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**TITLE: Calcium Signaling in Skeletal Muscle Atrophy: A Novel Role for the ERG1alpha K<sup>+</sup> Channel**

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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. We proposed to investigate the mechanism by which ERG1A increases intracellular calcium and the downstream effect of this on calpain enzyme-mediated proteolysis. To date, we have completed Major Task 2, determining that ERG1A does increase calpain activity mainly as a result of the increased calcium concentration and also a decrease in calpastatin protein abundance. We have completed approximately 40% of Major Task 1, determining that the increase in calcium is not a consequence of ERG1A modulation of L-type calcium channel gene expression or protein abundance, but that the source of the calcium is intracellular stores. In terms of the intracellular stores, we have shown that the source is not activation of the IP3 pathway. Thus, we will consequently investigate possible ERG1A modulation of ryanodine receptors and SERCA as the source of the calcium. Finally, we have completed about 50% of Major Task 3, having prepped samples for Next Generation Sequencing which has been completed. We now have an extremely large set of data to analyze to determine which genes are modulated in response to ERG1A.					
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# TABLE OF CONTENTS

	<b>Page</b>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	12
5. Changes/Problems.....	14
6. Products.....	15
7. Participants & Other Collaborating Organizations.....	18
8. Special Reporting Requirements.....	21
9. Appendices.....	22
A. Submitted Manuscript.....	22
B. Abstracts	
1.....	58
2.....	61
3.....	62
4.....	63

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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 more 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 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.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

skeletal muscle; atrophy; ether-a-gogo related gene (ERG1A); potassium channel; intracellular calcium concentration; calcium signaling; calpastatin; calpain enzymes; calcium channel; Cav1.1; C<sub>2</sub>C<sub>12</sub> cells; L-type calcium channel

3. **ACCOMPLISHMENTS:** *The 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.*

**What were the major goals of the project?**

*List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.*

**Major Task 1: Explore the mechanism responsible for the calcium signal increase that occurs in cultured C2C12 myotubes in response to HERG1A transfection. ~50% Completed.**

Subtask 1.A (Hockerman Lab): Determine the source of the HERG1A-mediated increase in intracellular calcium levels using Fura-2 assays and specific pharmacological treatments. 50% Completed.

Subtask 1.B.1 (Hockerman): Determine if there are HERG1A-mediated changes in Ca<sup>2+</sup> current density using electrophysiology and specific pharmacological treatments. 0% Completed.

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.

Subtask 1.C (Hockerman): Determine the effect of HERG1A on IP<sub>3</sub> levels in myotubes using an IP-ONE time-resolved fluorescence assay kit (Cisbio). *50% Completed.*

**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.**

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.*

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.*

**Major Task 3 (Pond): Determine the effect of HERG1A on gene expression in cultured C2C12 myotubes using qPCR arrays. 50% Completed.**

\*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 will need more time than originally planned to analyze and interpret this data.

### **What was accomplished under these goals?**

*For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

**Progress Report:** After a year of work, we are pleased to report that nice progress is being made on the project (described below):

Major Task 1 - ~50% complete  
Major Task 2 - 100% complete  
Major Task 3 - ~50% complete

### **Major Task 1. Nearly 60% Complete.**

Subtask 1.A (Hockerman Lab): ~50% complete. Using fura-2 calcium assays and the HERG blocker astemizole, we have determined that HERG expression causes an increase in basal intracellular calcium levels as well as an increase in calcium levels induced by depolarization with 100 mM KCl (figure 1).

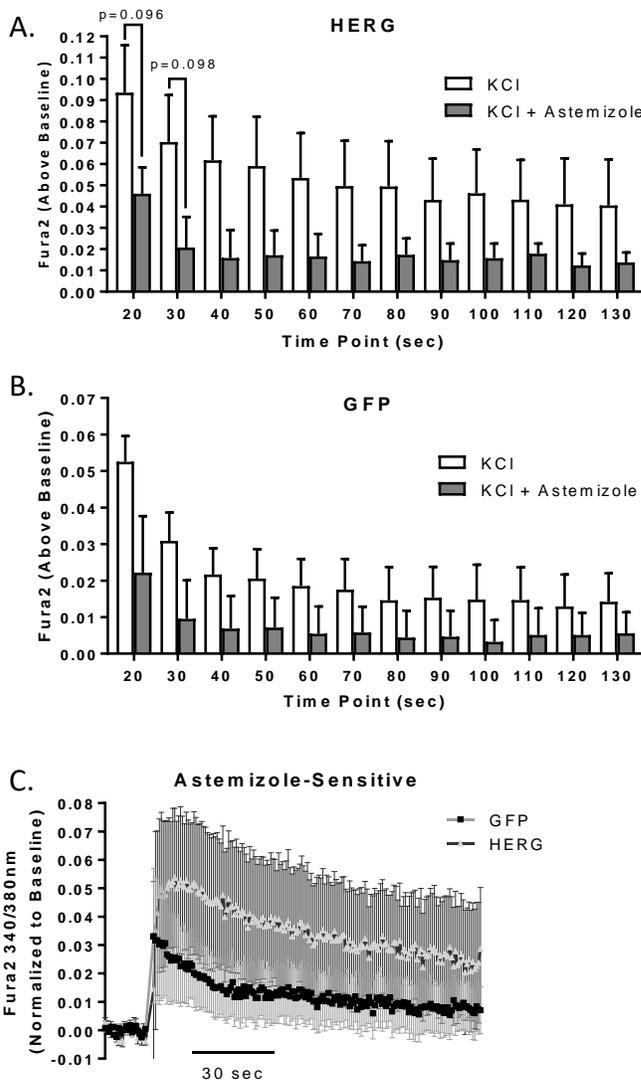


Figure 1. HERG expression promotes an increase in depolarization-induced intracellular calcium levels, and this increase is inhibited by the HERG blocker astemizole. A,B. Astemizole inhibits the calcium transient produced by depolarization in myotubes treated with either a HERG-encoded virus (A) or an appropriate control (GFP encoded only) virus (B), suggesting that the myotubes have native ERG1A channels. Indeed, we have confirmed this with immunoblot not shown). C. However, the astemizole inhibition of calcium transient is greater in the myotubes expressing HERG than in the control cells, showing that a portion of the calcium increase is unique to HERG.

Further, we have explored the source of the intracellular calcium and have shown that the increase in calcium is not a consequence of HERG modulation of L-type calcium channel function (figure 2). Additionally, the HERG-modulated increase in calcium is not a consequence of increased expression of Cav 1.X L-type calcium channel genes or protein abundances (figure 3; see Subtask 1.B.2 also).

However, the data show 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 (figure 4). We must now explore the potential role of RYR and SERCA in this increase in calcium to complete this subtask.

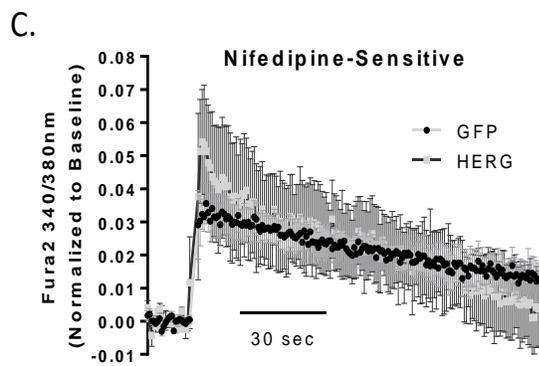
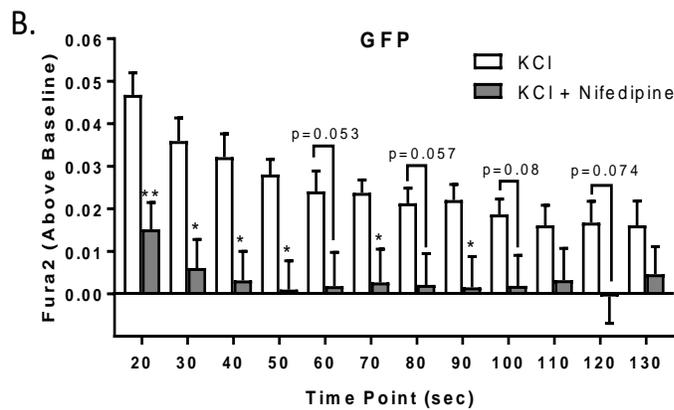
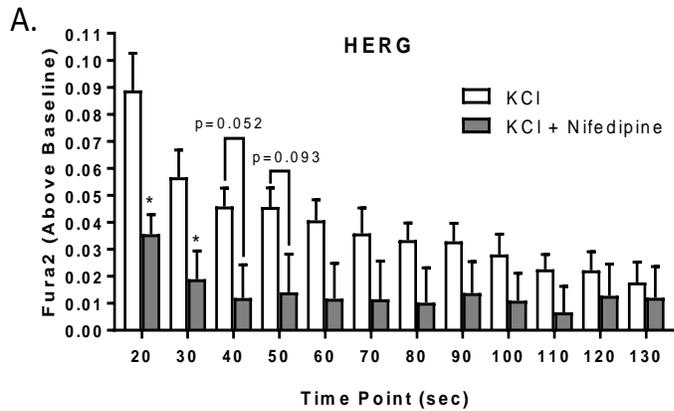


Figure 2. The transient increase in intracellular calcium levels that occurs with depolarization of HERG-treated myotubes is not sensitive to nifedipine. The increase in the transient  $[Ca^{++}]_i$  produced by depolarization in both HERG-expressing and control myotubes is inhibited by the specific L-type calcium channel blocker nifedipine (Fig. 2A,B), demonstrating that some component of the depolarization-induced increase in  $[Ca^{++}]_i$  is a result of the activity of an L-type calcium channel. The nifedipine has a strong blocking effect on  $[Ca^{++}]_i$  in the GFP control cells, knocking out 67.5% of the increase after 20s and then basically obliterating it (over 90-103% blocked) from 40s to 120s (Fig. 2B). This demonstrates that a large proportion of the increase in  $[Ca^{++}]_i$  that occurs in C2C12 myotubes in response to depolarization is a result of L-type channel activation. Nifedipine also has a strong effect on the

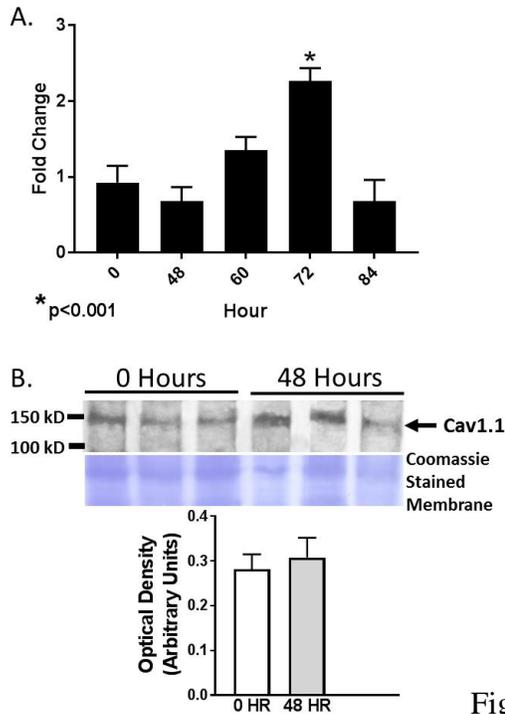


Figure 3. Although HERG expression produces a 2.1 fold change in gene expression levels of the Cav1.1 L-type calcium channel (A), there is no significant change in Cav1.1 protein abundance (B). There is no change in gene expression levels or protein abundances for the embryonic form of Cav1.1, nor for Cav1.2 or 1.3 (data not shown).

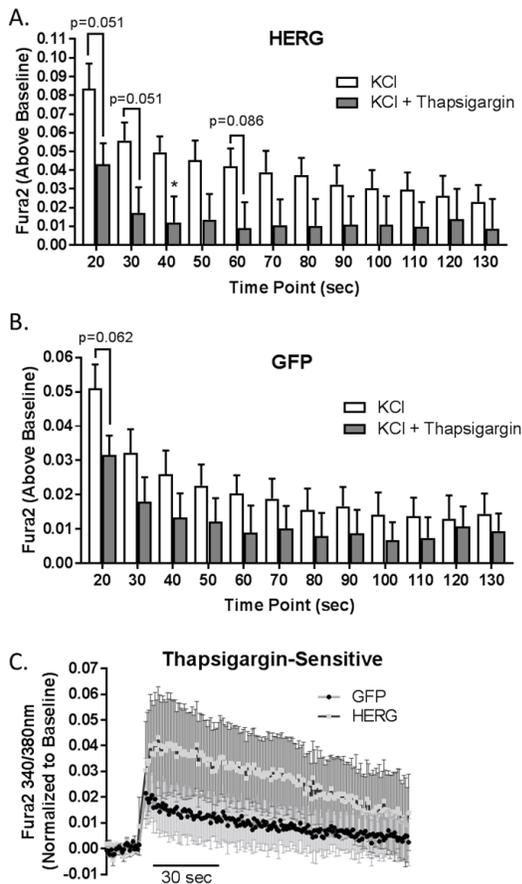


Figure 4. The transient increase in intracellular calcium levels that occurs with depolarization of HERG-treated myotubes is sensitive to thapsigargin, suggesting the source of the calcium is intracellular stores. The increase in the transient  $[Ca^{++}]_i$  produced by depolarization in both HERG-expressing and control myotubes is inhibited by the SERCA inhibitor thapsigargin (Fig. 4A,B), demonstrating that some component of the depolarization-induced increase in  $[Ca^{++}]_i$  is from internal stores. The thapsigargin has a mild blocking effect on  $[Ca^{++}]_i$  in the GFP control cells, knocking out 25.2% of the increase after 20s and then returning to near baseline levels (Fig. 4B). This demonstrates that there is an intracellular stores component of the response to depolarization. Thapsigargin, however, has a stronger effect on the  $[Ca^{++}]_i$  in the HERG transfected myotubes, knocking down about 95.2% of the current at 20s and then maintaining a significant decrease for up to 40s. Indeed, when the specific thapsigargin-sensitive currents are plotted (Fig. 4C), there is a difference in thapsigargin-sensitive currents of the HERG-treated and control myotubes beyond the initial 10-20 seconds post depolarization. Thus, HERG is likely significantly affecting some mechanism (e.g., IP<sub>3</sub>, RYR, SERCA, etc.) connected with modulation of internal stores.

Subtask 1.B.1 (Hockerman Lab): 0% Complete. The exploration of the effects of HERG on calcium current density have not been started yet. **The graduate student who has been working on this project has experienced some health issues and will be having surgery toward the end of July 2019.**

Subtask 1.B.2 (Pond): 100% Complete. The HERG-modulated increase in calcium is not a consequence of increased expression of Cav 1.X L-type calcium channel genes or protein abundances (figure 3 above).

Subtask 1.C (Hockerman): ~50% complete. In terms of the intracellular stores, we have data suggesting that the source is not activation of the IP3 pathway (to be repeated and validated). We will further explore this mechanism to definitively rule it out once the graduate student has recovered from her surgery.

**Major Task 2 (Pond). 100% Complete.** During this first year of the project, we validated our *in vitro* model of skeletal muscle atrophy and discovered that expression of human *ERGIA* in C<sub>2</sub>C<sub>12</sub> 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 **100% complete Major Task 2** of the proposal and have resulted in presentations and a manuscript which has been submitted to the journal *Skeletal Muscle* (see Appendix).

**Major Task 3 (Pond). ~50% Complete.** 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 have prepared the samples and the Purdue Genomics facility has completed the actual data collection (see Appendix). Now, with the guidance of the statisticians of the Purdue Bioinformatics Core, we must begin the task of analyzing and interpreting this massive amount of data. It will take longer to interpret and follow up on this larger set of data than we budgeted for the PCR arrays.

**Stated Goals Not Met:** We have just concluded the first year of the project and have 6 months more allotted to complete the work. We have completed Major Task 2 as proposed; however, the Hockerman lab has had a student injury/illness interfere with timely execution of Major Task 1. Further, the DOD reviewers recommended that we perform Next Generation Sequencing rather than PCR arrays to evaluate HERG-mediated effects on gene expression and this has resulted in a much larger data set to evaluate and follow up. Thus, we intend to apply for a 1 year no cost extension (as explained below).

**Major Task 1** has three subtasks: 1.A, 1.B.1, and 1.B.2. Subtask 1.B.2 is 100% complete and was completed within the timeframe proposed. Subtask 1.A is 50% complete although it was slated to be completed within the first 5 months of the award period. Additionally, subtask 1.B.1 has not been started although it was slated to be completed by the end of the first year of the grant period. Completion of Subtasks 1.A and 1.B.1 has been delayed because the graduate student responsible for (and familiar with) these projects has been experiencing some health issues which have interfered with her mobility. Indeed, she will be having surgery on her back in late July 2019 and will need further time off to recover at that point. In the interim, Dr. Pond's lab will begin work related to Subtask 1.A. The Pond lab has access to the proper equipment, but must develop the expertise to do so; therefore, completion of this Subtask will be further delayed while the Pond lab develops and validates the fura calcium assays. Subtask 1.B.1, however, will require equipment and expertise available in Dr. Hockerman's lab. Thus, we will continue this work once the graduate student is back to work in late August or early September. Thus, it will be necessary to work beyond the originally proposed time frame to complete this Subtask. Once the graduate student is back to work, she will also complete and write up her results for Subtask 1.C (which is about 50% complete at this point). Major Task 3 was amended as proposed based upon the excellent advice of the DOD reviewers. We had proposed performing PCR arrays to look at changes in gene expression in response to HERG channel expression in C2C12 myotubes; however, the DOD reviewers suggested that we could instead perform Next Generation Sequencing to do this. Indeed, Purdue University has a Genomics Core which performs this work. Thus, we prepared the samples and submitted them to the Genomics Core which has completed the physical analyses and generated a large amount of gene expression data. Indeed, they recently trained both Drs. Pond and Hockerman to use the DAVID gene pathways software to explore how best to interpret the gene expression patterns. This large amount of data will require time beyond that originally allotted for this to complete this Major Task and determine how best to proceed with the next steps of the research. Therefore, we intend to file for a one year no cost extension to complete Major Tasks 1 and 3.

**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."*

*Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

**PI.** Dr. Pond attended an international conference in Padua Italy (Padua Muscle Days, March 28-30, 2019, Padua, Italy) and a national meeting (Experimental Biology, April 6-9, Orlando, FL, USA) where she gave presentations to groups of researchers. The work was discussed with other experts in the field and Dr. Pond attended other presentations to increase her current knowledge of muscle biology. (See Appendix.)

**Co-PI.** Dr. Hockerman Nothing to report.

**Graduate Student.** Clayton Whitmore (Pond laboratory) worked on the project and defended his Masters thesis on October 25, 2018: *Investigation of ERGIA potassium channel modulation of calpain activity in C2C12 myotubes.*

**Graduate Student.** Emily Rantz (Hockerman laboratory) worked on the project performing IP1 assays and will work on the electrophysiology to measure calcium current density. The project has allowed her to apply her skills in single-cell Ca<sup>2+</sup> imaging to skeletal muscle biology.

**Undergraduate Student.** Luke Anderson, who has worked on culturing the cells and validated the *in vitro* model, further enhanced his laboratory and science skills by learning to perform enzyme assays, specifically the calpain assays. Luke presented a poster at the SIU Undergraduate Research Forum and has earned authorship on the paper supported by this grant and submitted to *Skeletal Muscle*. (See Appendix.)

**Undergraduate Student.** Omar Khader was a freshman this year and learned basic laboratory and safety skills. He completed a project related to the DOD grant and presented a poster at the SIU Undergraduate Research Forum. (See Appendix.)

**Anna-Jonesboro High School Senior.** Bryce Massey worked in the laboratory and learned basic lab

### **How were the results disseminated to communities of interest?**

*If there is nothing significant to report during this reporting period, state "Nothing to Report."*

*Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

The results of the project were presented at two different conferences by Dr. Pond, one of which was national and the other of which was international. Dr. Pond also judged a regional high school science fair where she discussed simple aspects of the project with students. One graduate student worked on the project and made a public presentation of his work as part of the defense of his Masters thesis. Two undergraduate students presented posters concerning aspects of this project at the Southern Illinois University Research Forum. A talented high school student was invited in to the lab to work and his work was discussed at the high school. A manuscript has been written by former graduate student Whitmore and Dr. Pond and submitted for publication to the peer reviewed journal *Skeletal Muscle*.

**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.”*

**Major Task 1:** Subtask 1.A. The Pond laboratory will develop and validate the fura calcium assays in their lab to confirm results discovered in the Hockerman laboratory and to make up for time lost as a result of the injury sustained by the Hockerman lab graduate student. Thus, the Pond lab will confirm that HERG expression increases intracellular calcium concentration and then investigate the source of the calcium increase by observing the effect on the HERG-mediated increase in calcium levels by certain pharmaceuticals: 1) U73122 [1  $\mu$ M; 30 min] to block phospholipase C - this will block the increase in calcium if the IP<sub>3</sub> signaling pathway is involved in the calcium increase; and 2) ryanodine [20  $\mu$ M; 30 min] to block RYR1 – this will prevent calcium release from stores through RYR receptor activation and thus prevent the HERG-mediated calcium increase if RYR receptors are involved.

Subtask 1.B.1. The Hockerman laboratory will complete this task and determine if there are HERG1A-mediated changes in L-type calcium current density using electrophysiology and specific pharmacological treatments.

Subtask 1.C. The graduate student from the Hockerman laboratory will resume and complete her studies of the subtask upon her return from her recovery. She will determine the effect of HERG1A on IP<sub>3</sub> levels in myotubes using an IP-ONE time-resolved fluorescence assay kit (Cisbio). Thus far, results with the IP-ONE assay are not consistent with Ca<sup>2+</sup> measurements in live myotubes. This has cast doubt on the usefulness of this assay in myotubes. It’s not clear why the IP-ONE assay is not detecting PLC activity that is clearly indicated by real time Ca<sup>2+</sup> measurements. One possibility is that IP<sub>1</sub>, the stable metabolite of IP<sub>3</sub>, does not accumulate as rapidly in myotubes as in other cell types. Thus, we will pursue the use of fluorescence-based indicators of PIP<sub>2</sub> levels in live myotubes to determine if HERG expression increases PLC activity, and a corresponding decrease in plasma membrane PIP<sub>2</sub>.

**Major Task 3:** The Pond and Hockerman laboratories have received Next Generation Sequencing data denoting the effect of HERG1A expression on overall gene expression in C2C12 myotubes. The PIs have received training in using software designed to explore this large data set. We will now train lab personnel in use of this software and use the data to look for patterns in gene expression affected by

4. **IMPACT:** *Describe 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.”*

*Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

It has not been reported that HERG has any effect on calcium level in skeletal muscle cells. Indeed, our labs were the first to report the detection of HERG in skeletal muscle and to tie it to atrophy. This work opens a new area of research for researchers interested in calcium signaling in skeletal muscle.

**What was the impact on other disciplines?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*

This work opens a new area of research for researchers interested in calcium signaling not only in skeletal muscle, but also in cardiac muscle and in cancer cells. Calcium misregulation is a serious concern in cardiac tissue. Indeed, HERG1A has been reported in malignant cells and appears to have some type of effects on cellular growth.

**What was the impact on technology transfer?**

*If there is nothing significant to report during this reporting period, state “Nothing to Report.”*

*Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

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.”*

*Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

The work on this project has included many young people at the high school and college levels. It is the hope that these people enjoyed their exposure to science and, if so, may 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**

*Describe any changes in approach during the reporting period and reasons for these changes.*

**There have been no changes made to the approach or plan** (as originally revised per reviewer request at the outset). However, we intend to ask for a one year no cost extension to complete Subtasks 1.A, 1.B.1, and 1.C (Hockerman) because of graduate student health issues and Major Task 3 (Pond) because the reviewer requested Next Generation Sequencing has produced more data than the PCR arrays originally proposed would have and we need more time to explore these data.

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

*Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

**Major Task 1.** Completion of Subtasks 1.A and 1.B.1 has been delayed because the graduate student responsible for (and familiar with) these projects has been experiencing some health issues which have interfered with her mobility. Indeed, she will be having surgery on her back in late July 2019 and will need further time off to recover at that point. In the interim, Dr. Pond's lab will begin work related to Subtask 1.A. The Pond lab has access to the proper equipment, but must develop the expertise to do so; therefore, completion of this Subtask will be further delayed while the Pond lab develops and validates the fura calcium assays. Subtask 1.B.1, however, will require equipment and expertise available in Dr. Hockerman's lab. Thus, we will continue this work once the graduate student is back to work in late August or early September. Thus, it will be necessary to work beyond the originally proposed time frame to complete this Subtask. Once the graduate student is back to work, she will also complete and write up her results for Subtask 1.C (which is about 50% complete at this point).

**Major Task 3** was amended as proposed based upon the excellent advice of the DOD reviewers. We had proposed performing PCR arrays to look at changes in gene expression in response to HERG channel expression in C2C12 myotubes; however, the DOD reviewers suggested that we could instead perform Next Generation Sequencing to do this. Indeed, Purdue University has a Genomics Core which performs this work. Thus, we prepared the samples and submitted them to the Genomics Core which has completed the physical analyses and generated a large amount of gene expression data. Indeed, they recently trained both Drs. Pond and Hockerman to use the DAVID gene pathways software to explore how best to interpret the gene expression patterns. This large amount of data will require time beyond that originally allotted for this to complete this Major Task and determine how best to proceed with the next steps of the research.

**Therefore, we intend to file for a one year no cost extension to allow for completion of Major Tasks 1 and 3.**

**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**

This project does not involve human subjects.

**Significant changes in use or care of vertebrate animals**

This project does not involve vertebrate animals.

**Significant changes in use of biohazards and/or select agents**

This project does not involve the 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).

None to report yet – one submitted (see Appendix).

- **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).

Nothing to Report.

- **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.

\***Pond AL**, Whitmore C, Pratt EE, Anderson LB, Rantz E, Salyer A, Carraro U, Wang W-H, Weillbaecher R, Davie JK, Hockerman GH. 2019. *The ERG1a K<sup>+</sup> 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, Weillbacher R, Davie JK, Hockerman GH. 2019. *The ERG1a K<sup>+</sup> channel increases intracellular calcium and calpain activity in C<sub>2</sub>C<sub>12</sub> 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.* Defended Masters Thesis on October 25, SIU-Carbondale.

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.

Khader O, Latour SM, Graham V, Pond AL. 2019. *The Effect of Erg1a Expression on Caspase Enzyme Activity in C<sub>2</sub>C<sub>12</sub> Myotubes.* Poster presentation given at SIU Undergraduate Research Forum, April 8, SIU.

- **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. Examples include:*

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*

- *other.*

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”.*

Example:

*Name: Mary Smith  
 Project Role: Graduate Student  
 Researcher Identifier (e.g. ORCID ID): 1234567  
 Nearest person month worked: 5*

*Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.  
 Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Amber L. Pond
Project Role:	PI
Researcher Identifier:	22836889X
Nearest person month worked:	5
Contribution to the Project:	Dr. Pond provided guidance and training to students working in her lab. She organized the work to be done and participated in the execution of the projects.
Funding Support:	SIU School of Medicine

Name: Dr. Gregory Hockerman  
Project Role: Co-PI  
Researcher Identifier: ORCID#: 0000-0001-6147-8604  
Nearest person month worked: 1  
Contribution to the Project: Dr. Hockerman provided guidance and training to students working in his lab. He organized the work to be done and participated in the execution of the projects.  
Funding Support: Purdue University

Name: Clayton Whitmore  
Project Role: Graduate Student  
Researcher Identifier:  
Nearest person month worked: 6  
Contribution to the Project: Mr. Whitmore worked in the lab, completing PCR and immunoblot work. Mr. Whitmore also wrote the original version of the manuscript submitted to *Skeletal Muscle*.  
Funding Support: Southern Illinois University – part of start-up package to ALP

Name: Emily Rantz  
Project Role: Graduate Student  
Researcher Identifier:  
Nearest person month worked: 3  
Contribution to the Project: Ms. Rantz works in the lab, performing fura-2 calcium assays, electrophysiology, and IPOne assays.  
Funding Support: R21 from NINDS/current project

Name: Luke Anderson  
Project Role: Undergraduate Student  
Researcher Identifier:  
Nearest person month worked: 5  
Contribution to the Project: Mr. Anderson maintains the lab and replenishes buffers, etc. He also helped perform the calpain assays and did the protein assays on the samples which allowed us to normalize the data.  
Funding Support: Mr. Anderson has been funded by the SIU Office of Research during the school year and by the DOD in the summer.

Name: Omar Khader  
Project Role: Undergraduate Student  
Researcher Identifier:  
Nearest person month worked: 2.5  
Contribution to the Project: Mr. Khader helps maintain the lab and cell culture facilities. He cultures cells and will be working with the fura calcium assays.  
Funding Support: DOD

Name:	Bryce Massey
Project Role:	High School Student
Researcher Identifier:	
Nearest person month worked:	1.0
Contribution to the Project:	Mr. Massey trained under Luke to learn how to maintain the lab and how to do immunohistochemistry.
Funding Support:	SIU

**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.”*

*Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*

*Provide the following information for each partnership:*

*Organization Name:*

*Location of Organization: (if foreign location list country)*

*Partner’s contribution to the project (identify one or more)*

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*

- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

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 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. **Manuscript submitted to *Skeletal Muscle*:**

**The ERG1a Potassium Channel Increases Basal Intracellular Calcium Concentration and Calpain Activity in Skeletal Muscle Cells**

Clayton Whitmore<sup>1,#</sup>, Evan Pratt<sup>2,#</sup>, Luke Anderson<sup>1,\*</sup>, Kevin Bradley<sup>1,\*</sup>, Sawyer M. Latour<sup>3,\*</sup>,  
Mariam N. Hashmi<sup>1,\*</sup>, A.K. Urazaev<sup>4</sup>, Rod Weilbacher<sup>5</sup>, Judith K. Davie<sup>5</sup>, Wen-Horng Wang<sup>6</sup>,  
**Gregory H. Hockerman<sup>7</sup>, Amber L. Pond<sup>1,8</sup>**

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#Graduate Students; \*Undergraduate Students

**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 (ERGIA)* K<sup>+</sup> channel has been shown to contribute to atrophy by up-regulating ubiquitin proteasome proteolysis in cachectic and unweighted mice and has also been implicated in calcium modulation in cancer cells.

**Methods:** We transduced C<sub>2</sub>C<sub>12</sub> myotubes with either a human *ERGIA* 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 two-way ANOVAs and SAS software.

**Results:** Expression of human *ERGIA* in C<sub>2</sub>C<sub>12</sub> 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. 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. 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.

**Conclusions:** Human *ERGIA* 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.

Key Words: ERG1A, skeletal muscle atrophy, calpains, calpastatin, intracellular calcium

## **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 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, 10, 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 ubiquitination 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  $\alpha$ -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 filament from the myofibril. 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<sub>2</sub>C<sub>12</sub> myotubes and consequently enhance calpain activity. Here, we perform

studies designed to explore this hypothesis and demonstrate that indeed, ERG1A enhances both intracellular calcium concentration and calpain activity.

## **METHODS AND MATERIALS**

### Cell Culture

C<sub>2</sub>C<sub>12</sub> 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<sub>2</sub> 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 into myotubes.

### Viral Transduction

Terminally differentiated C<sub>2</sub>C<sub>12</sub> myotubes were treated with 200 MOI virus to produce HERG1A protein after 48 hours. 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<sup>+</sup> channel (VQAd CMV Herg-GFP; ViraQuest). The cells were then incubated for 48 hours and monitored via fluorescence to verify that the transduction was successful.

### Resting Intracellular Ca<sup>2+</sup> Assay

C<sub>2</sub>C<sub>12</sub> myoblasts were cultured in DMEM supplemented with 10% FBS and 1% P/S and plated at a density of 5x10<sup>4</sup> 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 3-5 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  $\text{Ca}^{2+}$  measurements, the media was removed and myotubes were washed twice with 200  $\mu\text{L}$  PBS. 5  $\mu\text{M}$  Fura2-AM (Molecular Probes, Eugene, OR) was diluted in Krebs-Ringer HEPES buffer (KRBH), and each well of myotubes was incubated in 100  $\mu\text{L}$  of this solution for 1 h at RT. KRBH contained 134 mM NaCl, 3.5 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM  $\text{MgSO}_4$ , 1.5 mM  $\text{CaCl}_2$ , 5 mM  $\text{NaHCO}_3$  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 sec for a total of 15 sec 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 nm, and these data points were averaged to yield a resting 340/380 nm ratio, or resting  $\text{Ca}^{2+}$  level, for each well of myotubes. Seven independent calcium measurements were performed, with each experiment containing between 6-16 replicates, and the average 340/380 nm ratio  $\pm$  SE was calculated among all wells for GFP- and HERG-transduced myotubes.

#### Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted from  $\text{C}_2\text{C}_{12}$  myotubes using Trizol reagent (Life Technologies;

Carlsbad, CA) according to manufacturer's instructions followed by chloroform 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."

#### Antibodies

The following antibodies were used: Calpain-1 polyclonal antibody 3189-30T (BioVision, Milpitas, CA); Calpain-2 polyclonal antibody 3372-30T (BioVision, Milpitas, CA); Calpain-3 polyclonal antibody A11995 (ABclonal, Woburn, MA); Calpastatin polyclonal antibody A7634 (ABclonal, Woburn, MA); GAPDH polyclonal antibody ABS16 (Sigma, St. Louis, MO).

#### Western Blot

Membrane proteins were extracted from C<sub>2</sub>C<sub>12</sub> myotubes 48 hours after viral transduction 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, 1mM 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 minutes. The samples were then centrifuged for 2 minutes at 15,000 rpm. 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).

### Animals

All procedures were approved by the Southern Illinois University Carbondale (SIUC) Animal Care and Use Committee. A total of 75 ND4-Swiss Webster 7-8 week old male mice (Harlan-Sprague; Indianapolis, IN) were used. Animals were housed in SIUC vivarium facilities on a 12 hour light/dark cycle, monitored by lab animal veterinarians and provided food and water *ad libitum*.

### Plasmids

The mouse *Erg1a* (*Merg1a*) clone in pBK/CMV plasmid (21) 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  $\mu$ l sterile saline and then stimulated with 8 pulses at 200V/cm for 20 ms at 1 Hertz 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 ug) and a plasmid encoding *Renilla* reporter (5 ug) were injected into the left *Gastrocnemius* muscles of mice (n=40). An empty control plasmid (30 ug) and the *Renilla* reporter plasmid (5 ug) 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 5 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.

### 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 minutes and luminescence was measured using a Synergy H1 Hybrid Reader (BioTek; Winooski, VT). Luminescence was measured again 10 minutes later to ensure that the reaction had reached an end point after the first 10 minutes. 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 differentiated for four days and then transduced with either a HERG1A encoded adeno-virus or the same non-encoded virus as control (12 wells each). At 48 hours 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, 6 wells per viral treatment received buffer supplemented with the calpain inhibitor MDL28170 (50 µM). These were allowed to sit at room temperature for 5 minutes 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 minutes, 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 (13000xg; 3 minutes) 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 ul buffer/ug tissue. The sample homogenates were aliquoted and frozen at -80°C. Prior to assay, the homogenates were thawed and sample aliquots (40 ul) and positive control (purified porcine calpain) were added to wells of 96 well plates with assay buffer (40 ul) 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 Relative Light Units (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<sub>2</sub>C<sub>12</sub> Myotubes with a HERG1A-Encoded Adenovirus Results in**

**Elevated HERG1A Protein.** Immunoblot of equal protein aliquots from both non-virus treated C<sub>2</sub>C<sub>12</sub> myoblast and myotube lysates detects a 40.7% (p<0.01; n=6) 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<sub>2</sub>C<sub>12</sub> 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 (Figure 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<sub>2</sub>C<sub>12</sub> 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). Coomassie stained membrane confirms that equal amounts of protein were loaded into each well of the gel for immunoblot.

### **Transduction of C<sub>2</sub>C<sub>12</sub> Myotubes with a HERG1a-Encoded Adenovirus Results in**

**Decreased Myotube Area and Increased MuRF1 E3 Ligase Abundance.** 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 ( $\mu\text{m}^2$ ) of fluorescent myotubes from both sets is determined at both 48 and 72 hours after transfection, we discover that, similarly to mouse skeletal muscle fibers electro-transferred with *Merg1a* plasmid (24), 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 hours post transfection and by 19.3% at 72 hours post transfection ( $p < 0.01$ ). 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* (25), myotubes transduced with HERG1A exhibit increased levels of the UPP E3 ligase, MuRF1, but not the E3 ligase ATROGIN (Fig. 2B). The data demonstrate that HERG1A treatment of myotubes results in atrophy as it does in mouse skeletal muscle; and it appears to do so, at least in part, by an increase in abundance of the UPP E3 ligase MuRF1. Therefore, 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<sub>2</sub>C<sub>12</sub> myotubes with either a GFP- and HERG1A-encoded adenovirus or an appropriate control GFP-only encoded adenovirus. At 48 hours 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) 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  $\mu$ M MDL28170 to inhibit calpains or an equal volume of buffer vehicle.

We observed that basically the same amount of enzyme activity (control myotubes= $160.8 \pm 7.3$  and HERG1A expressing myotubes= $167.5 \pm 5.34$  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 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 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 (Figure 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.** 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 ( $p < 0.05$ ; 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 ( $p < 0.05$ ; Fig. 4B). It returns to day 0 control levels by day 5.

**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 calpain 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 hours 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). Calpain 2, when autolyzed and hence activated, appears as a doublet found at ~75kD (22). Interestingly, our results show that there is a 40.7% decrease ( $p<0.05$ ;  $n=6$ ) in total calpain 2 protein abundance in response to 48 hours of HERG1A treatment (Fig. 6). Calpastatin expression was not significantly affected by the HERG1A channel for up to 84 hours post-transduction (Fig.7A); however, calpastatin protein abundance declined by a statistically significant 31.7% (Fig. 7B;  $p<0.05$ ;  $n=6$ ). Additionally, there is a decrease in two of the three noted calpain 3 autocatalytic products (23; 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 ( $p<0.03$ ;  $n=6$ ). When the optical 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<sup>+</sup> channel is responsible for late phase repolarization of the cardiac action potential and was reported to be absent from skeletal muscle [21, 24]; however, the Pond and Hannon labs demonstrated that this protein is detectable in the atrophying skeletal muscle of mice and 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<sub>2</sub>C<sub>12</sub> myotubes transduced with either control or HERG1A-encoded adenovirus, we show that HERG1A expression also increases basal [Ca<sup>2+</sup>]<sub>i</sub> and calpain activity. There are numerous potential sources of the calcium that contributes to the increased [Ca<sup>2+</sup>]<sub>i</sub>. 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. The source of calcium is currently under investigation in our laboratories.

The explanation for the increased calpain activity may seem obvious – the increase in [Ca<sup>2+</sup>]<sub>i</sub>. However, we ectopically expressed mouse *erg1a* (*Merg1a*) in mouse *Gastrocnemius* muscle and homogenized the muscle, thereby disrupting the [Ca<sup>2+</sup>]<sub>i</sub> 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<sup>2+</sup>]<sub>i</sub> may not be the only factor that contributes to the ERG1A-induced increase

in calpain activity. Other possible contributors include increased calpain 1 and/or 2 protein and/or decreased calpastatin protein.

Calpains 1 ( $\mu$ -calpain) and 2 (m-calpain) are both classical calpains and are detected throughout the body, including skeletal muscle (25). Indeed, calpain activity has been demonstrated to contribute to muscle atrophy (25). 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 (26). 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 hours 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, 27]. 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 [28, 29]. 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 [23, 30]. Indeed, the absence or reduction of this protein has been shown to lead to limb-girdle muscular dystrophy type 2A (LGMD2A) in humans [27-33]. Studies suggest that calpain 3 takes part in remodeling of the sarcomere in response to cellular damage such as atrophy [30, 32, 33]. Interestingly, studies with calpain 3 knock out 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 [30]. Thus, calpain 3 appears to be protective against muscle loss and its protein abundance might be expected to be lower in an atrophic situation. 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 [27, 34]. 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 muscle atrophy, specifically calpain and UPP activities. Further study is needed to learn how ERG1A functions in skeletal muscle. Indeed, because of its role in cardiac action potential repolarization, ERG1A will likely never be a target for pharmacological treatments of atrophy, however, continuing study of this protein may reveal other possible targets to combat atrophy.

#### **List of 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

## Declarations

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

Consent for publication: Not applicable

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

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

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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 electrotransfer 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 *HERGIA* 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.

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Authors' information (optional): Not desired.

Table 1. Sequences of primers used for quantitative PCR.

<b>Primer Name (mouse)</b>	<b>Primer Sequence 5' – 3'</b>	<b>Size (bp)*</b>	<b>Tm (°C)</b>	<b>GC (%)</b>	<b>Amplicon Size (bp)*</b>
<b>Merg1a Forward</b>	cctcgacaccatcatccgca	20	59.6	55.0	145
<b>Merg1a Reverse</b>	aggaaatcgcaggtgcaggg	20	60.3	60.0	
<b>18S Subunit Forward</b>	cgccgctagaggtgaaattct	21	57.2	52.4	101
<b>18S Subunit Reverse</b>	agaacgaaagtcggaggttc	20	57.0	52.4	
<b>Calpain 1 Forward</b>	gctaccgtttgtctagcgtc	20	58.73	55.0	98
<b>Calpain 1 Reverse</b>	taactcctctgtcatcctctggt	23	59.99	47.83	
<b>Calpain 2 Forward</b>	ttttgtgcggtgtttgtcc	20	59.83	50.0	107
<b>Calpain 2 Reverse</b>	aactcagccacgaagcaagg	20	60.89	55.0	
<b>Calpain 3 Forward</b>	ttcacaggaggggtgacaga	20	60.11	55.0	122
<b>Calpain 3 Reverse</b>	ttcgtgccatcgtaatggag	21	61.01	52.38	
<b>Calpastatin Forward</b>	gccttgatgacctgataga	20	53.8	50.0	115
<b>Calpastatin Reverse</b>	gtgcctcaaggtaggtagaa	20	53.7	50.0	

\*bp = base pair

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Figure 1. Transduction of C<sub>2</sub>C<sub>12</sub> 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) 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<sub>2</sub>C<sub>12</sub> myotubes with a HERG1A-encoded adenovirus results in synthesis of HERG1A protein as demonstrated by immunoblot. Coomassie stained membrane (blue) reveals that equal amounts of cell lysate protein were loaded into each lane.

Figure 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 hours after transduction and a significant 19.3% smaller (p<0.01; n=3 experimental sets) at 72 hours after transduction. Scale bar=100 μm. Bars of the graph represent the mean myotube area (μm<sup>2</sup>) while the error bars represent the standard error of the mean. B. Immunoblot shows that transduction of C<sub>2</sub>C<sub>12</sub> 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 3 experiments.

Figure 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<sub>2</sub>C<sub>12</sub> 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<sub>2</sub>C<sub>12</sub> myotubes with a HERG1A-encoded adenovirus increases combined native calpain 1 and 2 activity a significant 31.9% (p<0.08; n=24) over control myotubes. All bars represent the mean while error bars represent the standard error of the mean.

Figure 4. Expression of mouse *erg1a* in mouse *Gastrocnemius* muscle increases *Merg1a* transcription and native calpain activity. 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.

Figure 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 hours after transduction ( $n=15$ ). B. Immunoblot demonstrates that there is no significant change in calpain 1 protein abundance at 48 hours 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.

Figure 6. Calpain 2 protein abundance decreases ( $p<0.05$ ;  $n=6$ ) 48 hours 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.

Figure 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 hours 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 hours after transduction. All bars represent the mean  $\pm$  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.

Figure 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 3 notable autocatalytic products: 114 kD (down 29.6%), 60 kD (down 29.2%), and 30 kD which was not affected. Bars represent the mean  $\pm$  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.

Figure 1.

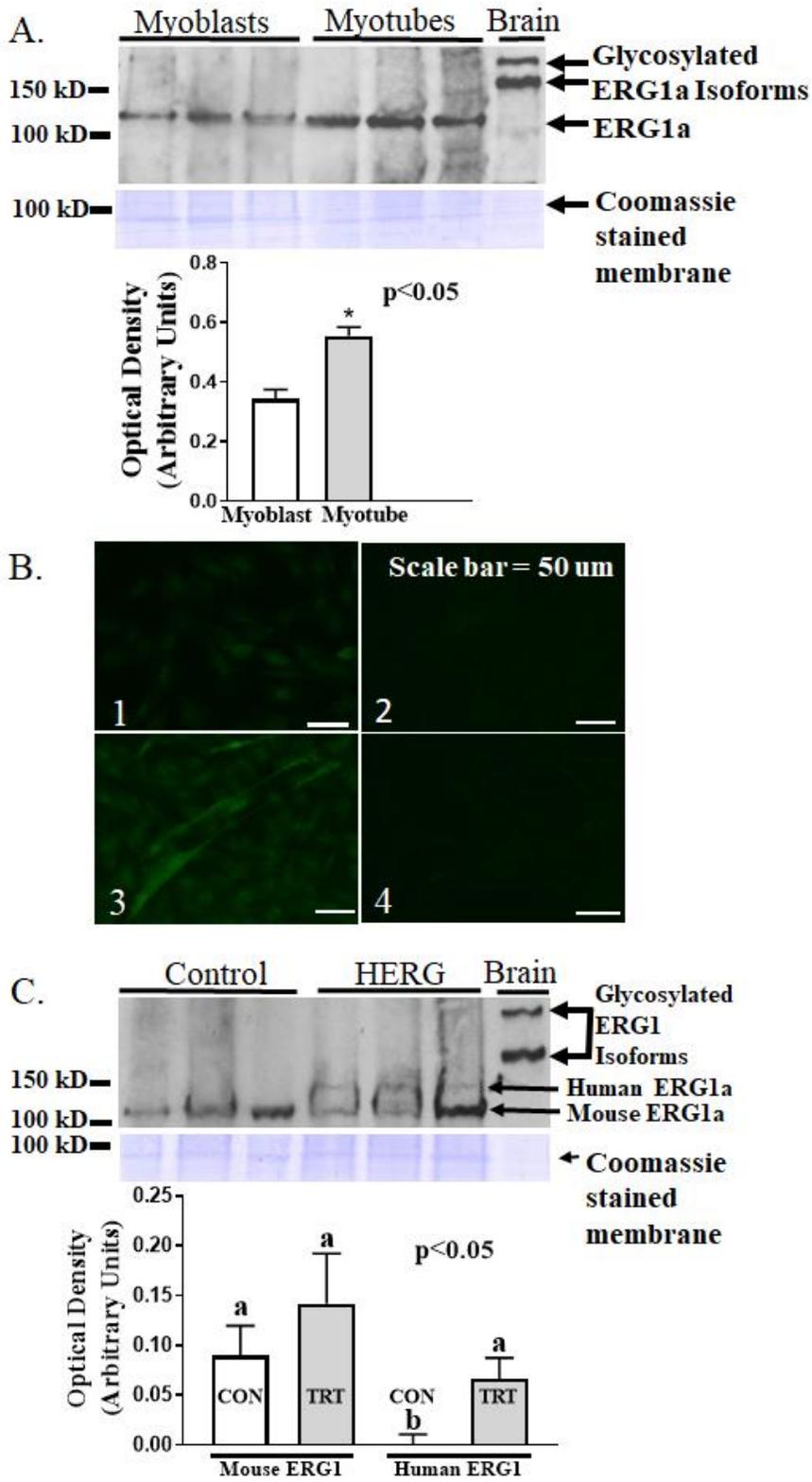
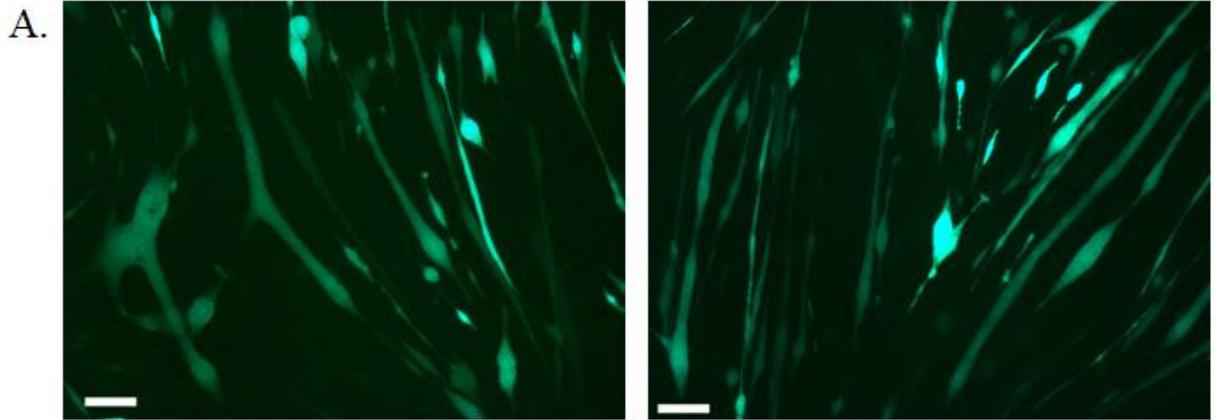


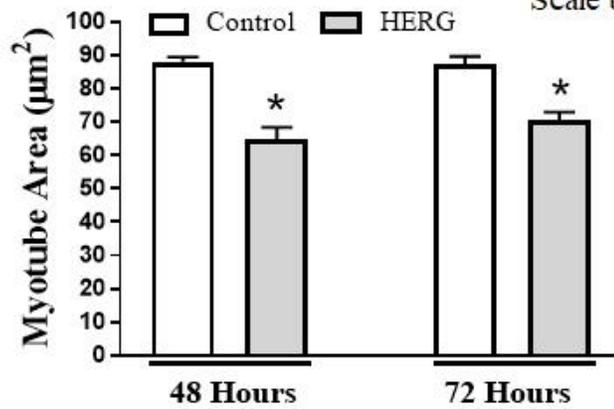
Figure 2.



Myotubes 48 hours after transfection with GFP encoded control adeno-virus.

Myotubes 48 hours after transfection with HERG and GFP encoded adeno-virus.

Scale bars = 100  $\mu\text{m}$ .



\* $p < 0.01$  within time point;  $n = 3$  experimental sets.

B.

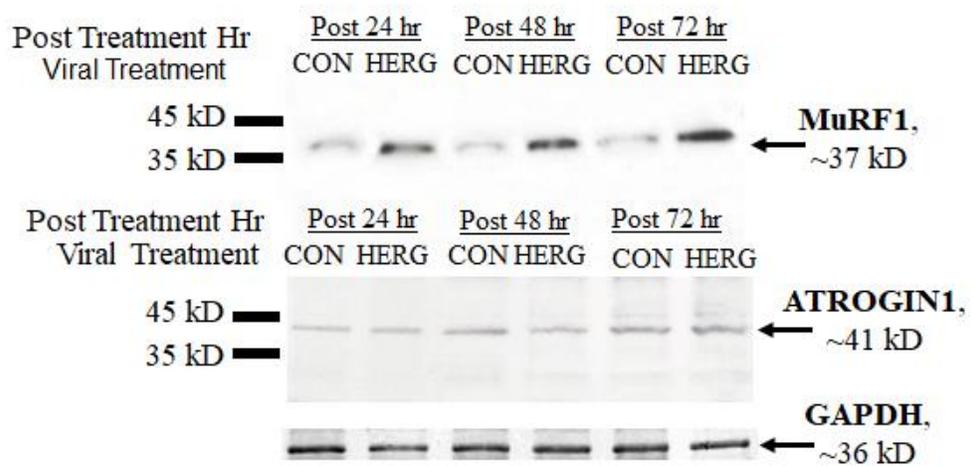


Figure 3.

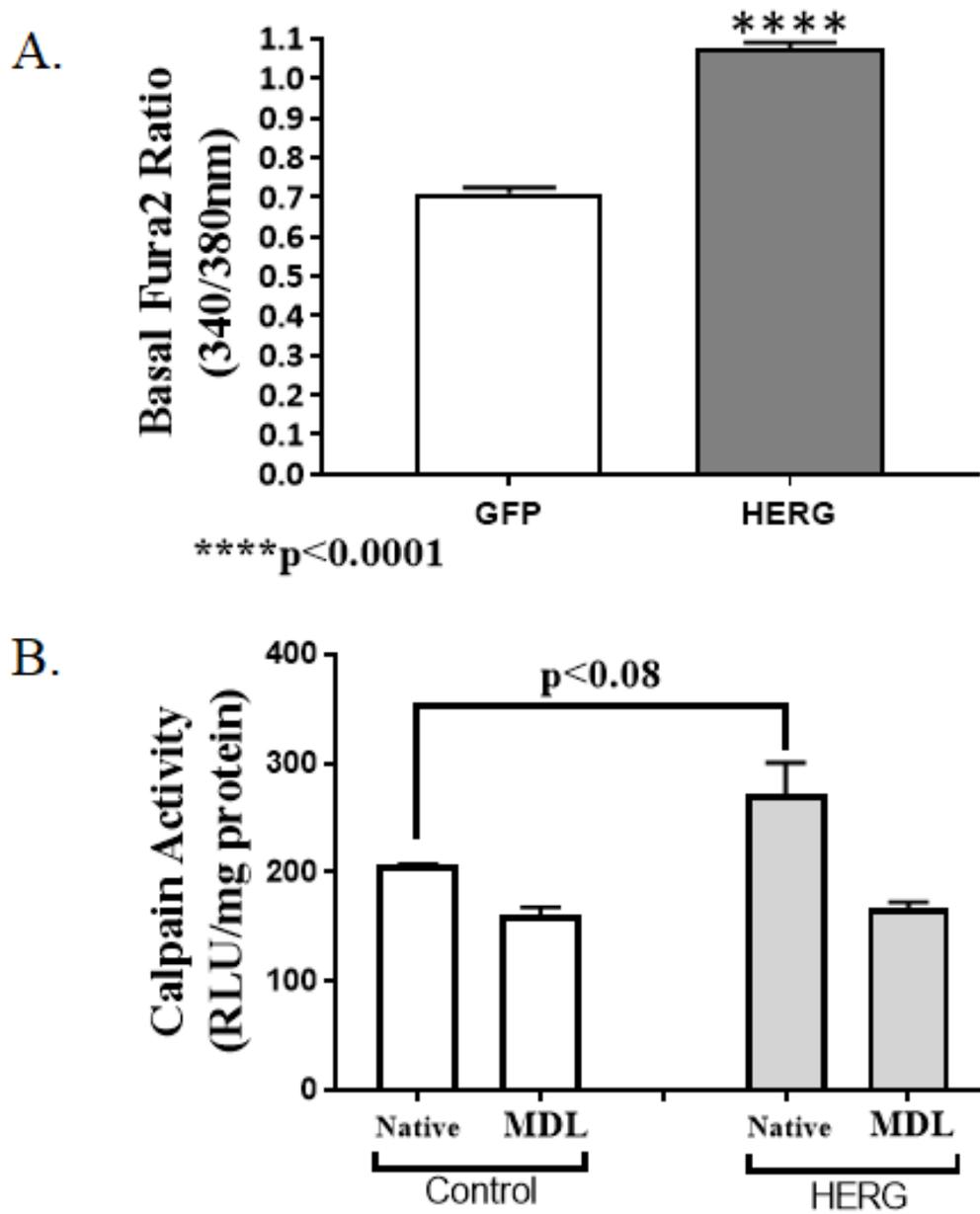


Figure 4.

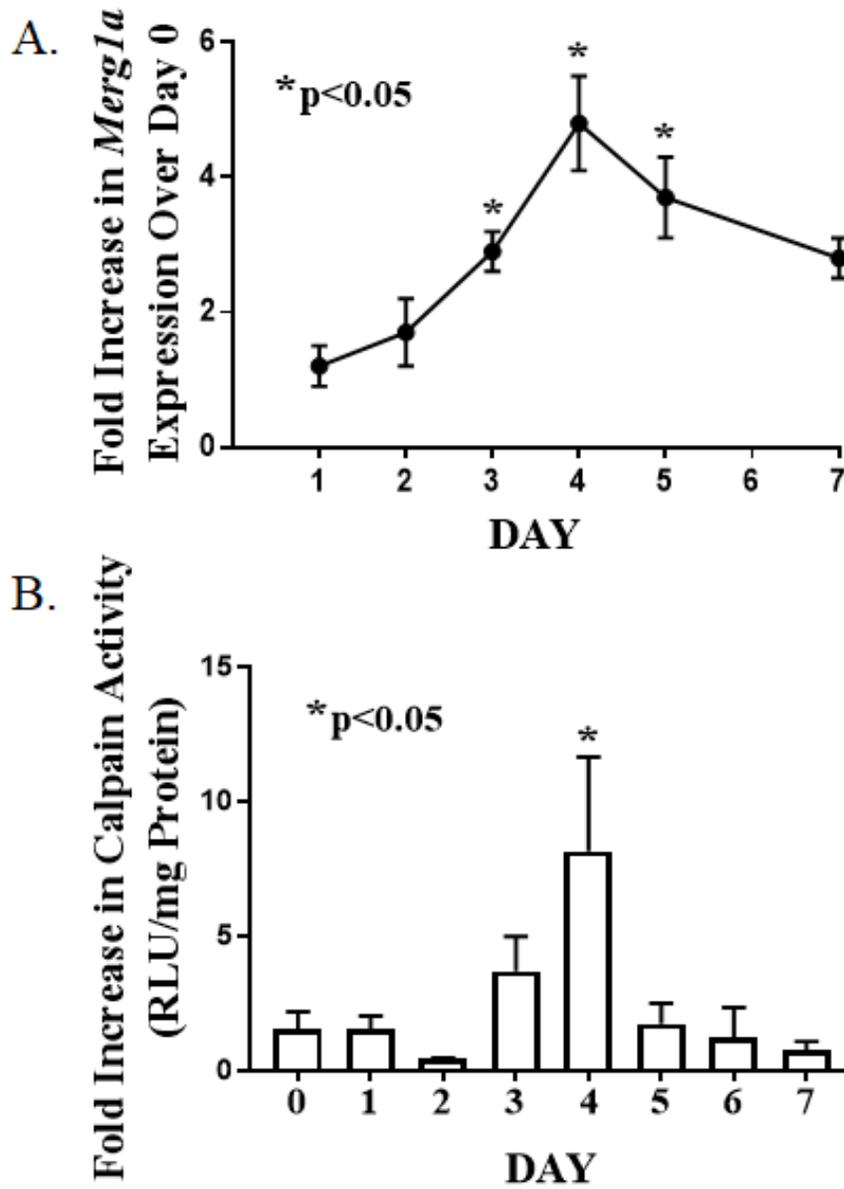


Figure 5.

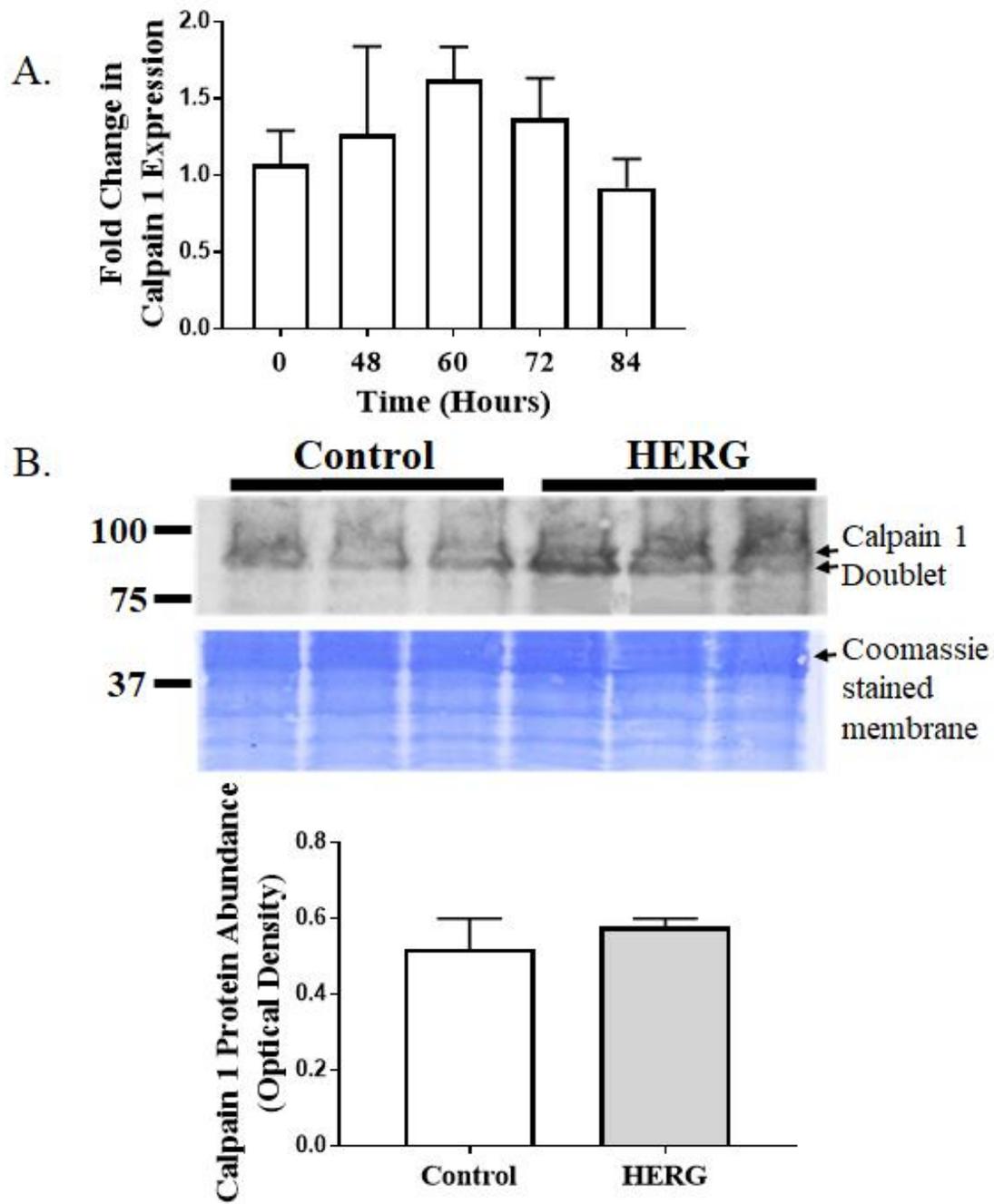


Figure 6.

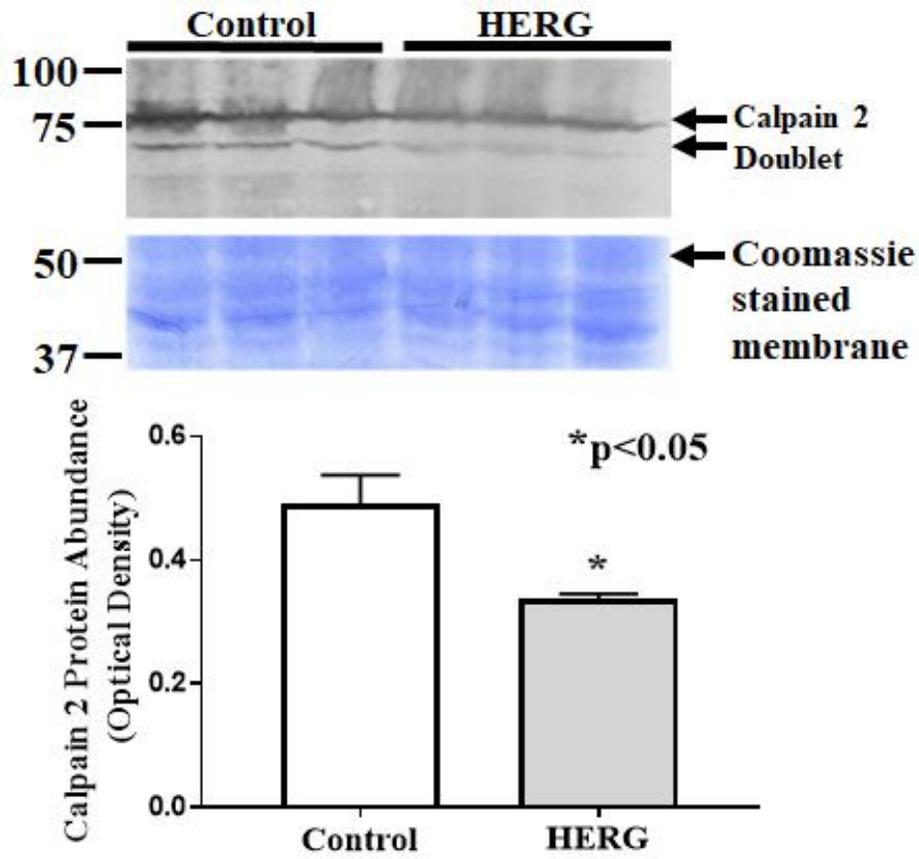


Figure 7.

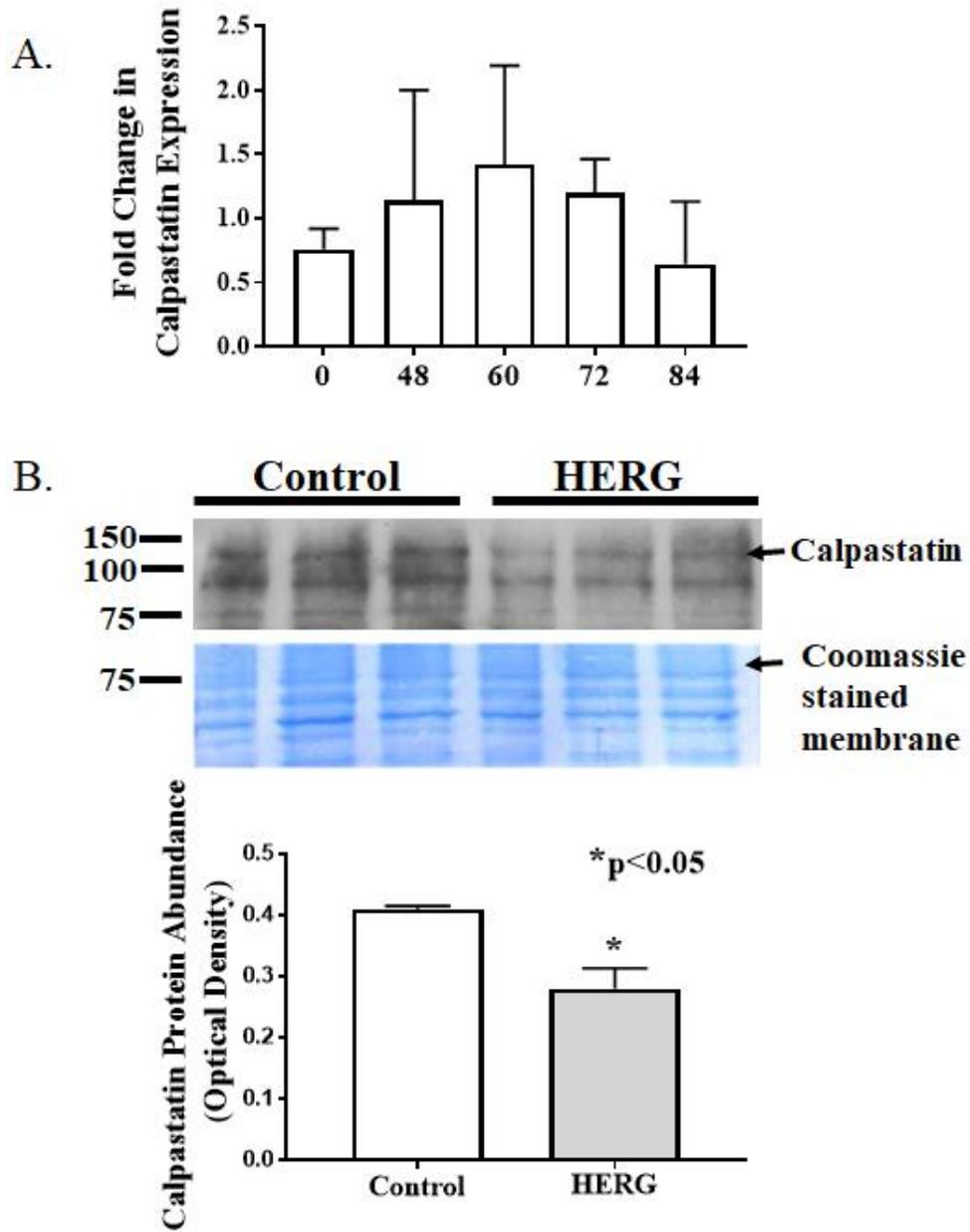
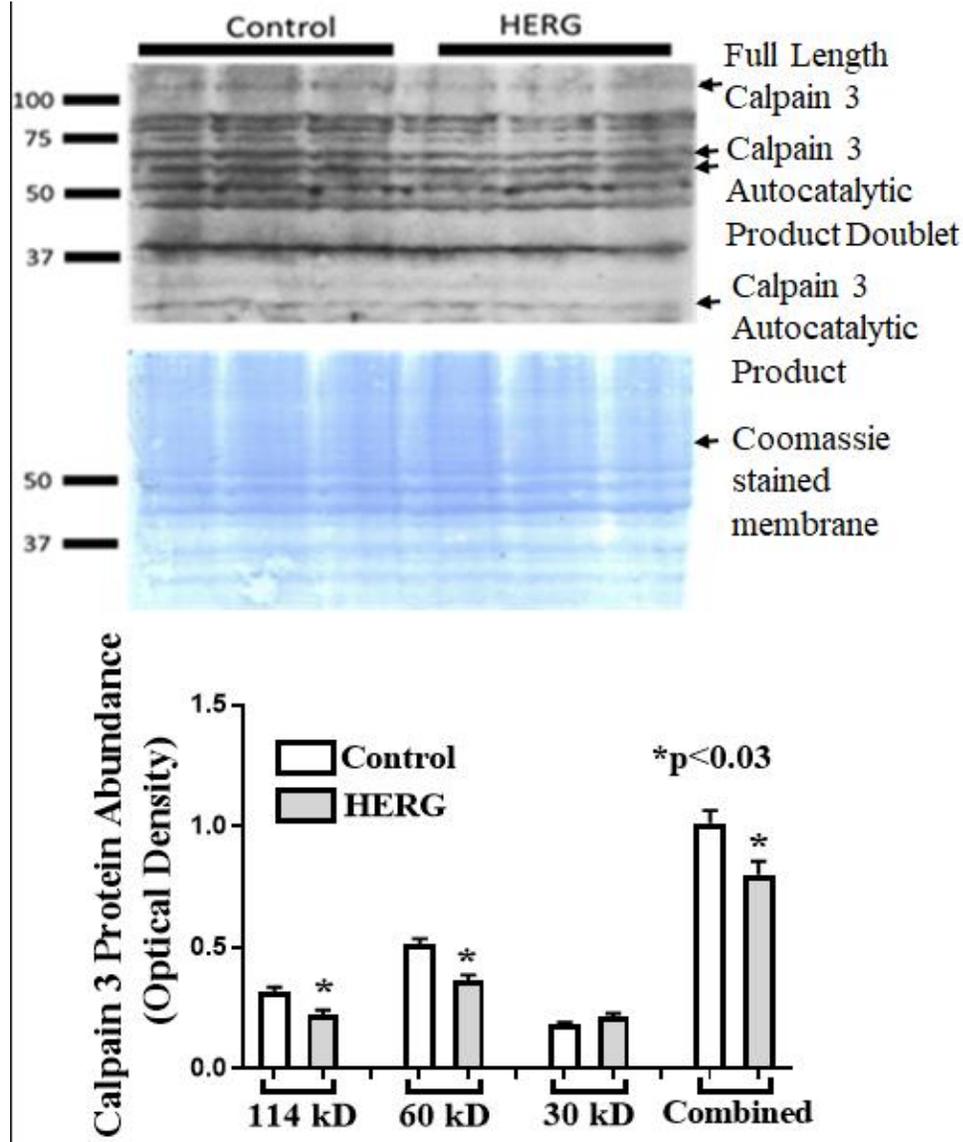


Figure 8.



## B. Abstracts.

### 1.

**Pond AL**, Whitmore C, Pratt EE, Anderson LB, Rantz E, Salyer A, Carraro U, Wang W-H, Weilbaeher R, Davie JK, Hockerman GH. 2019. *The ERG1a K<sup>+</sup> 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.

## **The ERG1a K<sup>+</sup> 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 Weilbaeher (5), Judy K. Davie (5), and Gregory H. Hockerman (2).

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Increased intracellular calcium concentration ( $[Ca^{2+}]_i$ ;  $\mu M$  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.<sup>1-4</sup> 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.<sup>1</sup> In earlier reports, we have shown that the *ether-a-gogo related gene* (ERG) *1a* K<sup>+</sup> channel is detected in atrophying skeletal muscle and that *mouse erg1a* (*Merg1a*) expression in mouse muscle upregulates ubiquitin proteasome proteolysis.<sup>5,6</sup> Because ERG1a is detected in the t-tubules of cardiac muscle,<sup>7</sup> 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<sup>2+</sup> 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

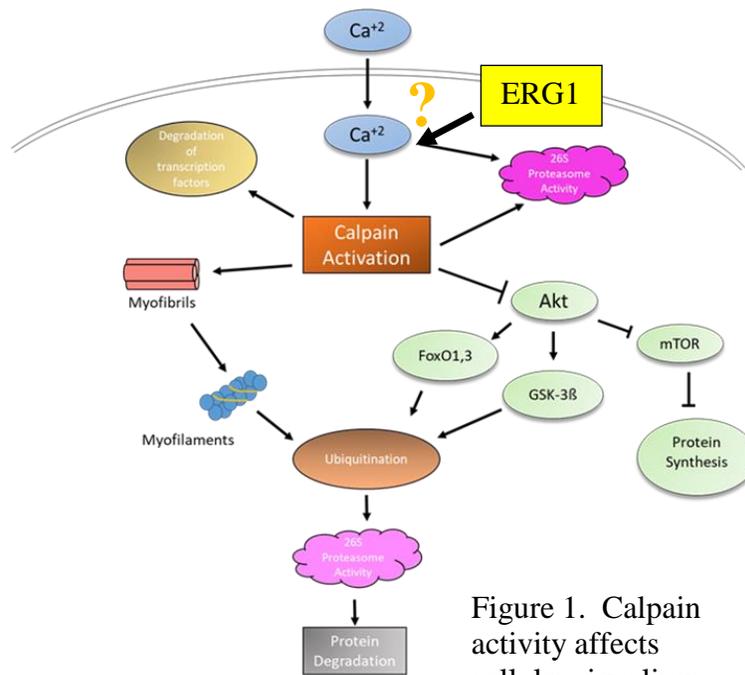


Figure 1. Calpain activity affects cellular signaling

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2.

**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<sup>+</sup> channel increases intracellular calcium and calpain activity in C<sub>2</sub>C<sub>12</sub> myotubes*. Poster presentation given at Experimental Biology Conference in Orlando, FL, April 8, 2019.

## **The ERG1a K<sup>+</sup> 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 Weilbaeher (5), Judy K. Davie (5), and Gregory H. Hockerman (2).

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In skeletal muscle increased intracellular calcium concentration ( $[Ca^{2+}]_i$ ;  $\mu M$  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<sup>+</sup> channel is upregulated in atrophying skeletal muscle and contributes to increased ubiquitin proteasome proteolysis. Here, using the ratiometric Ca<sup>2+</sup> 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, 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.

#### **ERG1a Abundance Varies by Skeletal Muscle in Mice**

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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  $\mu\text{m}$ ) 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, Latour SM, Graham V, Pond AL. 2019. *The Effect of Erg1a Expression on Caspase Enzyme Activity in C<sub>2</sub>C<sub>12</sub> Myotubes*. Poster presentation given at SIU Undergraduate Research Forum, April 8, SIU.

### **The Effect of *Erg1a* Expression on Caspase Enzyme Activity in C<sub>2</sub>C<sub>12</sub> Myotubes**

**Omar Khader<sup>1</sup>, Sawyer M. Latour<sup>2</sup>, Valerie Graham<sup>2</sup>, Amber L. Pond<sup>1</sup>**

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<sup>2</sup>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.