

5-22-2016

Murine Hypometabolism: A Putative Signaling Pathway.

Trevor Thomas Hollinger
Dickinson College

Follow this and additional works at: http://scholar.dickinson.edu/student_honors

 Part of the [Digestive System Commons](#), [Medical Physiology Commons](#), and the [Physiological Processes Commons](#)

Recommended Citation

Hollinger, Trevor Thomas, "Murine Hypometabolism: A Putative Signaling Pathway." (2016). *Dickinson College Honors Theses*. Paper 240.

This Honors Thesis is brought to you for free and open access by Dickinson Scholar. It has been accepted for inclusion by an authorized administrator. For more information, please contact scholar@dickinson.edu.

Murine hypometabolism: a putative signaling pathway.

By:

Trevor Hollinger

Submitted in partial fulfillment of the requirements for
Departmental Honors in the Department of Biology

Professor Chuck Zwemer, Ph. D., *Advisor*
Professor Thomas Arnold, Ph. D., *Reader*
Professor Mary Niblock, Ph. D., *Reader*

May 16, 2016

The Department of Biology at Dickinson College hereby accepts this senior honors thesis by Trevor Hollinger, and awards Departmental Honors in Biology.

Chuck Zwemer, Ph. D. (Advisor)

Date

Thomas Arnold, Ph. D. (Committee Member)

Date

Mary Niblock, Ph. D. (Committee Member)

Date

Kirsten Guss, Ph. D. (Department Chair)

Date

Department of Biology
Dickinson College, Carlisle, PA, USA

May 16, 2016

Title- Murine hypometabolism: a putative signaling pathway.

Author- Trevor Hollinger¹

¹Department of Biology, Dickinson College, Carlisle, PA, USA

Abstract

Hypometabolism is an important energy saving, highly regulated physiological response for some mammals; however, its mechanisms of control have yet to be fully described. It induces a reduction in metabolic rate, heart rate, blood pressure and body temperature. Although the degree and rate of body temperature decrease is subject to ambient temperatures, mammals still maintain control over the whole process. A major goal of hypometabolism appears to be maintaining energetic stores within the body as external food sources become sparse.

Uncovering the mechanisms by which these mammals control this process is of interest as organ and tissue damage following hypometabolism, is rarely, if ever present. 5'adenosine monophosphate (5'AMP) has been demonstrated in this lab and others to induce a hypometabolic state in mice. Other molecules, such as TAN-67, a highly specific δ -opioid receptor agonist is able to induce hypometabolism and protect the brain from hypoxic conditions. A putative connection between these signaling molecules is possible at 5'AMP-activated protein kinase (AMPK). AMPK has an intimate role in metabolic processes throughout the body including roles in signaling and managing metabolic organelles.

Understanding this mechanism could lead to potential therapeutic intervention in humans in areas following major surgery and organ transplantation. The aim of the present study is to test the hypothesis that murine hypometabolism induced by TAN-67 and 5'AMP respectively is characteristic of a signaling pathway homology. Male CD-1 mice were treated with one of the agonists: TAN-67 (4 mg/kg) or 5'AMP (400 mg/kg), followed by one of the antagonists, Naloxone (20 mg/kg) or Aminophylline (20 mg/kg), with saline serving as a control (n=3/group). During the trials measurements of flow of oxygen, flow of carbon dioxide and respiratory exchange ratio were followed for indirect calorimetry. Results indicate that 5'AMP, but not TAN-67 at the present dose was successful in inducing hypometabolism. Changes in dosage could be necessary to induce hypometabolism by TAN-67.

Introduction

Hypometabolism and its physiological effects

Hypometabolism is a well documented physiological response wherein mammals reduce their metabolic rate (MR), blood pressure (BP), heart rate (HR) and body temperature (T_b) for an extended period of time to save energy and food stores^{1,2}. MR in hypometabolism is affected in the liver and the mitochondria throughout all cells. It is believed that changes in oxidative metabolic levels in the liver and active mitochondrial suppression may be the root causes of successful MR reduction². A large surface area to volume ratio in mice allows for

rapid heat dissipation to the surrounding environment following metabolic changes². Despite the rapid change, these animals control their T_b throughout every stage of the arousal process, suggesting that they are able to control the return of blood to organs following hypometabolism². In hypometabolism, mice have been shown to reduce their HR significantly from 600 BPM to 200 BPM³. These factors combined allow for protection of internal organs throughout the process, as tissue and organ damage from ischemic and reperfusion injury (IRI) in these mammals is often not seen⁴. To mitigate the effects of apoptosis and necrotic tissue injury mammals induce a controlled hypometabolic state⁴. The animal's ability to sequester leukocytes and platelets in the liver and spleen could help reduce clotting effects further leading to tissue protection⁵. Protecting against clotting can be beneficial to these animals as the development of clots makes reperfusion more difficult and could lead to a stroke or hemorrhage of vascular tissue.

Hypometabolism is now being studied as a potential form of medical intervention in humans. It could provide medical personnel an opportunity to extend the window of time they have available to them to treat a patient in trauma. It is already being used in the form of therapeutic hypothermia following major surgery in order to limit major organ damage⁶. Likewise, unraveling the mechanisms that signal and regulate hypometabolism could improve the survival time of transplant organs as opposed to the current standard-of-care, ice and an ice chest⁴. An understanding of the signaling mechanisms and their potential crosstalk, in the long run, could unlock a deeper understanding of one of the three following situations: extending the "golden hour", adding to the time transplant organs can survive outside the body, and potential development of new drugs. The "golden hour" is a term that describes the hour following traumatic injury during which life saving medical intervention is most likely to be effective⁷. Extension of this time could give doctors the opportunity to save a patient, since following the "golden hour" survival expectancy drops significantly⁷. In transplant medicine the current way to keep an organ alive for transplant is to place it on ice until it is ready for insertion into the recipient. A large risk factor is IRI following transplantation. Considering hypometabolic mammals rarely suffer organ or tissue damage upon arousal, understanding the mechanisms of initiation and cessation could provide a safer method to transport transplantable organs. Finally, opioid based drugs while effective in inducing analgesia are extremely addictive and the list of side effects is lengthy. A patient's dependence on these drugs is as costly as it is dangerous⁸. If a connection between TAN-67 and 5'AMP, discussed below, is discovered it could provide pharmacologists with a new cellular target that could decrease dependence on these drugs.

Opiates and their signaling pathway

TAN-67 is a non-peptidic exogenous opioid expressing high δ -opioid receptor (DOR) affinity^{9,10}. Studies have demonstrated that TAN-67 has the ability to reduce brain infarct volume and neurologic deficit following medial cerebral artery occlusion¹¹. Furthermore, DOR agonist injection was able to significantly reduce infarct size by 68%⁵ and it has been

shown to protect the myocardium from IRI¹². In the same manner other DOR specific agonists have the ability to assist in ischemic preconditioning (IPC) and protection from IRI. Following the opioid treatment, naloxone reversed these protective effects¹². Naloxone is a broad range opioid receptor (OR) antagonist that has been used clinically for years¹³. It binds competitively to OR however consistent injection seems to be the most effective intervention style¹³. Depending on the serum concentration of opioid the risk of “re-narcotization” can be high¹³. Although the ligands in the studies regarding tissue protection were different, their activation point is the same and provides evidence that opioids are important in protecting against IRI. Interestingly, both isomers of TAN-67 demonstrated competing effects in mice¹⁰. The negative isomer gave antinociceptive behavior¹⁰. Previous studies demonstrating the protective effects by both isomers for organs show promise in the present study.

OR have broad functionality in mammalian life and all of their functions have yet to be completely documented. OR come in 3 main subtypes each coded for by a single gene, μ -opioid receptor (MOR), κ -opioid receptor (KOR) and δ -opioid receptor (DOR), all of which function as G-protein coupled receptors (GPCR)^{12,14}. These receptors function at three distinct areas in the nervous system at the peripheral, spinal and supraspinal levels¹⁴. Of the three, MOR is a common target in pharmaceutical applications for drugs such as morphine¹⁴. However, the list of side effects involved with MOR is substantial⁸. In the present study DOR is of specific interest. DOR has been shown to express at the cell surface making it a viable target⁸. One study found that an increased expression and specific agonism of DOR in the brain helped to protect the brain from IRI following medial cerebral artery occlusion⁴. This subtype is widely distributed in the mammalian central nervous system and has been located in the myocardium, ventricular and arterial tissue^{11,12}. In both cases its presence seems to allow opioid agonists to protect these tissues from ischemic infarction and IRI, respectively^{11,12}. Its distribution in vital organ systems suggests its importance in mediating the protective effects of opioids on these tissues. OR have shown an ability to crosstalk with adenosine receptors (AR) in the heart, to initiate cardioprotection^{15,16}.

OR have a wide range of functionality in mammalian life. They can respond to a range of signaling molecules which can be either endogenous or exogenous in origin¹³(Figure 1). The signaling of OR has a role in pain control, hedonic homeostasis, mood and well being¹³. Despite the numerous actions that are mediated through OR their structures are highly conserved¹³. The uniformity and selectivity of this phenomenon can be explained in part by the idea of the message and address model followed by OR. The message or efficacy of the drug is dictated by the lower portion of the binding site¹³. This region is the most conserved part and in many cases all OR can recognize the message of any ligand. However, the address or selectivity of the drug is dictated by the upper pocket which maintains much of the OR variability¹³. In the present study DOR is of interest as it is activated by the opioid TAN-67^{9,10,17}. Similarly recent studies have shown that there are 2 isoforms of DOR: DOR₁ and DOR₂¹⁷. One study predicted they had competing effects in relation to cardio protection,

however the study was unable to isolate which DOR was at work¹⁷. DOR has been shown in many studies to hold a central role in cardioprotection, reduction in brain infarct size and IRI^{5,11,12,17,18}.

Adenosine and its signaling pathways

5'AMP is a metabolite of adenosine triphosphate (ATP) and has been demonstrated in this laboratory and others to induce hypometabolism^{4,19}. Some of these labs have also reported that following treatment with 5'AMP the animals demonstrated an onset of hypothermia^{3,4,19}. Similarly, it has been described as a ligand that is able to allow non-hibernating species to enter hypothermia safely³. Mice housed in darkness for 24 hours continuously were triggered to enter hypometabolism and subsequent blood analysis showed increased levels of 5'AMP in the blood^{3,6}. This increase could lead to agonistic effects on AR, AMPK and adenylate kinase ultimately resulting in hypometabolism⁶. Significant reductions in HR can be brought on by the injection of 5'AMP from 600 to 200 BPM³. 5'AMP has the ability to determine whether nucleotides are salvaged or catabolized, and in many metabolic pathways it acts as a rate limiter³. Related genes coding for cellular transport proteins have been identified to act at some organelles especially the mitochondria³. Its targets are numerous but its ability to induce hypometabolism and hypothermia makes it an excellent ligand for the present study.

Exogenous 5'AMP putatively acting via AR has been shown in this laboratory and others to play a role in the physiological induction of hypometabolism¹⁹. Upon injection into the vascular space, 5'AMP is quickly converted to adenosine (2 msec) by a 5' ecto-nucleotidase (CD 73)²⁰ where it can then exert its signaling action²¹. It signals in two separate fashions: through AR and through affecting the intracellular AMP:ATP ratio (Figure 2). AR exists as four receptor subtypes one of which has been connected to hypometabolism¹. The A₁AR subtype has demonstrated an ability to decrease MR and HR, important reductions needed for hypometabolism¹. The action of this reduction is believed to be mediated by cellular hypometabolism. The AR2a and 2b subtypes increase cyclic AMP (cAMP), a second messenger of GPCR's, opening K⁺ channels and inducing an efflux of K⁺ into the extracellular space²¹. Second, adenosine can enter the cell through an equilibrative nucleoside transporter (ENT) where it has two fates. It can be converted back to 5'AMP by adenosine kinase or to inosine by deaminase²¹(Figure 2). In the 5'AMP form the AMP:ATP ratio is increased which signals AMPK(Figure 2). This induces both an increase in catabolic processes and a decrease in anabolic processes in an effort to restore ATP stores^{6,22,23}. Similarly A₁-specific antagonist injection triggers a hypometabolic mouse to quickly arouse¹. Aminophylline, an A₁ antagonist and intracerebroventricular injection of an adenosine antagonist trigger cessation of hypometabolism as well¹. This suggests the effects that come as a result of adenosine are centrally acting¹. AR plays a role in modulating the effects of endogenous opioids²⁴. Likewise, exogenous opioids crosstalk with AR in the same fashion as endogenous opioids¹⁶. OR and AR target similar pathways and when considered together their crosstalk mediates acute cardioprotection^{15,16}.

AMPK is a secondary messenger that exists as an obligate trimer maintaining a conserved functionality across numerous species^{22,23}. It contains one catalytic subunit accompanied by two regulatory subunits β and γ ^{22,23}. AMPK plays a large role in metabolic and protective signaling pathways⁴. It maintains an important role in adaptive responses in energetically stressed cells²². Its intimate connection with metabolic rate and its ability to be signaled by numerous physiological stressors, including 5'AMP, make it of particular interest. AMPK's roles are so broadly defined that higher order mammals have tissue specific roles for AMPK in organs such as the liver, muscle and fat²³. In skeletal muscle it can be a positive regulator of glucose transport, through GLUT 1, from glycolysis and fatty acid oxidation^{22,23}. Here, AMPK can also promote the shift from anabolic to catabolic pathways to match the need for a reduction in energy usage^{6,22,23}. In low energy states the allosteric binding of 5'AMP to AMPK allows AMPK to sense a change in the AMP:ATP ratio and initiate ATP production²². Furthermore, it is in control of the production and destruction of mitochondria, one of the gatekeepers of metabolism²³. It has been demonstrated as an important mechanism in mediating the protective effects of IPC on IRI⁶. In relation to hypometabolism it has been shown to be linked to the circadian clock control through specific phosphorylation²³. In one study, testing leukocyte-endothelial cell adhesions, the investigators developed an AMPK^{-/-} KO transgenic mice colony that provides evidence that the absence of AMPK is not a lethal deletion²⁵. This suggests that its role is potentially regulatory in nature. Along with its broad control of metabolic activity, its possible role as a second messenger for hypometabolism is promising. Unfortunately due to time constraints the present study was not able to evaluate AMPK in the proposed signaling pathway. However, its broad functionality and clear connection to metabolic pathways make it worth including in future studies.

Aims of the Study

The mechanisms involved in signaling hypometabolism's initiation and cessation are complex and have yet to be fully documented. However the literature on TAN-67 and 5'AMP suggest potential links to a shared common pathway. Both demonstrate an ability to reduce damage to vital organs in times of low oxygen stress and/or impaired perfusion. Also, when injected with a specific antagonist for their targets their protective effects are halted or reversed. Organ protection appears to be a hallmark of hypometabolism, and whether or not these ligands are responsible for solely mediating the protection or hypometabolism as a whole is unclear. Similarly, the important role that AMPK plays in the modulation of metabolic processes suggests its potential link to hypometabolism. Its conserved ability across species to sequester or propagate metabolism is suggestive of its potential role in maintaining hypometabolism. The mechanisms involved in this process, at the present time, are elusive but the aim of this study is to explore the potential links that may exist in these ligands and second messengers. This study will evaluate the hypothesis that, murine hypometabolism induced by TAN-67 and 5'AMP respectively is characteristic of a signaling pathway homology.

Methods

Animals- Experiments were approved and carried out in accordance with the Dickinson College Institutional Animal Care and Use Committee. Male white CD-1 mice were purchased (Taconic Farms, Derwood, MD) and housed (n=2-3/cage) in a temperature controlled environment with free access to food and water. Mice were housed with a timer controlled 12h light and dark cycle. All mice were between 20 g and 40 g. All experiments were performed between 0800 and 1700 hours. Mice were euthanized with carbon dioxide gas at the completion of their trial.

Drug Preparation- 5' AMP, Naloxone and TAN-67 (400 mg/kg, 20 mg/kg and 4 mg/kg^{5,26}, respectively) were obtained and prepared in solution (Sigma Aldrich, St. Louis, MO, CAS# 18422-05-4 and 51481-60-8, respectively, Tocris, Minneapolis, MN, Cat.# 0921). Sterile saline was used as a solvent for all drugs. Aminophylline (20mg/kg) was obtained in solution (Moore Medical, Farmington, CT, MFG# 5921-01). Sterile saline was used as a placebo. Dosage did not exceed 1% of the animal's mass.

Experimental Design- Metabolism was measured by indirect calorimetry. The experimental apparatus was designed with two parallel chambers with matched volumes. One side was run as a blank while the other held the test subject. Data on inspired oxygen (F_{iO_2}), expired oxygen (F_{eO_2}) and expired carbon dioxide (F_{eCO_2}) (AEI Technologies, Pittsburgh, PA) and flow (V_I) (Aalborg, Orangeburg, NY) were recorded continuously. Inspired CO_2 (F_{iCO_2}) was entered as a constant at 0.003. Data sources interfaced with an MP 150 analog to digital recording device (Biopac Systems Inc., Goleta, CA) and were collected with AcqKnowledge 4.4 (Biopac Systems Inc., Goleta, CA). Simultaneously data was plotted against manually entered equations to give values of oxygen consumption (VO_2), carbon dioxide production (VCO_2) and respiratory exchange ratio (RER). Equations are presented below.

Statistical Analysis- Data from the AcqKnowledge software was manually analyzed and placed into a Graph-Specific Journal. Samples of data were taken every 5 minutes with each sample measuring a 30 second mean of the given data points (sample rate= 50Hz). Data for time of sample, VO_2 , VCO_2 , RER and time of interval sampled were recorded in the journal. Data was then transferred to analysis software. 2-way ANOVA was performed on Graph Pad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA).

Equations-

$$V_{O_2} = \frac{V_I \left(F_{iO_2} - \left(\frac{(1 - F_{iO_2} - F_{iCO_2})}{(1 - F_{eO_2} - F_{eCO_2})} \right) (F_{eCO_2}) \right)}{mass}$$

$$V_{CO_2} = V_I \frac{\left(\left(\frac{(1 - F_{iO_2} - F_{iCO_2})}{(1 - F_{eO_2} - F_{eCO_2})} \right) (F_{eCO_2}) \right) - F_{iCO_2}}{mass}$$

$$RER = \frac{V_{CO_2}}{V_{O_2}}$$

Experimental Procedure- Mice were assigned to 1 of 6 different experimental protocols (n=3/group). All protocols were identical with the exception of drug injections. Mice were inserted into the measurement apparatus and followed for 30 minutes (Baseline), given first injection then followed for 30 minutes (Treatment 1), and given second injection then followed for 30 minutes (Treatment 2). All drugs were administered by an intraperitoneal injection. Trials totaled 90 minutes from start to finish. The baseline was the same across all trials. Treatment 1 and Treatment 2 differed and were as follows- TAN-67:Naloxone, TAN-67:Aminophylline, 5'AMP:Naloxone, 5'AMP:Aminophylline, 5'AMP:Saline and Saline:Saline.

Results

Saline and Saline- Data were not significantly different across all time points. Calculated values of VO_2 , VCO_2 and RER remained constant throughout the trials. Small increases in VO_2 and VCO_2 were noted at times of injection, however they fell back to baseline reading after 5 to 10 minutes (Figure 3, 4, 5, 6).

5'AMP VO_2 Trials- All baseline readings from 0 to 30 minutes for all trials demonstrated no significant separation from each other nor when compared to Saline:Saline (Control). Injection of 5'AMP induced an almost instantaneous drop in VO_2 . Within 5 minutes of injection VO_2 had dropped significantly ($p < 0.001$) from $100 \text{ mlO}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ to below $50 \text{ mlO}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. VO_2 remained low with minimal to no standard deviation at all points after injection 1 to injection 2. From 65 to 90 minutes every point remained significantly separated from the control ($p < 0.05$). However there was one exception at 85 minutes where no significant separation was seen between control and 5'AMP:Aminophylline (Figure 3). Similarly the second treatments of Naloxone and Aminophylline at 60 to 90 minutes did not separate from each other and remained on a similar positive slope.

5'AMP VCO_2 Trials- All baseline readings from 0 to 30 minutes for all trials demonstrated no significant separation from each other nor compared to the control. From 40 to 60 minutes all trials demonstrated a significant drop in VCO_2 ($p < 0.001$). Within 10 minutes post injection 1, VCO_2 had dropped from $75 \text{ mlCO}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ to $20 \text{ mlCO}_2 \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ where it remained until 60 minutes at time of injection 2. All points within that range presented small standard deviations. From 60 to 80 minutes all points remained statistically separated from the control ($p < 0.05$). However from 80 to 90 minutes no significant separation was present

with the exception of the 5'AMP:Saline data (Figure 4). The animals treated with naloxone or aminophylline at 60 minutes were no different from the control data at those two time points.

5'AMP RER Trials- All points from 0 to 30 minutes showed no significant difference from control RER. From 40 to 90 minutes the 5'AMP:Saline trial was the only group to remain significantly separated from the control RER ($p < 0.05$). Groups of 5'AMP:Naloxone and 5'AMP:Aminophylline were not significantly separated from the control across all time points. The only exception was at 50 minutes where an aminophylline point returned values that were significantly different from the control (Figure 5) ($p < 0.05$).

TAN-67 VO₂, VCO₂ and RER Trials- In all measurement calculations of VO₂, VCO₂ and RER, no significant separation from the control was seen. All calculations followed the same trends as the control. The trials using the condition TAN-67:Naloxone (n=1) did not present a standard deviation and could not be used for statistical analysis. Similarly the condition TAN-67:Aminophylline (n=2) was not able to return statistical data. In the RER calculations the two experimental TAN-67 conditions were higher than the control however they failed to demonstrate a separation (Figure 6).

Discussion

These data did not support the hypothesis as tested. The insufficient data from the TAN-67 trials made comparisons difficult. The trials that included 5'AMP demonstrated a consistent significant drop in VO₂, VCO₂ and RER, suggesting the potential role of this agonist in inducing hypometabolism.

These data were consistent with previous literature on 5'AMP agonism of hypometabolism^{4,6,15,16,19,21,27}. These same studies utilized a dose dependent model and incorporated dosages of 800 mg/kg 5'AMP, double the present study's dosage¹⁹. This suggests the potential to raise dosage and see more rapid induction of hypometabolism. These data were unable to discern which of the many diverse signaling pathways is at work in this process. Whether it is a combination of pathways or just one remains to be determined. In terms of the antagonists used it is difficult to determine the extent of their effect on reversing 5'AMP's action. Aminophylline is the direct antagonist of 5'AMP however only a moderate reversal was noticed in the VCO₂ data at 80 to 90 minutes. These data were inconsistent with previous literature which suggested a more immediate effect^{1,19}. It is suggested that in future studies of this nature, a larger dosage of aminophylline be used and the mice be followed for a longer period of time following injection. This could help to determine if the changes in VCO₂ could be matched in VO₂ as well as help to show if the effects seen in these data were simply based on the dilution of the previous 5'AMP serum concentrations. In the 5'AMP trials utilizing naloxone in treatment 2, a similar increase in VCO₂ was recorded as in the aminophylline trials. This suggests its putative ability to reverse

hypometabolism induced by 5'AMP. However, similar to the aminophylline trials, further studies with a higher dosage of naloxone are suggested to determine the causation of the effects. It is possible these effects were simply based on a dilution of serum 5'AMP levels.

In the trials utilizing TAN-67 as treatment 1, these data did not show any difference across any measurements suggesting TAN-67 at this dosage was unable to induce hypometabolism. The dosage (4 mg/kg) selected at the start of the experiment was selected because literature utilizing TAN-67 and other DOR specific agonists such as DADLE suggested cardio-protective effects at that dose^{5,26}. Upon further literature searches for studies of a similar nature, studies were found that utilized dosages of 10-25 mg/kg TAN-67^{26,28}. This suggests that the dosages that were originally found were only effective at inducing local protection. The hypometabolic response that was observed it is believed to be a function of a systemic response to the drugs delivered. Similarly, from those studies and the present study it was unclear if TAN-67 can cross the blood brain barrier (BBB). One study did suggest a potential for BBB passage however further studies are needed²⁶. Due to the literature available at the time and in the interest of animal safety the present study did not go beyond the literature TAN-67 dose values for fear of a potential over dosage. However, now that higher dosages have been identified it is suggested that further studies utilize the higher dose to potentially induce hypometabolism.

Although the hypothesis was not supported, the 5'AMP data has presented a potentially exciting new fuel source selection process taking place in these mice. The data on RER in these trials has presented values following 5'AMP injection that are much lower than what is generally seen. Prior to injection all mice returned RER values between 0.7 and 0.9, suggestive of fatty acid and/or protein catabolism to produce energy. This is consistent with previous literature as these mice are in a fasted state at the time of day these trials were underway²⁹. Following the 5'AMP injection, RER values dropped to the range of 0.5 which is suggestive of ketone body usage^{29,30}. Although serum ketone levels were not tested, the low RER suggests the potential for a shift in fuel source selection while in a hypometabolic state.

Ketones are a protective fuel source utilized in mammals in times of starvation³⁰⁻³². Studies have shown that ketone body concentrations increase to 5-7 mM in starving man^{30,31}. Similarly the brain often prefers ketones over glucose as 60% of the brain's energy can come from ketones³². This dynamic fuel source enters the Krebs Cycle at Acetyl-CoA and upon ATP production gives off less CO₂ per unit of O₂ consumed than fats, proteins or carbohydrates. This means that in the presence of O₂ this process has the potential to assist in the survival of a mammal plagued by a low energy state^{29,32}. The mice in the present study were provided with sufficient oxygen to fill the requirement for O₂ in this process. The data demonstrated that VO₂ drops significantly, while simultaneously, the VCO₂ demonstrated a similar decrease. These conditions were consistent with the conditions necessary to induce ketone production in the liver³².

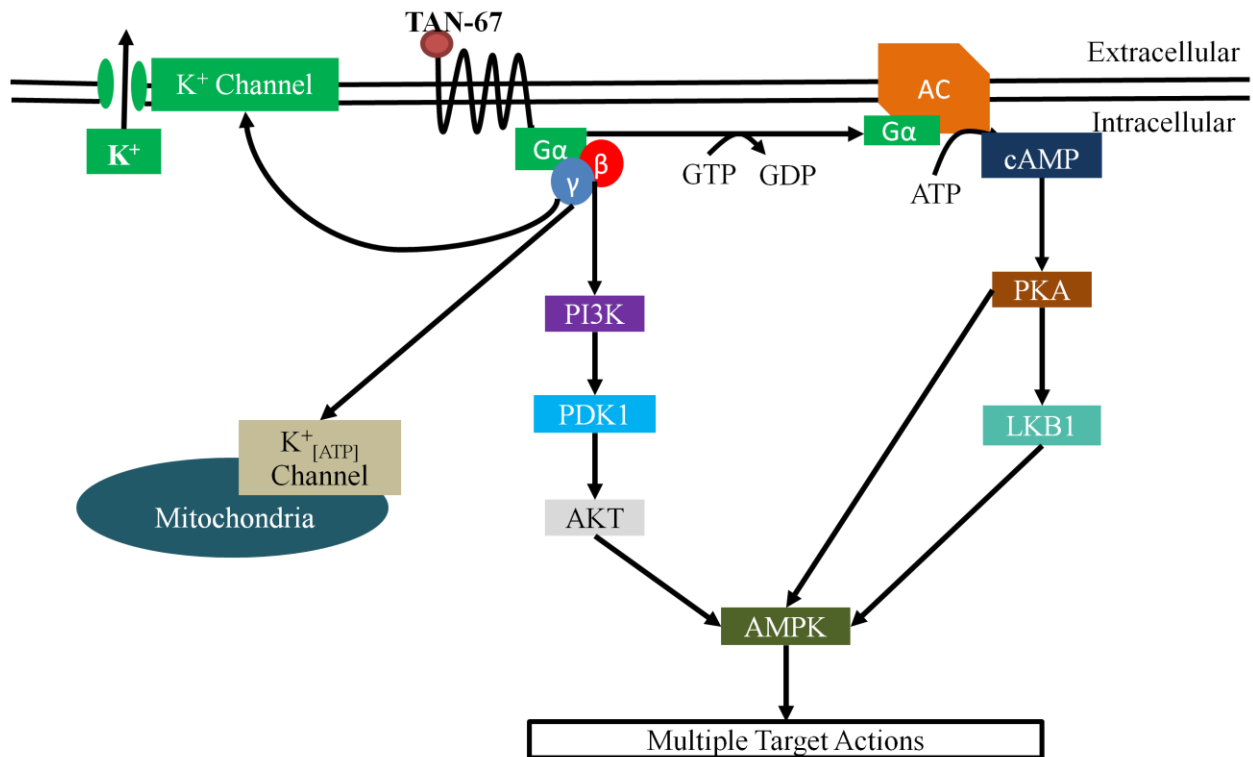
It is important to point out that this form of ketone production differs from the highly feared diabetic ketoacidosis³². In diabetic ketoacidosis the body's ability to produce ketones from free floating fatty acid is hindered by insulin insufficiency or resistance³². In that state, ketone levels could reach 20 mM inducing a dramatic drop in bicarbonate and a large acidification of blood pH to 6.9³². The ketogenic strategy suggested in the present study is not one of a pathogenic nature. With the exception of a potential gouty crisis, patients are generally asymptomatic³². The asymptomatic nature is only sustainable for an acute time frame. Increases in the free fatty acid necessary to produce ketones in the liver, have potentially negative side effects if not managed³². Most notably, increases in LDL cholesterol leading to atherosclerosis and the potential onset of diabetes³².

Although these data did not support the hypothesis, a potential change in energetic usage in hypometabolism may have been uncovered. Further tests are needed to confirm the presence of the protective ketones in this state of hypometabolism. Utilizing higher dosages of TAN-67 could prove to be a key factor to producing a systemic as opposed to a local effect.

Acknowledgements

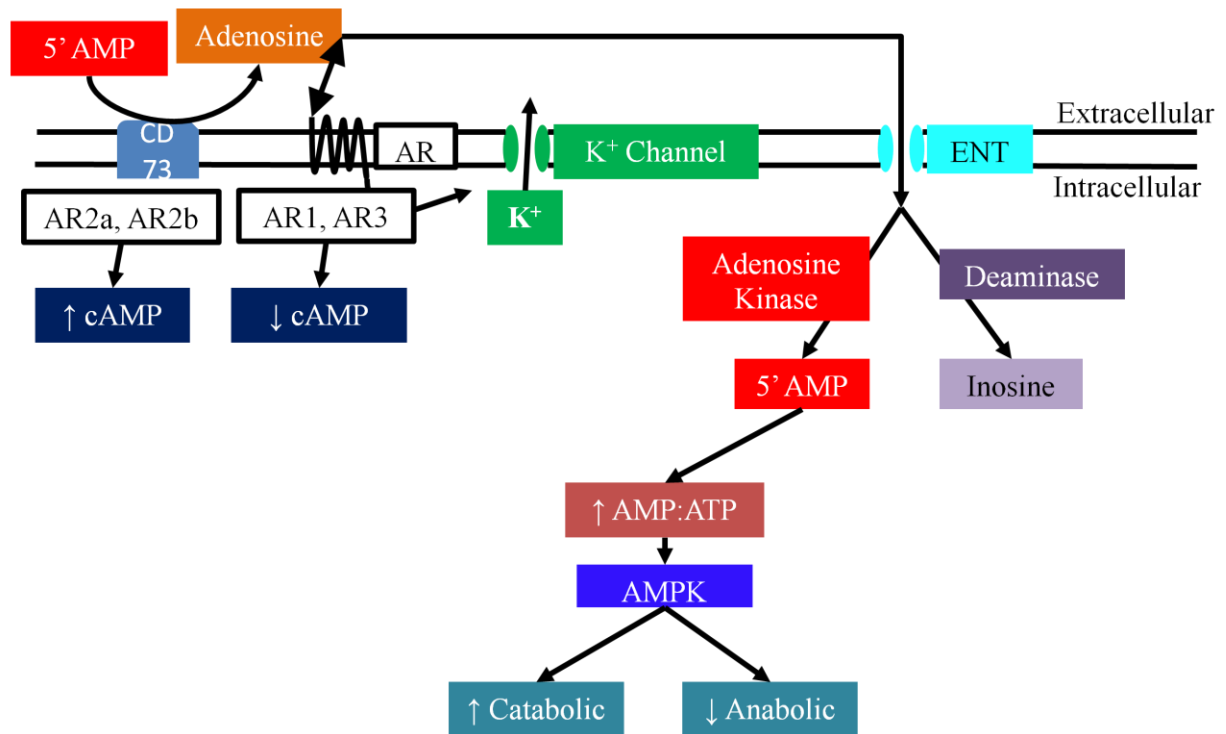
Funding for the present study was provided by the Science Executive Committee and the Office of the Dean of Dickinson College. The author would also like to thank Shelly Boback and Katie Landis for assistance with animal care, and Jim Kuenzie for his help with troubleshooting instrumentation issues. Finally, the author would like to thank Professor Zwemer for his assistance and guidance throughout this research.

Figures



Adapted from Al-Hasani *et. al.* (2011)

Figure 1. Opioid signaling pathway. Opioids acting through DOR can induce the production of $K^+_{[ATP]}$ channels at both the mitochondrial and cellular membrane. This gives the potential for hyperpolarization of the cell hindering action potentials. Similarly through numerous second messengers opioids can signal AMPK a gatekeeper of metabolic processes.



Adapted from Eltzschig (2009)

Figure 2. 5'AMP signaling through multiple pathways. A large bolus of exogenous 5'AMP is rapidly converted to adenosine by CD73 where it can signal in one of two ways: 1) through AR causing an increase or decrease in cAMP which will either open or close K⁺ channels, respectively. 2) it can transverse into the cell via an equilibrative nucleoside transporter (ENT) where it has two fates, conversion to inosine or back to 5'AMP. Conversion back to 5'AMP increases the AMP:ATP ratio signaling AMPK to induce catabolism and inhibit anabolism. This is performed in an effort to reestablish energy stores.

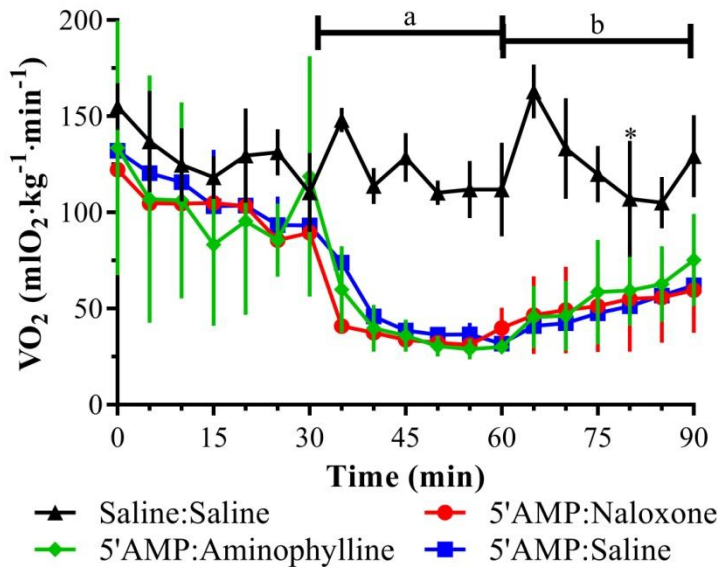


Figure 3. VO₂ trials utilizing 5'AMP as first treatment. All groups demonstrated no separation between 0 and 30 minutes indicating consistent baseline readings. At 30 minutes all groups demonstrated a significant drop in VO₂ as a result of 5'AMP injection. At 60 minutes, upon injection 2 (naloxone, aminophylline, or saline) a significant difference was still present. All three options for treatment 2 showed no separation between each other. One exception is at 80 minutes (*) where the Saline:Saline did not separate from 5'AMP:Aminophylline. These data suggest 5'AMP can reduce VO₂ while the antagonists as dosed were unable to reverse the drop in VO₂. (a, p<0.001)(b, p<0.05). Data presented as mean±SD.

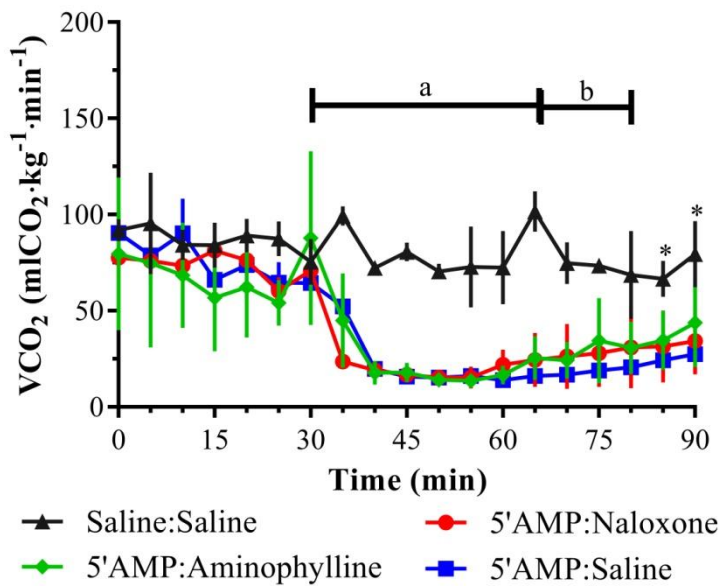


Figure 4. VCO₂ for trials utilizing 5'AMP as first treatment. All groups demonstrated no separation between 0 and 30 minutes indicating consistent baseline readings. At 30 minutes all groups demonstrated a significant drop in VCO₂ as a result of 5'AMP injection. At 60 minutes, upon injection 2 (naloxone, aminophylline, or saline) a significant difference was still present. All three options for treatment 2 showed significant separation from Saline:Saline until 85 minutes to 90 minutes (*, p<0.05). At these points 5'AMP:Saline was the only group to remain statistically separate from Saline:Saline. These data suggest 5'AMP can reduce VCO₂ while the antagonists as dosed were slow to reverse the drop in VCO₂. (a, p<0.001)(b, p<0.05). Data presented as mean±SD.

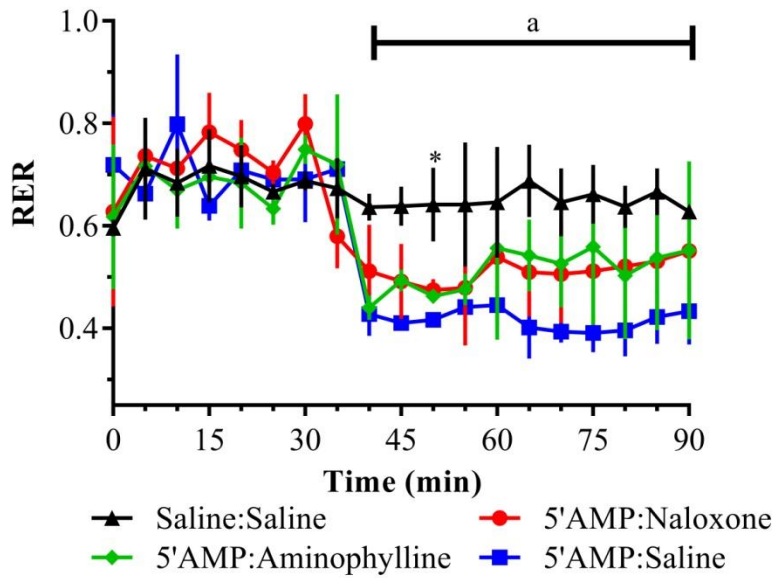


Figure 5. RER for trials utilizing 5'AMP as first treatment. Saline:Saline and baseline readings from 0 to 30 minutes returned an RER between 0.7 and 0.8 suggesting fats and protein catabolism during the time of trials. Upon injection of 5'AMP all mice dropped their RER to 0.5 or below suggesting a move towards ketones as a fuel source. 5'AMP:Saline was the only trial to remain significantly separated from Saline:Saline(a, $p < 0.05$). The only exception being at 50 minutes where 5'AMP:Aminophylline showed significant separation from Saline:Saline (*, $p < 0.05$). These data suggest a potential shift to a more protective fuel source in hypometabolic mice. Data presented as mean \pm SD.

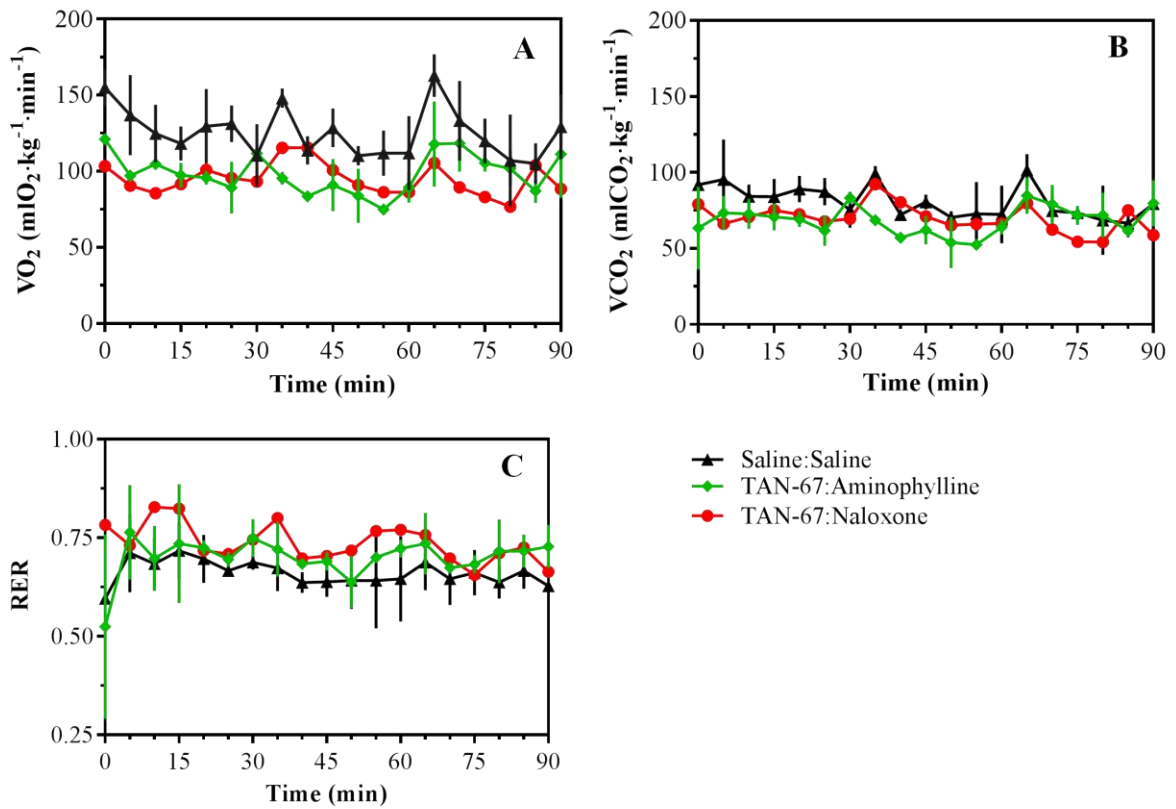


Figure 6. Trials utilizing TAN-67 as first treatment in male CD-1 mice. A) Calculations of VO_2 . B) Calculations of VCO_2 . C) RER in dimension less units. Panels A and B did not demonstrate a separation from the Saline:Saline data. These results were mirrored in panel C as no difference in RER was demonstrated. These data suggest that at the given dosage TAN-67 was unable to induce hypometabolism. Data presented as mean \pm SD.

References

1. Iliff BW, Swoap SJ. Central adenosine receptor signaling is necessary for daily torpor in mice. *Am J Physiol Regul Integr Comp Physiol*. 2012;303(5):R477-R484.
2. Staples JF. Metabolic suppression in mammalian hibernation: The role of mitochondria. *J Exp Biol*. 2014;217(12):2032-2036.
3. Lee CC. Is human hibernation possible? *Annu Rev Med*. 2008;59:177-186.
4. Bouma HR, Verhaag EM, Otis JP, et al. Induction of torpor: Mimicking natural metabolic suppression for biomedical applications. *J Cell Physiol*. 2012;227(4):1285-1290.
5. Govindaswami M, Brown SA, Yu J, et al. Delta 2-specific opioid receptor agonist and hibernating woodchuck plasma fraction provide ischemic neuroprotection. *Acad Emerg Med*. 2008;15(3):250-257.
6. Dugbartey GJ, Bouma HR, Strijkstra AM, Boerema AS, Henning RH. Induction of a torpor-like state by 5'-AMP does not depend on H₂S production. *PLoS One*. 2015;10(8):e0136113-e0136113.
7. Vanderschuren M, McKune D. Emergency care facility access in rural areas within the golden hour?: Western cape case study. *Int J Health Geogr*. 2015;14:5-5.
8. Al-Hasani R, Bruchas MR. Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology*. 2011;115(6):1363-1381.
9. Fujii H, Kawai K, Kawamura K, et al. Synthesis of optically active TAN-67, a highly selective delta opioid receptor agonist, and investigation of its pharmacological properties. *Drug Des Discov*. 2001;17(4):325-330.
10. Nagase H, Yajima Y, Fujii H, et al. The pharmacological profile of delta opioid receptor ligands, (+) and (-) TAN-67 on pain modulation. *Life Sci*. 2001;68(19-20):2227-2231.
11. Tian XS, Guo JC, Zhu M, Li MW, Wu GC, Xia Y. Delta-opioid receptor activation rescues the functional TrkB receptor and protects the brain from ischemia-reperfusion injury in the rat. *PLoS ONE*. 2013;8(7).
12. Tanaka K, Kersten JR, Riess ML. Opioid-induced cardioprotection. *Curr Pharm Des*. 2014;20(36):5696-5705.

13. Granier S, Manglik A, Kruse AC, et al. Structure of the δ -opioid receptor bound to naltrindole. *Nature*. 2012;485(7398):400-404.
14. Haddou TB, Malfacini D, Calo G, et al. Exploring pharmacological activities and signaling of morphinans substituted in position 6 as potent agonists interacting with the μ opioid receptor. *Molecular Pain*. 2014;10(1):48-58.
15. Peart JN, Gross GJ. Adenosine and opioid receptor-mediated cardioprotection in the rat: Evidence for cross-talk between receptors. *Am J Physiol Heart Circ Physiol*. 2003;285(1):H81-H89.
16. Peart JN, Headrick JP. Adenosinergic cardioprotection: Multiple receptors, multiple pathways. *Pharmacology and Therapeutics*. 2007;114:208-221.
17. Deo SH, Johnson-Davis S, Barlow MA, Yoshishige D, Caffrey JL. Repeated delta(1)-opioid receptor stimulation reduces delta(2)-opioid receptor responses in the SA node. *AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY*. 2006;291(5):H2246-H2254.
18. Borlongan CV, Hayashi T, Oeltgen PR, Su T, Wang Y. Hibernation-like state induced by an opioid peptide protects against experimental stroke. *BMC Biol*. 2009;7:31-31.
19. Swoap SJ, Rathvon M, Gutilla M. AMP does not induce torpor. *Am J Physiol Regul Integr Comp Physiol*. 2007;293(1):R468-R473.
20. Bynoe MS, Viret C, Yan A, Kim D. Adenosine receptor signaling: A key to opening the blood-brain door. *Fluids Barriers CNS*. 2015;12:20-20.
21. Eltzschig HK. Adenosine: An old drug newly discovered. *Anesthesiology*. 2009;111(4):904-915.
22. Laderoute KR, Amin K, Calaoagan JM, et al. 5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. *Molecular and Cellular Biology*. 2006;26(14):5336-5347.
23. Mihaylova MM, Shaw RJ. The AMP-activated protein kinase (AMPK) signaling pathway coordinates cell growth, autophagy, & metabolism. *Nat Cell Biol*. 2012;13(9):1016-1023.
24. Lopes SC, da Silva A, Virginia L., Arruda BR, et al. Peripheral antinociceptive action of mangiferin in mouse models of experimental pain: Role of endogenous opioids, KATP-channels and adenosine. *Pharmacology, Biochemistry and Behavior*. 2013;110:19-26.

25. Spencer Gaskin F, Kamada K, Zuidema M, Jones A, Rubin L, Korthuis R. Isoform-selective 5'-AMP-activated protein kinase-dependent preconditioning mechanisms to prevent postischemic leukocyte-endothelial cell adhesive interactions. *Am J Physiol Heart Circ Physiol*. 2011;300:1352-1360.
26. Maslov LN, Lishmanov YB, Oeltgen PR, et al. Comparative analysis of the cardioprotective properties of opioid receptor agonists in a rat model of myocardial infarction. *Acad Emerg Med*. 2010;17(11):1239-1246.
27. Peart J, Headrick JP. Adenosine-mediated early preconditioning in mouse: Protective signaling and concentration dependent effects. *Cardiovasc Res*. 2003;58(3):589-601.
28. van Rijn R, Brissett DI, Whistler JL. Dual efficacy of delta opioid receptor-selective ligands for ethanol drinking and anxiety. *J Pharmacol Exp Ther*. 2010;335(1):133-139.
29. Zwemer CF, Song MY, Carello KA, D'Alecy L. Strain differences in response to acute hypoxia: CD-1 versus C57BL/6J mice. *J Appl Physiol*. 2007;102(1):286-293.
30. Veech RL. The therapeutic implications of ketone bodies: The effects of ketone bodies in pathological conditions: Ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. *Prostaglandins Leukotrienes Essential Fatty Acids*. 2004;70(3):309-319.
31. Veech RL, Chance B, Kashiwaya Y, Lardy HA, Cahill GF, J. Ketone bodies, potential therapeutic uses. *IUBMB Life*. 2001;51(4):241-247.
32. Veech RL. Ketone ester effects on metabolism and transcription. *J Lipid Res*. 2014;55(10):2004-2006.