6.99E-8
Ubiquitin-like modifier activating enzyme 7	2.6	0.005
SMAD specific E3 ubiquitin protein ligase 2	1.5	0.05
Interferon induced protein 44	5.4	2.44E-11
Interferon inducible GTPase1	5.3	0.018
Interferon-induced protein with tetratricopeptide repeats 3	2.2	5.62E-5
Interferon-induced protein with tetratricopeptide repeats 1	2.0	1.11E-05
Interferon, alpha-inducible protein 27	1.4	0.08
Interferon activated gene 28	21.4	0.2
Myosin, heavy polypeptide 4, skeletal muscle	2	0.08
Folliculin interacting protein 2	0.7	0.06

3. SIU Undergraduate Research Forum 2021

Investigation of the Mechanism by which the ERG1 Potassium Channel Increases Intracellular Calcium Concentration

Omar Khader, GH Hockerman, Amber L. Pond

Anatomy Department, Southern Illinois University School of Medicine, Carbondale, IL 62901

Skeletal muscle atrophy occurs with injury, disease and starvation, and with natural aging, contributing to human morbidity and mortality. Muscle atrophy can be caused by imbalances or defects in pathways modulating intracellular calcium level, which plays a crucial role in signaling and the excitation-contraction process in muscle fibers which cause movement. Because we have shown that the ERG1a potassium channel contributes to muscular atrophy, we hypothesized that it would also contribute to imbalances of intracellular calcium levels which are known to modulate protein degradation in atrophic muscle. In order to test this, we transduced cultured C2C12 myotubes with either ERG1-encoded adenovirus or an appropriate control adenovirus and determined that indeed intracellular calcium concentration is higher in ERG1-expressing myotubes than in control cells. We need to determine the source of this calcium increase. To test for the source of the calcium concentration increase, we treated the control and ERG1-expressing myotubes with nifedipine, an L-type calcium channel blocking agent, and found no difference in intracellular calcium concentration, demonstrating that L-type channels do not contribute to this increase in calcium. Further, we treated the transduced myotubes with thapsigargin, a reagent which blocks SERCA and thus calcium reuptake into intracellular stores, and found that the intracellular calcium levels were not increased in the ERG1 expressing myotubes (relative to control cells), strongly suggesting that the source of the calcium increase is indeed intracellular stores. At this point, we hypothesized that the intracellular source is likely release of calcium from endoplasmic reticulum stores through IP3 receptors. However, IP1 assays reveal that IP3 does not increase in response to ERG1, suggesting that this signaling pathway is not involved. Further research is necessary to determine if the increase in intracellular calcium concentration is a result of modulation of IP3 or ryanodine receptors or potentially other ion channels. It is the hope of the researchers that investigation of this pathway will produce basic information which may lead to discovery of a more efficient therapy for skeletal muscle atrophy.