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Acidity Enhances the Ability of 5-Aminoimidazole-4-carboxamide Ribonucleotide to Increase Respiration and Lipid Metabolism in *Daphnia magna*

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ABSTRACT. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), a structural analog of adenosine monophosphate (AMP), promotes oxidative remodeling in muscle cells. AICAR activates AMP-dependent protein kinase (AMPK), thus increasing lipid metabolism, respiration, and mitochondrial counts. This process is called oxidative remodeling, which enhances the physical endurance of mice. To test this drug on an invertebrate that is genetically similar to humans, we used the small water crustacean *Daphnia magna*, which is sensitive to changes in water conditions. We tested the effects of pH on the efficacy of AICAR using two methods. One method measured oxygen consumption of *Daphnia* in oxygen chambers. The other method determined lipid levels of *Daphnia* through fluorescent tagging of lipids. The results showed that when exposed to AICAR at pH 6.58, *D. magna* consumed more oxygen and had lower overall levels of lipids, which is consistent with the expected effects of AICAR, such as increased respiration and lipid metabolism.

Key words: 5-Aminoimidazole-4-carboxamide ribonucleotide, *Daphnia magna*, pH, Lipid, Respiration

INTRODUCTION

Daphnia magna is a transparent planktonic crustaceans commonly referred to as the "water flea," which is often used in studies about the pollution of freshwater lakes that are sensitive to environmental factors.[1] Here we used these organisms to test the effects of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) as a function of pH. For this purpose, we used oxygen chambers to determine the rate of respiration and the fluorescent dye Nile Red to stain lipids. The oxygen chambers measure the change in voltage per second, which is inversely proportional to oxygen consumption and reflects cellular metabolism.

AICAR induces the breakdown of lipids in favor of glucose. Therefore, we can monitor lipid levels at different pH values by tagging them with a fluorescent dye. The effects of AICAR on *D. magna* are relevant to its future human use, because *D. magna* is the most genetically similar invertebrate to humans.[2] Thus, mechanisms within *D. magna* are similar to those of humans, and this evolutionary relationship can be applied to our findings for future human use of AICAR for treatment of type 2 diabetes.

AICAR is used to preserve blood flow during cardiac surgery and has recently caught the attention of scientists who envision its potential for increasing endurance during exercise and for possibly treating diabetes.[3] AICAR is a structural analog of AMP, which is a byproduct of the hydrolysis ATP that occurs when the cell uses energy, such as during exercise.[3] AMP then activates AMP-dependent protein kinase (AMPK), which favors lipid oxidation over the metabolism of sugars. Also known as an "exercise in a pill",[4] AICAR "tricks" the recipient into thinking that exercise is finished. AICAR does this by specifically targeting skeletal muscles. In response to exercise, organisms produce additional myoglobin that carries oxygen in the muscles for increased respiration, lipid metabolism, and mitochondrial counts, which is called oxidative remodeling in muscles. AICAR specifically "tricks" the organism by acting as a chemical mimic of AMP. Therefore, activating AMPK increases oxidative remodeling.

In an attempt to examine the effects of AICAR on endurance, a group of scientists led by Ronald Evans studied AICAR in mice, specifically the peroxisome proliferator-activated receptor delta (*PPARδ*) gene that regulates fatty acid metabolism. These investigators discovered that AMPK increases the transcription of *PPARδ*, which is involved in the regulation of oxidative myofibers (carry oxygen in muscles), therefore enhancing physical endurance.[5] This, in turn,

increases lipid metabolism and endurance such that the mice are dubbed "marathon mice." The reasons for increased endurance are unknown. A prominent theory is that increased production of mitochondria increases the "energetic capacity"[6] of muscles. Further, endurance capability is limited by consumption of carbohydrates such that the shift from carbohydrate to lipid metabolism induced by AICAR may potentially increase endurance.

To determine how pH affects AICAR absorption, we dissolved AICAR in acidic, neutral, and basic solutions (pH 6.58, 7.25, and 8.51, respectively). We determined if acidity improved the absorption of AICAR by *D. magna*, because changes in pH reflect natural changes in freshwater lakes and the influence on the absorption of drugs.[7]

We hypothesized that as the pH decreases, absorption of AICAR by *D. magna* will increase, therefore increasing the rate of cellular respiration and decreasing the levels of lipids. To test this hypothesis, we treated *D. magna* with dilutions of AICAR to determine the AICAR concentration at each pH consistent with viability, while allowing us to observe the effects on their internal mechanisms. The optimum concentration of AICAR was 32 μM, which was used to measure its effects on cellular respiration by determining oxygen consumption.

Oxygen consumption can be used to determine the rate of cellular respiration. We measured lipid levels using the fluorescent dye, Nile Red, which tags lipids.[8] Nile Red is a highly lipophilic lysochrome that is used to stain triglycerides, lipoproteins, and fatty acids. We therefore expected to detect higher overall levels of oxygen consumption in *D. magna* treated with AICAR, as well as lower lipid levels, because AICAR oxidizes lipids but not glucose. Moreover, we expected that at an acidic pH, AICAR would have more drastic effects because of increased absorption by *D. magna*. Thus, the pKa of AICAR is low, and AICAR is therefore more soluble at a lower pH and more efficiently asborbed into *D. magna*. We later determined that our results could not be attributed to this reasoning, although they indicated that variance from the optimal pH range (pH 7.2–8.5), at which *D. magna* naturally lives, increased the absorption of AICAR from the environment.

Testing AICAR on *D. magna* may provide valuable insights on its effects on humans. *D. magna* possess the same basic mechanisms as humans, and testing the effects of AICA on *D. magna* may better predict its effects on humans. AICAR induces ATP production and transcription of the *PPAR∂* gene in muscles, which forces the body to reap the benefits of virtual physical activity. Such an effect can maintain the health of people with disabilities that render them physically incapable of exercising. AICAR can be administered to patients with type-2 diabetes, which is often caused by poor diet or insufficient physical activities. Therefore, AICAR's ability to trigger the benefits of exercise can be incredibly useful.[9] For example, when AICAR is chronically administered to mice, the amounts of glycogen stores in skeletal muscles and the glucose transport protein GLUT4 increase, thus increasing maximum insulin-stimulated glucose transport and translocation of GLUT4. Therefore the activation of AMPK by AICAR may lead to improved insulin action.

EXPERIMENTAL

Viability Assay and Dose Curves

The serial dilutions of AICAR allowed us to eventually determine the concentration of required for optimal survival of *Daphnia*. AICAR was diluted two-fold in 0.5 μL (0.006%) of dimethyl sulfoxide (DMSO) at pH from 1 μM to 128 μM. To determine the optimum concentration of AICAR for treating *D. magna*, we administered various concentrations of the drug and conducted a viability assay by monitoring the heart rate of the *D. magna* before and after treatment. Depression slides were used to isolate one *D. magna* per dilution, and then 10 μl of each diluted drug was pipetted into the glass depression slides for 8 s. The heart rate was determined for 15 s. After determining that a manual clicker was not suitable for counting the rapid heart rate of *D. magna*, we used a marker to make a dot on a sheet of paper for each heartbeat. This process was repeated twice for three trials per each pH value, including a control with no drug for each trial.

Dose-response curves were generated according to the results of the viability assay, pH, and the average heart rate of *D. magna* at each pH. The standard deviation of each concentration at each pH was calculated and included in the graphs to show the average heart as a function of pH. The final curves were then generated according to the percentage change of heart rate at a resting state compared with that after AICAR administration. Resting heart rates were determined by counting the beats per minute (bpm) of *D. magna* in the absence of AICAR.

Cellular Respiration with Oxygen Chambers

We used oxygen chambers to measure oxygen consumption at 15 s, 2 min, 5 min, and 10 min to determine the effect of AICAR on cellular respiration. Using LabChart software connected to oxygen chambers, we determined the change in voltage, which is inversely proportional to oxygen consumption, at pH 6.58, pH 7.25, and pH 8.51 with and with-

out AICAR. Three trials were conducted at each pH, and the average slopes of oxygen consumption at each pH were compared to determine the optimal pH for AICAR absorption.

Lipid Staining

After exposing the organisms to the optimal concentration of AICAR (32 μM) at 15 s, 2 min, 5 min, and 10 min as well as a control without drug at pH 6.58, pH 7.25, and pH 8.51, we treated the *D. magna* samples with the lipophilic stain Nile Red (Alemán-Nava et al, 2016). This procedure measures the effect of AICAR on lipid levels at different pH. The Nile Red stock solution (25 μg/mL) in dimethyl sulfoxide (DMSO) was diluted to 1 μg/mL using moderately hard water (MHW) containing 96 mg/L $NaHCO_3$, 4 mg/L KCl, 60 mg/L $MgSO_4$, and 78.8 mg/L $CaSO_4 \cdot 0.5H_2O$.[8] After washing the organisms three times for 3–5 min in MHW, we transferred three *D. magna* to a Petri dish and removed excess MHW. We then added 100 μl of the appropriate drug dilution at the corresponding pH, except for the controls, to the Petri dish with *D. magna*. We waited for the corresponding exposure time and removed as much excess liquid as possible. We then immediately added 100 μl of the diluted Nile Red to the Petri dish and then covered it with an aluminum box to incubate it in the dark for 1 h. After incubation, *D. magna* was washed with fresh MHW for 3–5 min, placed on a slide, and then observed using a fluorescent microscope equipped with an NIB blue-light filter. The samples were then imaged using a filter for yellow fluorescence. If slides required preservation, the samples were stored in fresh MHW overnight.

RESULTS

Viability Assay and Dose Curves

AICAR was dissolved in DMSO to prepare a stock solution of 128 μM. with 7.4 ml of water and 0.5 μl of DMSO. DMSO is toxic and potentially fatal to *D. magna.* The percentage of DMSO in the stock solution was 0.007%, which most likely would have no toxic effect. Therefore, we did not control for the effects of DMSO.

To determine the optimal concentration at which AICAR is best absorbed and effective, we generated dose-response curves (*Fig.* 1) to show the effect of AICAR vs the percentage change in heart rate, which reduced and potentially eliminated the confounding effect of different resting heart rates. The three curves show an AICAR-dependent general increase in the percentage change relative to resting heart rate. The heart rates increased after exposure for 8 s.

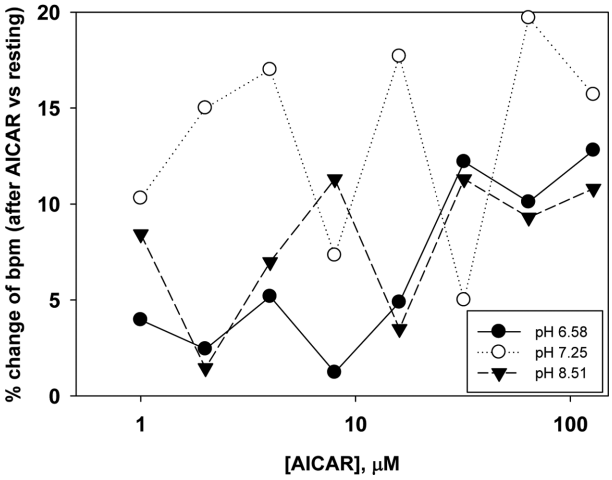

***Figure* 1.** Percentage change of bpm in *D. magna* increases as AICAR concentration increases at varying pH. The average resting heart rate of six *Daphnia* at each pH served as a standard to calculate the percentage change of bpm from the resting bpm. This final dose-response curve was used to determine the optimal sublethal concentration of AICAR. For all pH levels, increased AICAR concentrations generally increased the percentage change relative to the average resting bpm.

The curve for pH 6.58 was similar to a sigmoid (S)-curve, suggesting that AICAR was most active at this pH. Inconsistencies in the S-curve may be explained by the inaccuracies in our method for determining bpm as well as the physiological differences in the *D. magna* population (not all were in the same stage of the life cycle). We transferred *D. magna* that appeared the same size, which is not equivalent to the same stage of development. Moreover, *D. magna* are extremely sensitive to light and temperature, and the may experience stress if heated or exposed to intense light such as that delivered by the dissection microscope.[10] When observing the organisms with the microscope using intense light sources, we may have induced stress and possibly caused an increased heart rate. This would have led to inconsistent data, because the increase in heart rate may have been caused by a factor other than AICAR.

We used 32 μM AICAR for the remainder of the experiment to test respiration rates and lipid metabolism (*Fig.* 1). We found that 128 μM AICAR yielded the most significant effects, but we decided against using this concentration, because we hypothesized that >100 μM may have potentially or irreversible effects. We found that 32 μM had the next greatest effect (*Fig.* 1) (bpm: 12.20% at pH 6.58, 5.00% at pH 7.25, and 11.30% at pH 8.51). This concentration significantly affected heart rate, but was sublethal and did not induce irreversible effects. However, there was a significant difference in these findings between pH 7.25 and the other

pH values. The data acquired at pH of 7.25 were unpredictable and inconsistent (*Fig.* 1). Therefore, we excluded the data for 32 μM AICAR, used pH 7.25 pH as a control, and focused on data acquired at pH 8.51 and pH 6.58 (*Fig.* 1).

Lipid Metabolism

D. magna at basic pH emitted fluorescence throughout the body. The carapaces, appendages, and caudal spines fluoresced, which was not as intense at pH 6.58 or pH 8.51. As the pH varied, lipids concentrated around the esophagus, brain, digestive gland, midgut, and other parts of the gut. Further, as the pH deviated from the neutral optimal pH in the controls, fluorescence intensity was lower compared with that for the same exposure. At suboptimal pH, fluorescence was less intense compared with that at neutral pH, and fluorescence was more intense at pH 8.51 compared with pH 6.51. Increased exposure time at each pH led to a clear concentration of lipids around vital organs and an increase in lipid droplets. There were droplets of lipids inside the organisms (*Fig.* 2c and 2n). *D. magna* often stores lipids as droplets, which enlarge under chemical stress.[8] The droplets were not present in the controls and only in some of the AICAR-treated *D. magna*. We assumed that the formation of lipid droplets was caused by AICAR-induced stress. Further, some of the organisms were not stained and emitted faint greenish-yellow fluorescence vs the expected bright orange-yellow. This may be explained by the death of the organisms before completing AICAR treatment, most likely caused by injuries incurred during the transfer of the organisms between steps.

Oxygen Consumption and Cellular Respiration

The change in volts per second at each pH, which represents the rate of oxygen consumption, was significantly affected by initial AICAR administration before stabilizing after 15 s. Oxygen consumption can represent cellular respiration. At pH 6.58, the average rate of change in oxygen consumption of *D. magna* treated with AICAR during the first 15 s was -3.27×10^{-4} volts per second, which later stabilized between -6.77×10^{-5} and -7.66×10^{-5} volts per second (*Fig.* 3).

D. magna exhibited similar behavior at pH 8.51, with a rate of change of -3.00×10^{-4} during the first 15 s of AICAR exposure, before stabilizing to values between -1.07×10^{-4} and -8.25×10^{-5} volts per second (*Fig.* 5). At pH 7.25, the rate of oxygen consumption did not significantly change (initial rate, -1.04×10^{-4} during the first 15 s before stabilizing to -7.01×10^{-5} to 7.19×10^{-5} volts per second (*Fig.* 4). These data indicate that *D. magna* likely responded to stress, which enabled them to sense increased oxygen intake, which restored the normal rates of oxygen consumption and cellular metabolism. The stress response of *Daphnia* explains

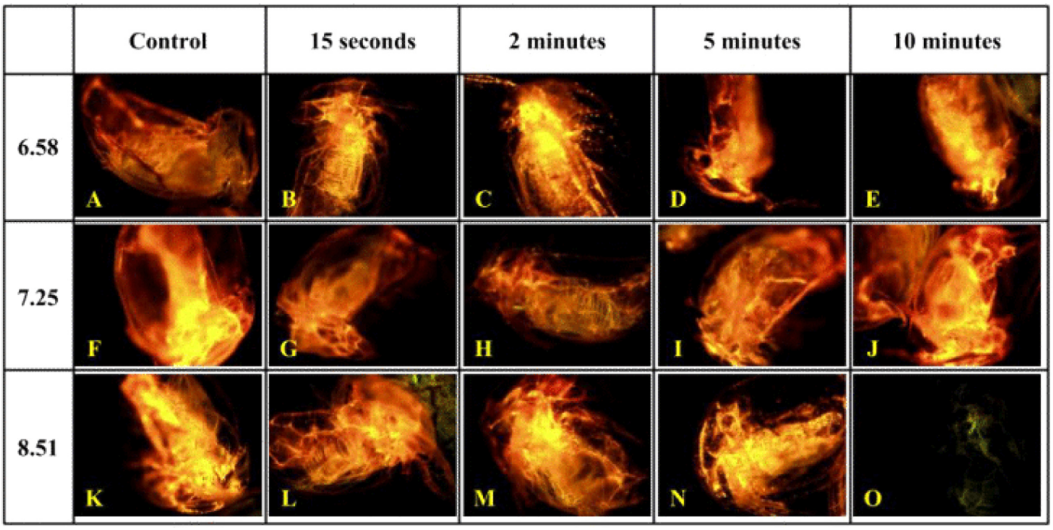

***Figure* 2.** Deposition of lipids increases outside of vital organs following exposure to AICAR at acidic pH. After at least 1 h of exposure to Nile Red dye following AICAR treatment, lipids within the *D. magna* were observed. Nile Red is lipophilic, thus the lipids within the organisms emit orange/yellow fluorescence. Panels A–E, pH 6.58; F–J, pH 7.25;, and K-O, pH 8.51. Panels A, F, and K show the controls. *D. magna* were observed at exposure times of 15 s, 2 min, 5 min, and 10 min. As the exposure time increased at each pH, fluorescence increased. Fluorescence intensities at pH 8.51 and pH 7.25 were significantly higher compared with that at pH 6.58 pH. At lower pH, the lipids were less widely distributed, instead accumulating around the brain, digestive gland, esophagus, midgut, and upper end of the gut. The fluorescence intensities were highest at pH 8.51. Lipid droplets that were observed in the controls and in only in some AICAR-treated *D. magna* that emitted, dim yellow-green fluorescence.

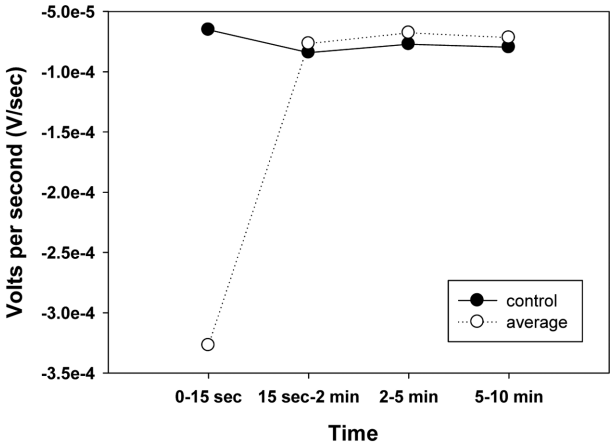

***Figure* 3.** Oxygen consumption immediately increases significantly following exposure to AICAR at pH 6.58. AICAR-treated Daphnia at pH 6.58 consumed high levels of oxygen upon immediate exposure. Untreated *Daphnia* consumed oxygen at a relatively constant rate. AICAR-treated *Daphnia* eventually returned to normal rates of oxygen consumption. The rate of oxygen consumption was higher at pH 6.58 compared with that at pH 8.51.

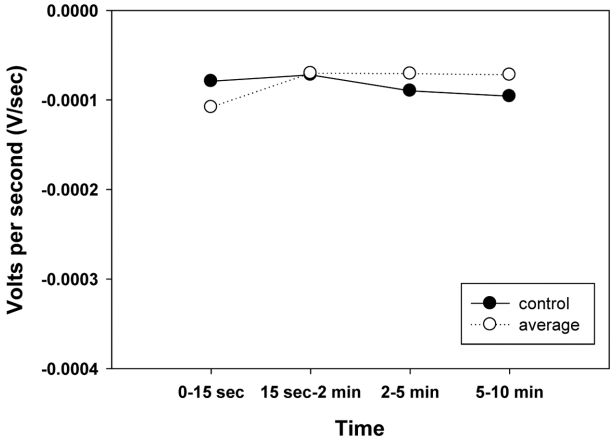

***Figure* 4.** Oxygen consumption immediately and slightly increases after exposure to AICAR at pH 7.25. AICAR-treated *Daphnia* at a pH 7.25 consumed oxygen at a constant rate compared with untreated *Daphnia* at the same pH. Although the rate of oxygen consumption was slightly greater during the first 15 s of exposure, there was little variance from normal oxygen consumption at that pH. Oxygen consumption by AICAR-treated *Daphnia* decreased further as exposure increased, similar to that of untreated Daphnia.

the change in the slope of the graph (*Fig.* 4). The slopes were similar after 15 s, indicating restoration of stable oxygen intake after acute exposure to AICAR.

These results can be attributed to suboptimal pH that does not maintain homeostasis. Thus, *D. magna* would incorporate more AICAR, increasing its effect on oxygen consumption and cellular respiration, This may not occur as

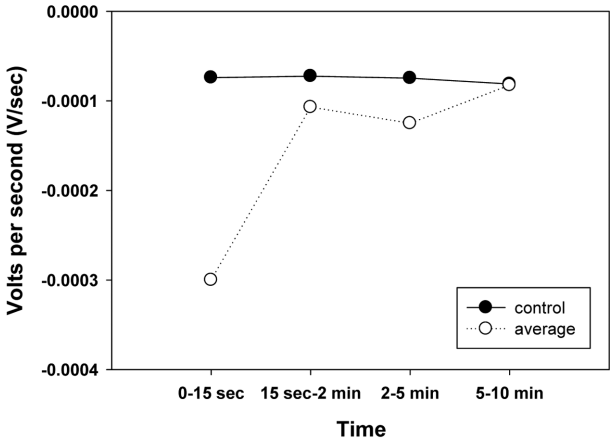

***Figure* 5.** Oxygen consumption significantly increases immediately after exposure to AICAR at pH 8.51. *D. magna* consumed high levels of oxygen immediately (15 s) after exposure to AICAR. In contrast, the control showed very little change in oxygen consumption. Oxygen consumption by AICAR-treated *Daphnia* returned to normal after prolonged exposure (>15 s).

drastically in *D. magna* at pH 7.25, because they maintain homeostasis, thus eliminating excess absorption of AICAR. However, oxygen consumption was greater at pH 6.58 than at pH 8.51. We preliminarily attributed this difference to the acidity of the AICAR treatment and assumed increased uptake occurred in an acidic solution.

However, this may not be accurate, because AICAR was effective a pH 8.51. Therefore, the difference in efficacies of AICAR on cellular respiration at pH 6.58 and pH 8.51 can be attributed to greater deviation from the optimal range at pH 6.58. Thus, AICAR was most effective at acidic or basic pH, because stress induced by altered pH significantly affected cellular respiration.

The initial results of the control trial in the oxygen consumption assay at pH 8.51 was inaccurate because of the significant fluctuation of oxygen consumption between -1.02×10^{-4} and -8.24×10^{-5} volts per second. Further, the initial results were inaccurate, because the control should not induce stress that affected the rate of oxygen consumption, as in the initial control trial. Therefore, conducted another trial and achieved more reproducible results. After repeating the trial for the control at pH 8.51 without AICAR, there was a clearer distinction between the control data and the average of three AICAR trials. Oxygen consumption without AICAR was more reproducible in this trial, as indicated by the slopes of the curves. Thus, when comparing the control value to the average slopes of the three AICAR trials, there was a significant increase in oxygen consumption during the first 15 s of drug administration.

DISCUSSION

AICAR is Best Absorbed in at Acidic pH

The data shown in *Fig*. 1 support our hypothesis that an acidic environment will optimize the capability of AICAR to increase cellular respiration and lipid metabolism. The curve for pH 6.58 curve was S-shaped, without significant fluctuations, suggesting that the drug was more effective at lower pH. This confirms our hypothesis that AICAR is absorbed most efficiently by *D. magna* in an acidic environment. The results of the oxygen consumption assays were consistent with this hypothesis, because there was far greater oxygen intake upon immediate exposure for 15 s at acidic pH compared with that of untreated organisms (*Fig*. 3).

To determine if AICAR or pH had the greatest effect on *D. magna*, we considered how lipid metabolism and heart rate were affected. After determining the lipid levels at each pH and exposure, we found that acidic pH increased the depletion of lipids, indicating the higher efficacy of AICAR at acid pH. Basic pH led to a breakdown of lipids to an extent that was greater compared with that of AICAR at the neutral optimal pH, and there were not drastic changes such as those found at acidic pH (*Fig*. 2). These findings agree with our original hypothesis that acidic pH increases the efficacy and absorption of AICAR by *D. magna.*

AICAR Generally Demonstrates Greater Efficacy at Non-neutral pH

Overall, we detected increased efficacies of AICAR treatment of *Daphnia* at acidic and basic pH compared with that at neutral pH (*Figs*. 3, 4, and 5). The increased oxygen consumption at an acid pH was greater compared with that at basic pH. However, basic pH increased the efficacy of AICAR, albeit not as drastically compared with acidic pH. This finding was unexpected.

After analyzing the data, we believe that any disruption of the homeostasis leads to greater absorption of substances in the environment. Although AICAR was better absorbed at acidic pH, this is because we used pH 8.51, which is closer to the natural optimal range for *Daphnia* (pH 7.2 to pH 8.5 vs pH 6.58). At basic and acidic pH, we detected lower fluorescence throughout the body and an accumulation of the remaining lipids around the brain, esophagus, midgut, digestive gland, and upper half of the gut. There was not a significant effect of pH 8.51 on lipid levels compared with that of 6.58 pH, which did increase the efficacy of AICAR.

Further Applications of AICAR Absorption

AICAR, although not tested in humans, has the potential to provide the benefits of exercise, increasing the endurance of professional athletes, and treating patients with diabetes. AICAR, which has been tested only on mice, increases respiration and promotes lipid metabolism. Here we show that AICAR had the same effects on *D. magna.* Humans and *D. magna* share similar genes and basic mechanisms. Therefore, humans may experience the same effects of AICAR as those shown here for *D. magna*, and AICAR may be used to treat invertebrates as well.

The development of AICAR, which is particularly useful for treating humans, specifically patients with diabetes. For example, type-2 diabetes often occurs because of lack of physical exercise, and AICAR may be utilized to confer the beneficial effects of exercise. Moreover, AICAR increases the activity of insulin. Enhancing the activity of insulin may cure diabetes, because the primary cause of type-2 diabetes is the body's inability to properly utilize insulin for glucose metabolism. A study of AICAR's influence on insulin utilization in rats indicates its potential for treating diabetes.[9]

We conclude that the results of our study provide insights into how to optimize the efficacy of AICAR, with the goal of treating humans. We discovered that AICAR worked most effectively at acid pH. These results may help design a strategy for administering AICAR to diverse species, including humans.

CONCLUSION

In this work, we report the effect of pH on 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) efficacy using small water crustacean *Daphnia magna*. We measured the amount of oxygen consumption and lipid levels of the Daphnia through fluorescent tagging of lipids. Our results showed that under the influence of AICAR at pH 6.58, *D. magna* consumed more oxygen and overall had lower levels of lipids in their bodies. This indicates that AICAR works most effectively in acidic environments.

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