Effect of Prodrug 39 on ZMP Accumulation and AMPK Activity in C2C12 Myotubes and Mouse Gastrocnemius

Avery N. Allen, Jared S. Ashby and David M. Thomson
Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT

Abstract

The discovery of AMP-activated protein kinase's (AMPK) beneficial roles in the metabolic regulation of skeletal muscle and other tissues has made it a target for pharmacologic activation. There are currently very few effective AMPK direct activators used in research and even fewer in clinical trials. Prodrug-39 (P39) has recently emerged as a potential AMPK direct activator. The prodrug is composed of phosphorylated ZMP, an AMP mimetic, attached to a functional group designed to aid the transportation of the molecule across cell membranes. Once in the cell, phosphodiesterase enzymes cleave the bond between the functional group and ZMP, increasing free ZMP levels. In theory, this should mimic elevated AMP levels and activate AMPK. In preliminary studies, P39 only mildly activated AMPK while activating mTORC1, which is critical in stimulating protein synthesis and muscle growth. Appropriate AMPK and mTORC1 activation may be of great value in treating several pathologies, including muscle wasting disorders like sarcopenia. Therefore, the purpose of this study is to further elucidate P39's acute in-vitro (using C2C12 cells) and in-vivo (using C57BL/6J mice gastrocnemii) effects on ZMP accumulation and AMPK activation within skeletal muscle fibers.

Background

- AMPK is considered a master regulator of skeletal muscle metabolic pathways and is a key player in the benefits of exercise [1].
- Increased AMP:ATP, after exercise, allosterically aids in the activation of AMPK [2].
- P39 seeks to mimic the raised AMP:ATP ratio by increasing the intracellular concentration of the AMP mimetic ZMP.
- **This study was designed to verify P39's proposed mechanism of action as well as its ability to increase AMPK activation.**

Methods

Does P39 Increase Intracellular ZMP Concentrations?

- Creation of a HPLC standard for ZMP, AMP, ADP, ATP, NAD+, and IMP metabolites. Create a "master mix" containing each of the metabolites and use all standards to evaluate elution time and identify absorbance patterns of known metabolites.
- Treat mature C2C12 myotubes in 12 55mm culture dishes with 2 mL of DM (ctrl), DM + 500 uM P39, DM + 1mM P39, and DM + 1mM AICAR (positive ctrl) and allow incubation for 3 hours. Harvest once incubation is complete.
- Run 100uL of each lysate through reverse phase HPLC and quantify presence of each metabolite in cell lysates.

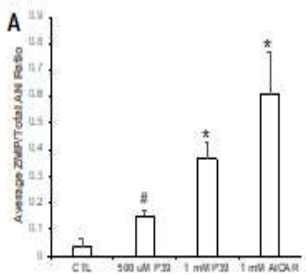
What is the Optimal Concentration of P39 for mTOR and AMPK activation in Myotubes?

- Prepare mature C2C12 myotubes in four 6-well plates.
- Treat mature C2C12 cells with DM (ctrl), DM + 10uM P39, DM + 100uM P39, DM + 500uM P39, DM + 1mM P39, or DM + 1mM AICAR and incubate for 1 hour. Harvest cells once incubation is complete.
- Measure protein concentration of the lysates via DC protein assay and prepare 1 mg/ml Western blot loading samples.
- Probe for the phosphorylated and total forms of S6, S6k, 4EBP1, AMPK, ACC, Raptor, TBC1D1, and AS160 via Western blot.
- Analyze Western blot results and run statistics.

What is the Optimal P39 Time Course For Maximum AMPK Activation in C57BL/6J Mice

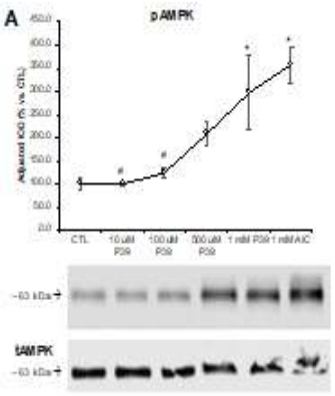
- Begin mouse fast 4 hours before sacking
- Prepare sterile P39 (50 mg/kg) and inject appropriate amount (based on mouse weight) intraperitoneally at proper time point.
- P39 Treatment time points include (each correspond with a saline injection of the same duration as control): 15 min, 30 min, 60 min, 120 min, 240 min. Time points were assessed in both sexes.
- Following time point, mice were euthanized and sacked for heart, blood, gastrocnemius, tibialis anterior, soleus, quadricep, liver, fat, and brain tissue.
- Right gastrocnemius was homogenized from each replicate and DC assays were performed to prepare 1.5 mg/ml Western Blot loading samples.
- Western blots performed to probe for phosphorylated forms of ACC, AMPK, 4E-BP1, Raptor, S6K1, and S6 proteins.
- Analyze blots and run statistics

Does P39 Increase Intracellular ZMP Concentrations?



(A) Relative ZMP/Total Adenine Nucleotide peak area in C2C12 myotubes after 3 hours of treatment as measured by HPLC. In treatments where ADP and AMP levels could not be quantified, only ATP was used as the denominator. One way ANOVA analysis indicated significant differences between treatments, and while subsequent Tukey analysis only indicated one significant difference to the control (AICAR), a student's t-test indicated more. Results presented are from the student's t-test (* = significant ($p \leq .05$) vs. CTL; # = significant ($p \leq .05$) vs. AICAR).

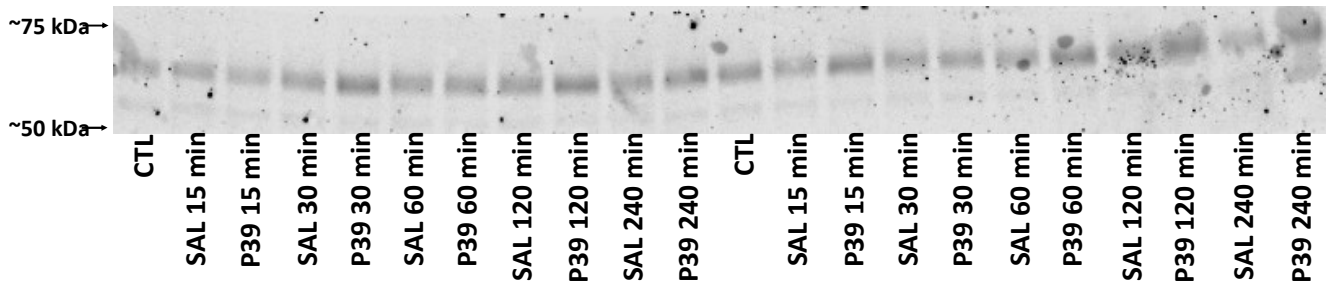
What is the Optimal Concentration of P39 for mTOR and AMPK activation in Myotubes?



(A) AMPK phosphorylation in C2C12 myotubes after 1 hour of treatment. (* = significant ($p \leq .05$) difference vs. CTL; # = significant ($p \leq .05$) difference vs. AICAR treatment)

What is the Optimal P39 Time Course For Maximum AMPK Activation in C57BL/6J Mice?

Male C57BL/6J Right Gastrocnemius Phosphorylated AMPK Signaling (n=2)



Conclusion

- The administration of P39 increased [ZMP] in C2C12 cells as shown in HPLC data.
- Administration of 500 uM P39 showed significant AMPK activation in C2C12 myotubes.
- P39 appears to activate AMPK more than control at many timepoints in vivo (needs to be verified with statistical analysis).
- AMPK activation in vivo seems to increase at longer time points such as 120 or 240 minutes (needs to be verified with statistical analysis).

Future Directions

- Does P39 cause intracellular ZMP levels to increase in vivo?
- Quantify in vivo data for AMPK and mTOR activity at different time points.
- Elucidation of P39 effects on mTOR activation.

References:
1. Lantier, L., Fentz, J., Mounier, R., Leclerc, J., Treebak, J. T., Pehmøller, C., Sanz, N., Sakakibara, I., Saint-Amand, E., Rimbaud, S., Maire, P., Marette, A., Ventura-Clapier, R., Ferry, A., Wojtaszewski, J. F., Foretz, M., & Viollet, B. (2014). AMPK controls exercise endurance, mitochondrial oxidative capacity, and skeletal muscle integrity. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 28(7), 3211–3224. https://doi.org/10.1096/fj.14-250449
2. Thomson D. M. (2018). The Role of AMPK in the Regulation of Skeletal Muscle Size, Hypertrophy, and Regeneration. International journal of molecular sciences, 19(10), 3125. https://doi.org/10.3390/ijms19103125

Contact info:
Avery Allen email: allenavery95@gmail.com
David Thomson email: david_thomson@byu.edu

Acknowledgements:
This work was funded by the BYU College of Life Sciences CURA program