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Murine Hypometabolism: A Putative Signaling Pathway.

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Murine hypometabolism: a putative signaling pathway.

By:

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Title- Murine hypometabolism: a putative signaling pathway.

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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 reduction[2]. A large surface area to volume ratio in mice allows for

rapid heat dissipation to the surrounding environment following metabolic changes[2]. 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 hypometabolism[2]. In hypometabolism, mice have been shown to reduce their HR significantly from 600 BPM to 200 BPM[3]. 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 seen[4]. To mitigate the effects of apoptosis and necrotic tissue injury mammals induce a controlled hypometabolic state[4]. The animal's ability to sequester leukocytes and platelets in the liver and spleen could help reduce clotting effects further leading to tissue protection[5]. 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 damage[6]. 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 chest[4]. 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 effective[7]. Extension of this time could give doctors the opportunity to save a patient, since following the "golden hour" survival expectancy drops significantly[7]. 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 dangerous[8]. 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 occlusion[11]. Furthermore, DOR agonist injection was able to significantly reduce infarct size by 68%[5] and it has been

shown to protect the myocardium from IRI[12]. 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 effects[12]. Naloxone is a broad range opioid receptor (OR) antagonist that has been used clinically for years[13]. It binds competitively to OR however consistent injection seems to be the most effective intervention style[13]. Depending on the serum concentration of opioid the risk of "re-narcazation" can be high[13]. 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 mice[10]. The negative isomer gave antinocioceptive behavior[10]. 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 levels[14]. Of the three, MOR is a common target in pharmaceutical applications for drugs such as morphine[14]. However, the list of side effects involved with MOR is substantial[8]. In the present study DOR is of specific interest. DOR has been shown to express at the cell surface making it a viable target[8]. 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 occlusion[4]. 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 origin[13](Figure 1). The signaling of OR has a role in pain control, hedonic homeostasis, mood and well being[13]. Despite the numerous actions that are mediated through OR their structures are highly conserved[13]. 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 site[13]. 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 variability[13]. 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: DOR_1 and DOR_2[17]. One study predicted they had competing effects in relation to cardio protection,

however the study was unable to isolate which DOR was at work[17]. 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 safely[3]. 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 hypometabolism[6]. Significant reductions in HR can be brought on by the injection of 5'AMP from 600 to 200 BPM[3]. 5'AMP has the ability to determine whether nucleotides are salvaged or catabolized, and in many metabolic pathways it acts as a rate limiter[3]. Related genes coding for cellular transport proteins have been identified to act at some organelles especially the mitochondria[3]. 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 hypometabolism[19]. 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 action[21]. 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 hypometabolism[1]. The A_1AR subtype has demonstrated an ability to decrease MR and HR, important reductions needed for hypometabolism[1]. 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 space[21]. 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 deaminase[21](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 A_1-specific antagonist injection triggers a hypometabolic mouse to quickly arouse[1]. Aminophylline, an A_1 antagonist and intracerebroventricular injection of an adenosine antagonist trigger cessation of hypometabolism as well[1]. This suggests the effects that come as a result of adenosine are centrally acting[1]. AR plays a role in modulating the effects of endogenous opioids[24]. Likewise, exogenous opioids crosstalk with AR in the same fashion as endogenous opioids[16]. 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 pathways[4]. It maintains an important role in adaptive responses in energetically stressed cells[22]. 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 fat[23]. 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 production[22]. Furthermore, it is in control of the production and destruction of mitochondria, one of the gatekeepers of metabolism[23]. It has been demonstrated as an important mechanism in mediating the protective effects of IPC on IRI[6]. In relation to hypometabolism it has been shown to be linked to the circadian clock control through specific phosphorylation[23]. 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 deletion[25]. 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. Aminophyilline (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_{iO2}), expired oxygen (F_{eO2}) and expired carbon dioxide (F_{eCO2}) (AEI Technologies, Pittsburgh, PA) and flow (V_I) (Aalborg, Orangeburg, NY) were recorded continuously. Inspired CO_2 (F_{iCO2}) 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-

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

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

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

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 intraperitonial 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 $mlO_2 \cdot kg^{-1}min^{-1}$ to below 50 $mlO_2 \cdot kg^{-1}min^{-1}$. 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 $mlCO_2 \cdot kg^{-1}min^{-1}$ to 20 $mlCO_2 \cdot kg^{-1}min^{-1}$ 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 VO_2, VCO_2 and RER Trials- In all measurement calculations of VO_2, VCO_2 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 VO_2, VCO_2 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 dosage[19]. 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 VCO_2 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 VCO_2 could be matched in VO_2 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 VCO_2 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 where 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 needed[26]. 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 underway[29]. 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 starvation[30-32]. 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 ketones[32]. This dynamic fuel source enters the Krebs Cycle at Acetyl-CoA and upon ATP production gives off less CO_2 per unit of O_2 consumed than fats, proteins or carbohydrates. This means that in the presence of O_2 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 O_2 in this process. The data demonstrated that VO_2 drops significantly, while simultaneously, the VCO_2 demonstrated a similar decrease. These conditions were consistent with the conditions necessary to induce ketone production in the liver[32].

It is important to point out that this form of ketone production differs from the highly feared diabetic ketoacidosis[32]. In diabetic ketoacidosis the body's ability to produce ketones from free floating fatty acid is hindered by insulin insufficiency or resistance[32]. In that state, ketone levels could reach 20 mM inducing a dramatic drop in bicarbonate and a large acidification of blood pH to 6.9[32]. 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 asymptomatic[32]. 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 managed[32]. Most notably, increases in LDL cholesterol leading to atherosclerosis and the potential onset of diabetes[32].

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.

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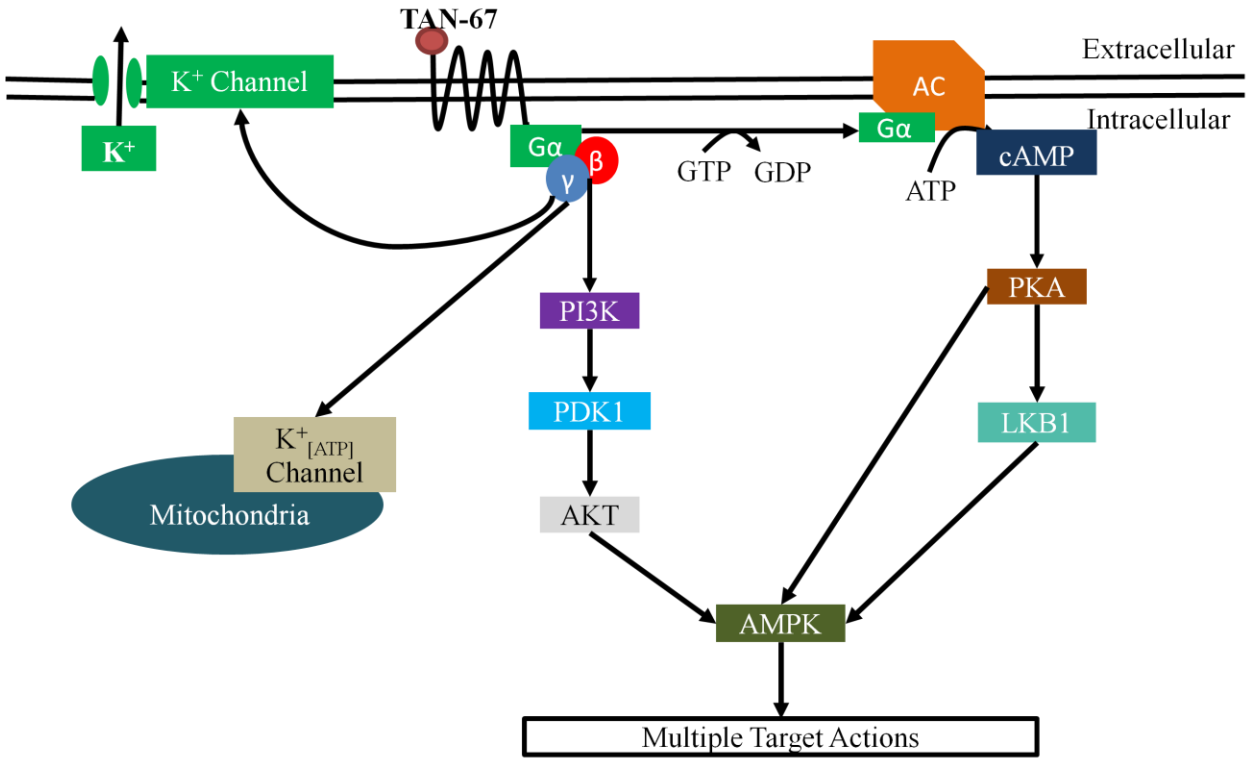
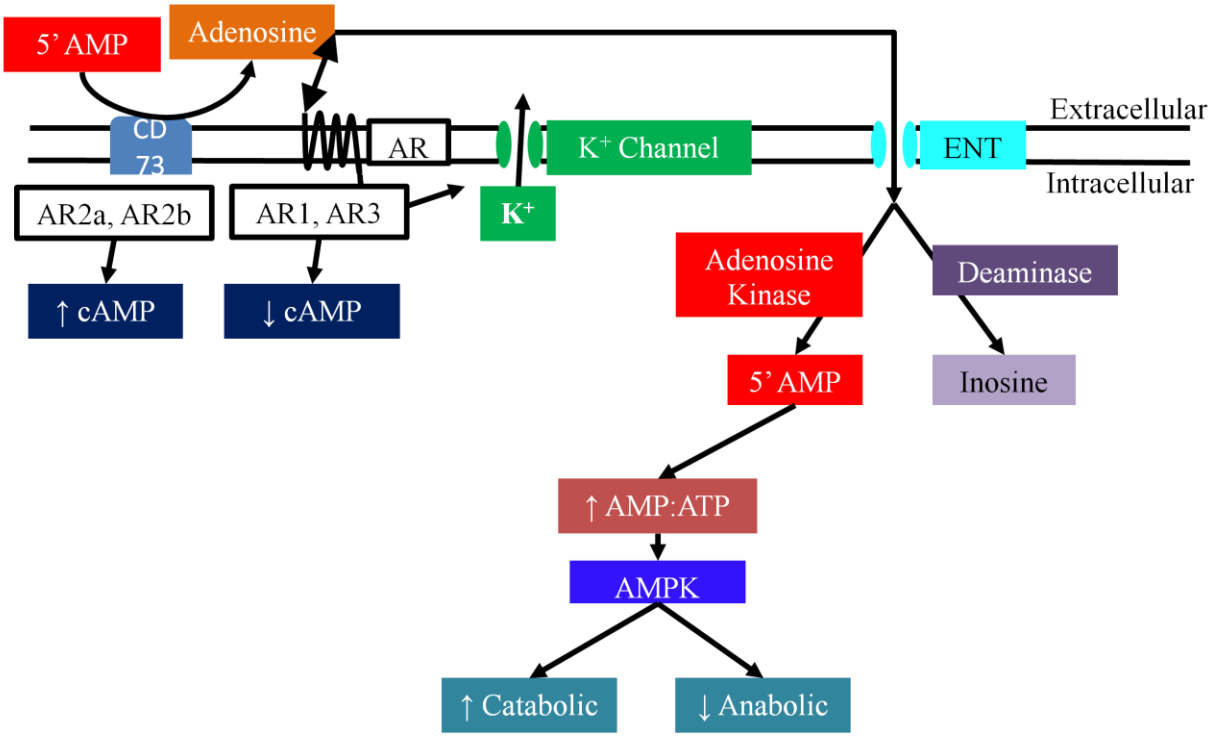
Figures



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.

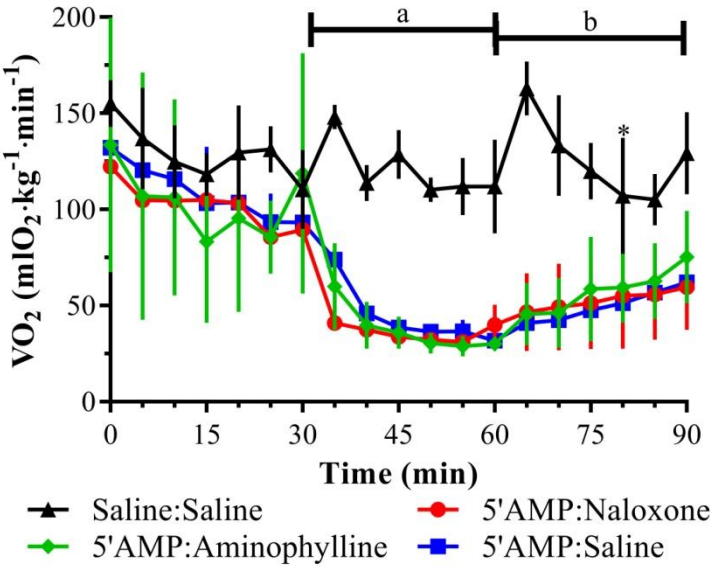


Figure 3. VO_2 trials utilizing 5'AMP as first treatment. All groups demonstrated no separation between 0 and 30 minutes indicating consistent baseline readings. At 30 minutes all groups demonstrated a significant drop in VO_2 as a result of 5'AMP injection. At 60 minutes, upon injection 2 (naloxone, 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 VO_2 while the antagonists as dosed where unable to reverse the drop in VO_2. (a, $p<0.001$)(b, $p<0.05$). Data presented as mean±SD.

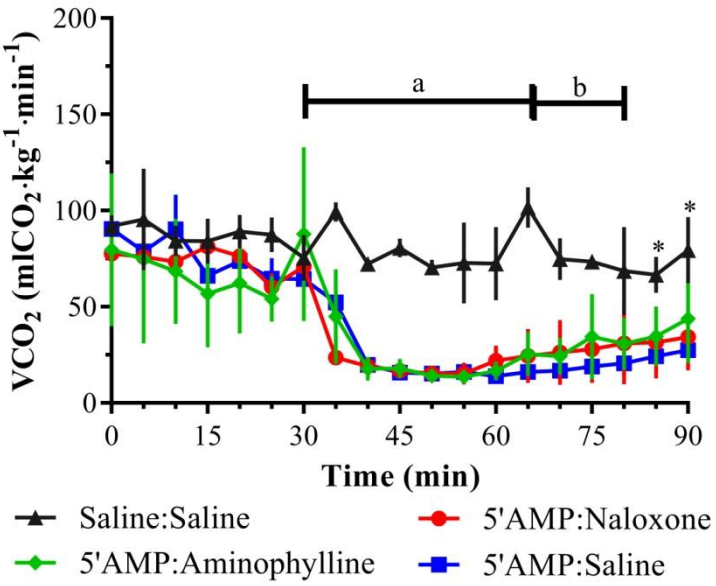


Figure 4. VCO_2 for trials utilizing 5'AMP as first treatment. All groups demonstrated no separation between 0 and 30 minutes indicating consistent baseline readings. At 30 minutes all groups demonstrated a significant drop in VCO_2 as a result of 5'AMP injection. At 60 minutes, upon injection 2 (naloxone, 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 VCO_2 while the antagonists as dosed were slow to reverse the drop in VCO_2. (a, $p<0.001$)(b, $p<0.05$). Data presented as mean±SD.

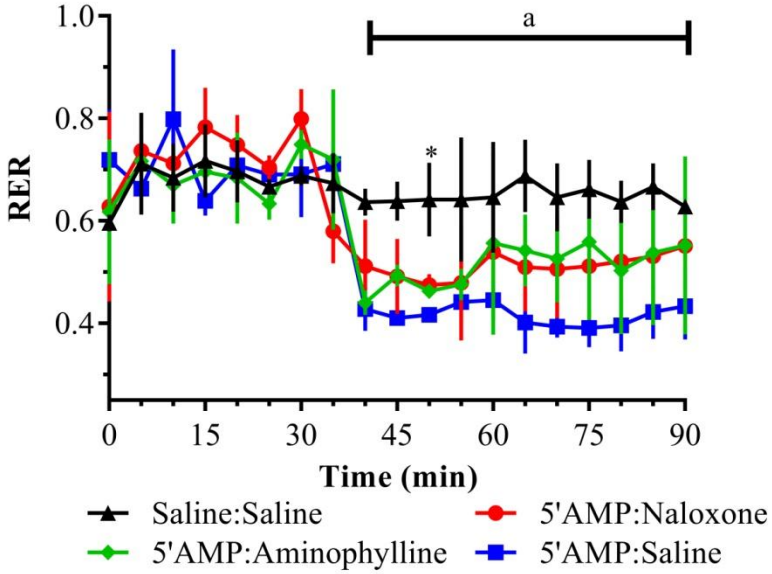


Figure 5. RER for trials utilizing 5'AMP as first treatment. Saline:Saline and baseline readings from 0 to 30 minutes returned an RER between 0.7 and 0.8 suggesting fats and protein catabolism during the time of trials. Upon injection of 5'AMP all mice dropped their RER to 0.5 or below suggesting a move 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:Aminphylline showed significant seperation from Saline:Saline (*, $p<0.05$). These data suggest a potential shift to a more protective fuel source in hypometabloic mice. Data presented as mean±SD.

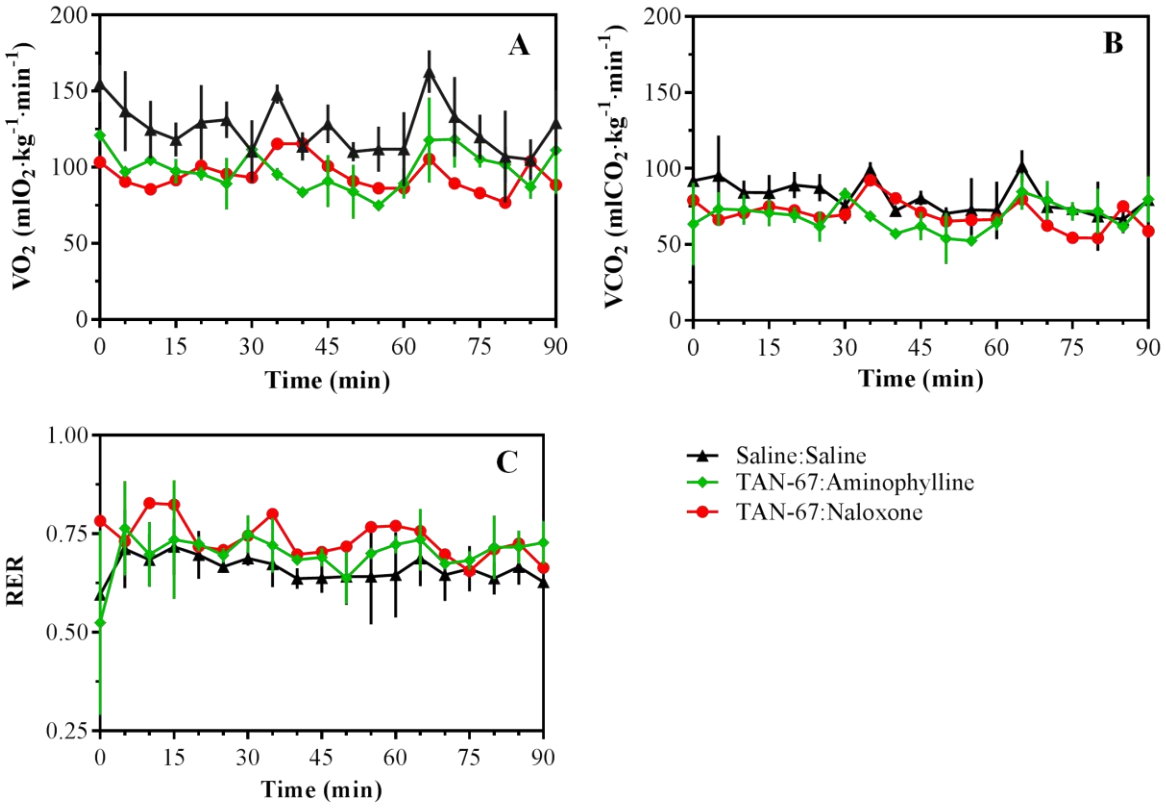


Figure 6. Trials utilizing TAN-67 as first treatment in male CD-1 mice. A) Calculations of VO_2. B) Calculations of VCO_2. C) RER in dimension less units. Panels A and B did not demonstrate a separation from the Saline:Saline data. These results were mirrored in panel C as no difference in RER was demonstrated.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

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