The relationship between 5-aminoimidazole-4-carboxamide-ribotide and AMP activated protein kinase activity in the perfused mouse heart

Li Zhang§, Markus Frederich§, Huamei He and James A. Balschi

NMR Laboratory for Physiological Chemistry, Division of Cardiovascular Medicine, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School
Boston, MA 02115 USA

Running Title: ZMP activation of AMPK in the heart

Address correspondence to: James A. Balschi, Ph.D., 221 Longwood Ave., BLI 247, Boston, MA 02115 USA Phone (617) 732-6996 Fax (617) 732-6990
Email: jbalschi@rics.bwh.harvard.edu

§Both authors contributed equally to this work.
Abstract

AMP-activated protein kinase (AMPK) is a cellular energy sensor whose activity responds to AMP concentration ([AMP]). An agent that activates AMPK in cells is 5-aminoimidazole-4-carboxamide-1-riboside (AICAruboside). Phosphorylated AICAruboside or AICArubotide (ZMP) is an AMP analog. It is generally assumed that ZMP accumulation does not alter [AMP]. Additionally, the effect of AICAruboside on AMPK activity of the heart is uncertain. Two hypotheses were tested in the isolated mouse heart: one, sufficient [ZMP] forms to increase AMPK activity; and, two, [ZMP] accumulation increases [AMP]. Perfusion of isolated mouse hearts with Krebs-Henseleit buffer containing 0.15 to 2 mM [AICAruboside] resulted in [ZMP] of 2 to 8 mM. ZMP accumulation reduced [phosphocreatine], which increased cytosolic [AMP]. In hearts with [ZMP] less than ~3 mM, in vivo AMPK allosteric activity effects of ZMP were observed; AMPK phosphorylation and [AMP] were not increased. With [ZMP] between 3 to 5 mM in vitro AMPK activity and phosphorylation increased with unchanged [AMP]. This occurred in hearts perfused with [AICAruboside] of 0.25 mM for 48 min and 0.5 mM for 24 min. The [ZMP] resulting in 50% AMPK activity (covalent phosphorylation of AMPK) was 4.1 ± 0.6 mM. Hearts with [ZMP] greater than 5 mM displayed increased [AMP] and AMPK activity that was not different from hearts with similar [AMP] with no [ZMP]; the A0.5 of AMP was 5.6 ± 1.6 µM. Thus, in mouse hearts AICAruboside was metabolized to [ZMP] adequately to increase AMPK activity. Higher [ZMP] also increased cytosolic [AMP], which affects AMPK activity.

Keywords: Signal transduction, Acetyl-CoA carboxylase, AICAruboside, (5’-aminoimidazole-4-carboxamide-1-b-D-riboside), AICAR
ZMP activation of AMPK in the heart

Introduction

AMP-activated protein kinase (AMPK) and AMPK kinase (AMPKK) comprise a protein kinase cascade that has been highly conserved throughout evolution (10, 11). The AMPK cascade acts as a cellular low fuel warning system (10). Increases in AMP concentration ([AMP]) increase AMPK activity (7, 8). Once activated AMPK functions to either conserve ATP or promote alternative methods of ATP generation. One way that AMPK stimulates ATP synthesis in the heart is by the phosphorylation of acetyl-CoA carboxylase (ACC). The phosphorylation inhibits ACC activity, which lowers malonyl-CoA formation. Lower malonyl-CoA, in turn, relieves the inhibition of carnitine palmitoyl transferase 1 (CPT1), the primary transporter and control point for fatty acyl-CoA entry into the mitochondrion. This results in an acceleration of the β-oxidation of fatty acids and the generation of ATP.

Increased AMPK activity has been elicited by use of ischemia, hypoxia or metabolic inhibitors to alter cellular energetics and, hence, increase [AMP]. A pharmacological agent, 5-aminoimidazole-4-carboxamide-riboside (AICAriboside; Z-riboside), has been used to increase AMPK activity in a wide range of tissues and cell types including adipocytes (4) and skeletal muscle (17, 22). AICAriboside does not directly activate AMPK. AICAriboside must be phosphorylated to the active agent 5-aminoimidazole-4-carboxamide-ribotide (AICAribotide; ZMP). ZMP accumulation in INS cells did not alter total AMP and ATP content but did increase AMPK activity (12). Total AMP content was also unchanged in gastrocnemius muscles with ZMP formation accompanied by increased AMPK activity (22). ZMP accumulation, however, decreased PCr in rat white quadriceps muscle (30). This raises the possibility that AICAriboside
metabolism can reduce [PCr]. We have demonstrated that reductions in [PCr] increased cytosolic [AMP] in the heart (7, 8).

ZMP, a structural analog of AMP, increases AMPK activity in vitro. The [ZMP] that affect AMPK activity are 50 times higher than the [AMP] required for increased AMPK activity. In vitro measurements of the concentrations required for half-maximal (A0.5) allosteric activity of AMPK have found the following: first, in the presence of 4 mM ATP the A0.5 for AMP was 29 ± 14 µM while the A0.5 for ZMP was 1.5 ± 0.6 mM (4); and, second, the A0.5 for ZMP was ~ 5 mM in the presence of 3 mM ATP (16). Two measurements of the A0.5 for ZMP for phosphorylation of AMPK in vivo have been reported. In hepatocytes the A0.5 of ZMP for phosphorylation of AMPK was approximately 2 mM (4). A ZMP/ATP of about 0.2 was required for half-maximal activation of AMPK in INS-1 cells (12).

Reports of the effects of AICAraboside treatment upon the AMPK activity of the heart have been contradictory. Russell et al reported that an in vivo arterial [AICAraboside] of 0.9 mM increased myocardial AMPK activity (23). But Longnus et al reported that perfusion of isolated rat hearts with 0.8 and 1.2 mM [AICAraboside] did not increase AMPK activity or phosphorylation (20). We have confirmed that AICAraboside does not increase AMPK activity in isolated rat hearts (M. Frederich and J. Balschi, unpublished observations). Longnus reported, however, that the phosphorylation of Ser 79 of acetyl-CoA carboxylase (ACC), an AMPK target, did increase with AICAraboside perfusion. Since AMPK phosphorylation was not increased these results are consistent with an allosteric increase in AMPK activity in vivo.
The present study tests two hypotheses in the isolated mouse heart: one, AICAr iboside metabolism forms sufficient [ZMP] to increase AMPK activity and phosphorylation; and two, ZMP accumulation alters cellular energetics and increases cytosolic [AMP]. This study defines the relationship among [ZMP], [AMP] and AMPK activity in isolated mouse hearts perfused with a range of [AICAr iboside]. Phosphocreatine (PCr), ATP and ZMP were measured in vivo using $^{31}$P NMR spectroscopy. HPLC also measured Z nucleotide content. AMPK activity and phosphorylation of the hearts were measured in vitro. Cytosolic [AMP] was determined in vivo using [PCr], [ATP] and intracellular pH measurements (7).
Materials and Methods

Preparation of Isolated Perfused Hearts- Hearts of male C57BL6 mice (24-26 g) were isolated and perfused in the isovolumic Langendorff model. The Krebs-Henseleit buffer (KH) perfusate contained 118 mM NaCl, 5.3 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, 0.5 mM Na⁺ EDTA, 10 mM D-glucose, 0.5 mM pyruvate and was equilibrated with 95% O₂ and 5% CO₂, with a resultant pH of 7.4. Specific metabolic inhibitors and AICArriboside were added to this perfusate as noted. A pressure transducer connected to the left ventricle balloon measured the left ventricle pressure and heart rate (HR). Hearts were electrically paced using a Grass stimulator (Grass, Quincy, MA). LV pressures were recorded with a PowerLab system (ADInstruments, Colorado Springs, CO 80906). The animal protocol was approved by the Harvard Medical Area Standing Committee on Animals and followed the recommendations of NIH and the American Physiological Society guidelines for the use and care of laboratory animals.

Metabolic Inhibitors- Addition of 0.3 mM 2-bromo-octanoic acid (BrO) (Aldrich, Milwaukee, WI) to the KH perfusate irreversibly inhibits the β-oxidation of long chain fatty acids, which arise from endogenous triacylglycerol (26). Perfusion with 0.4 mM amino-oxyacetate (AOA) (SIGMA, St. Louis, MO), a reversible inhibitor of the malate-aspartate shuttle (25), results in near complete inhibition of glucose oxidation (1).

Study Design and Experimental Protocols to vary [AMP] and [ZMP]- Figure 1 diagrams the perfusion protocols used to vary [AMP] (panel A) and [ZMP] (panel B) in the mouse heart. Details and the number of hearts in the perfusion groups are given in the figure legend.
ZMP activation of AMPK in the heart

$^{31}$P NMR Spectroscopy of Isolated Perfused Hearts- $^{31}$P NMR free induction decays were acquired at 161.8 MHz using a Varian Inova spectrometer (Varian Inc., Palo Alto, CA). Typically, 208 FIDs were averaged over 8 min using $60^\circ$ pulses and a recycle time of 2.4 sec. The frequency of the inorganic phosphate (Pi) resonance was measured using Varian software (VNMR 6.1C). The resonance areas of phosphocreatine (PCr), ATP, ZMP were quantified using Bayesian Analysis software (G.L. Bretthorst, Washington University, St. Louis, MO). Bayesian Analysis software uses a direct statistical analysis of the free induction decay amplitudes, which corresponds to the resonance area (3). Saturation factors for resonances were determined from fully relaxed spectra, recycle time 15 sec.

Heart $^{31}$P Metabolite Content and Calculation of AMP- The concentrations of the $^{31}$P NMR-visible metabolites of hearts were derived using the $\gamma$-resonance of ATP as an internal standard. The area of the $\gamma$-resonance of ATP at the beginning of each protocol was set to 10.0 mM. The areas of other resonances, corrected for variable saturation, were then calculated on this basis. Intracellular pH ($\text{pH}_i$) was calculated from the frequency of intracellular Pi relative to the frequency of PCr. The creatine kinase equilibrium expression and $^{31}$P NMR measurements of [PCr], [ATP], and [$H^+$] were used to calculate cytosolic [ADP] (19). Cytosolic [AMP] was calculated using the adenylate kinase equilibrium expression (7, 19). ZTP and ATP peaks overlap in the spectrum. To correct for loss of ATP and the gain in ZTP, the final NMR measured [ATP] of hearts perfused for 48 min with AICAruboside were corrected as follows. The initial [ATP] of each heart in the [AICAruboside] groups was scaled by multiplication with the following factors: 0.25 mM (0.96); 0.5 mM (0.92), 1.0 mM (0.83), 1.5 mM (0.71), and 2.0 mM (0.69). These factors were derived from a line fit to the HPLC measurements of ATP content of hearts.
ZMP activation of AMPK in the heart

perfused with AICArabinoside (Table 1). The corrected value was then used as the final [ATP] measurement.

**HPLC Analysis**- Adenine nucleotides (ATP, ADP, AMP) and Z nucleotides (ZMP, ZDP, and ZTP) were measured in the perchloric acid extracts of freeze clamped hearts using methods described by Sabina et al (24). The results are expressed as nmol × mg protein⁻¹. Protein was determined using the method of Lowry et al (21).

**Measurement of AMP-Activated Protein Kinase Activity**- Total AMPK activity was measured using the method of Dagher et al (5) modified by Frederich et al (8). No AMP was added to the assay. AMPK activity was quantified as the incorporation of ³²P from [γ-³²P] ATP (10 GBq/ mmol, NEN, Boston, MA) into a synthetic peptide with the specific target sequence for AMPK, the SAMS-peptide, amino acid sequence HMRSAMSGLHLVKRR, (American Peptides, Sunnyvale, CA, USA). Radioactivity was measured using a liquid scintillation counter (Tri-Carb 2100TR, Packard Biosciences, Meriden, CT). Protein content in the solution containing the resuspended (NH₄)₂SO₄ pellet was determined using the Bradford method (2).

The isoform specific activity of AMPK in heart tissue was measured (8) after immunoprecipitation of the α₂ subunits by anti-AMPK-α₂ antibodies coupled to protein A agarose beads (Upstate Biotechnology, Lake Placid, NY), activity was measured as the incorporation of ³²P into the SAMS peptide. Protein content was determined in the homogenization buffer and was adjusted to a content of 0.5 mg/ml prior to immunoprecipitation.
Measurement of the phosphorylation of α Thr\textsuperscript{172} of AMPK and Ser79 of ACC-
Phosphorylation was measured using western immunoblots as described by Frederich et al (8).
The primary antibodies were anti-phospho-AMPK-α polyclonal antibody (Thr\textsuperscript{172}; Cell Signaling
Technology, Beverly, MA, USA) and anti-phospho-Acetyl-CoA Carboxylase polyclonal antibody (S-79; Upstate, Lake Placid, NY, USA). Signal detection was facilitated with enhanced chemiluminescence (ECL Kit, Amersham, Piscataway, NJ, USA). The membrane signals were quantitated using an image scanning densitometer (Bio-Rad Model GS-700 Imaging Densitometer and Bio-Rad Quantity One 4.2.1).

Statistical Analysis- The data are presented as the mean ± one standard deviation (SD) unless otherwise indicated. Statistical computations were performed with Statistica (Version 6.1, StatSoft Inc, Tulsa, OK 74104). An analysis of variance (ANOVA) was used to compare measurements among all groups. A post-hoc Fishers Protected Least Significant Difference was used for comparison of the means. Differences were declared statistically significant if p<0.05. GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego California USA was used for graphs and fitting of the measurements of AMPK activity, [AMP] and [ZMP] to the appropriate equation.
Results

Functional consequences of AICAruboside perfusion in the mouse hearts- AICAruboside has an inotropic effect on the isolated mouse heart. Left ventricular pressures were as follows: one, during KH perfusion end diastolic = 9 ± 2 mmHg and systolic = 90 ± 8 mmHg; two, after 20 min of AICAruboside end diastolic 12 ± 2 mmHg (p < 0.05 vs. KH perfusion) and systolic 108 ± 18 mmHg (p < 0.05); and, three, after 40 min AICAruboside end diastolic 14 ± 3 mmHg (p < 0.05) and systolic 121 ± 22 mmHg (p < 0.05). During KH perfusion coronary flow was 1.7 ± 0.2 ml min⁻¹; it increased to 2.0 ± 0.2 ml min⁻¹ (p < 0.05) after 20 min of AICAruboside and 2.4 ± 0.3 ml min⁻¹ at 40 min (p < 0.05).

HPLC measured nucleotide content of the heart following perfusion with AICAruboside for 48 min- The adenine nucleotide (ATP, ADP, and AMP) and Z nucleotide (ZMP, ZDP, and ZTP) contents were measured by HPLC (Table 1). Substantial ZTP was formed. Perfusion with [AICAruboside] of 1.5 and 2.0 mM for 48 min decreased ATP content. Because the phosphorus nuclei of ZTP resonate at the same frequency as those of ATP, NMR was not able to detect this. Thus the NMR peak area at the ATP frequency increased during the protocol, reporting the sum of ATP and ZTP. Final NMR determined [ATP] areas were corrected to reflect the HPLC measured decline (see methods).

³¹P NMR measured metabolite content of the heart during perfusion with AICAruboside- Perfusion of the isolated mouse heart with KH containing AICAruboside resulted in the appearance of a new peak at 6.2 ppm in the ³¹P NMR spectrum, which was assigned to ZMP (Figure 2A). To confirm the identity of this resonance as ZMP, extracts were combined from three
hearts after perfusion with 1 mM AICArriboside KH. The $^{31}$P NMR spectrum of the extract exhibited a peak at 6.4 ppm. Addition of authentic ZMP to the extract increased the peak at 6.4 ppm (Figure 2B). The slight difference in resonance frequencies of the ZMP peaks in the heart and in the heart extract probably resulted from a difference in pH.

Hearts perfused with KH containing a range of AICArriboside from 0.15 mM to 2.0 mM exhibit increasing rate of [ZMP] growth (Figure 3, panel A). The maximum [ZMP] resulted from 48 min perfusion with KH containing 0.5 to 2.0 mM AICArriboside.

The [ZMP] growth during AICArriboside was accompanied by reduction of [PCr] (Figure 3, panel B). Removal of AICArriboside from KH perfusion medium results in a reduction of [ZMP] and a restoration of [PCr]. In separate experiments 48 min perfusion with AICArriboside KH was followed by AICArriboside KH with 50 µM 5-iodotubercidin, an inhibitor of adenosine kinase (15). This caused [ZMP] to decrease (Figure 3, panel C). This indicates that AICArriboside is continuously phosphorylated to form ZMP by adenosine kinase while ZMP is continuously dephosphorylated, probably by cytosolic 5’nucleotidase, to form AICArriboside (28).

AICArriboside is phosphorylated by adenosine kinase in a reaction that requires ATP. As a result ZMP sequesters phosphoryl groups. Since PCr functions, in part, as a reservoir of phosphoryl groups that maintains [ATP] via the creatine kinase reaction, the sequestration of phosphoryl groups by ZMP decreases PCr. Because of the near equilibrium of the creatine kinase reaction, reduction in [PCr] increases cytosolic [ADP]. In turn, the near equilibrium of
the adenylate kinase reaction converts the increased cytosolic [ADP] into an increase in cytosolic [AMP]. Sufficient accumulation of [ZMP] resulted in increased cytosolic [AMP] (Table 2A). Note that HPLC measured total AMP (and ADP) content did not change (Table 1). Most AMP and ADP are bound to macromolecules in the myocyte and are not detected by NMR experiments. If the total AMP content in the well oxygenated heart were cytosolic, the [AMP] would be ~300 µM not the ~1 µM cytosolic [AMP] that we calculate. $^{31}$P NMR spectroscopy, in combination with the creatine kinase and adenylate kinase equilibrium expressions, provides the best estimates for the metabolically active cytosolic [AMP] and [ADP]. We believe that AMPK activity responds to the cytosolic [AMP] not total AMP.

A primary goal of this study was to characterize the effect of [ZMP] on AMPK activity in the heart. Since [ZMP] accumulation in the heart increases [AMP], control hearts were treated with metabolic inhibitors to increase [AMP] without [ZMP] (Table 2B).

**AMP-Activated Protein Kinase Activity: Relationship between total AMPK activity and cytosolic [AMP] (Figure 4A and B)-** Binding of AMP to the $\gamma$ subunit of AMPK increases AMPKK catalyzed phosphorylation of AMPK $\alpha$ Thr$^{172}$. Phosphorylation of $\alpha$ Thr$^{172}$ increases AMPK activity. Rapid freezing preserves the physiological phosphorylation status of AMPK. The *in vitro* activity measurements, therefore, reflect the *in vivo* phosphorylation state or the degree of covalent modification of AMPK. The dependence of the *in vitro* total AMPK activity on the *in vivo* cytosolic [AMP] was determined by fitting the individual measurements from hearts to the equation,
ZMP activation of AMPK in the heart

1) \[ v = \frac{V_{\text{max}} \times [\text{AMP}]}{A_{0.5} + [\text{AMP}]} \]

where \( v = \) AMPK activity, \( A_{0.5} = [\text{AMP}] \) at 50% AMPK activation, \( V_{\text{max}} = \) maximal activity.

Fitting the measurements of all hearts, with all [AICAraboside] and without AICAraboside, separately to equation 1 (Figure 4A) yields the following values (best fit ± standard error): first, for the hearts perfused without AICAraboside, \( A_{0.5} = 5.5 \pm 1.6 \) µM, \( V_{\text{max}} = 9.6 \pm 1.0 \text{ pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1} \); and, second, for the hearts perfused with AICAraboside \( A_{0.5} = 1.9 \pm 0.7 \) µM, \( V_{\text{max}} = 7.5 \pm 0.8 \text{ pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1} \). The \( A_{0.5} \) for the two hearts groups were different (\( p=0.025 \)); the \( V_{\text{max}} \) were not different (\( p = 0.11 \)). The reduction in apparent \( A_{0.5} \) for AMP of the AICAraboside hearts signals that the presence of cytosolic ZMP is increasing AMPK activity (Figure 4B). The lower apparent \( A_{0.5} \) does not reflect a change in the sensitivity of AMPK to AMP binding. The effects of [ZMP] are most prominent below 5.5 µM (the \( A_{0.5} \) for AMP in the absence of ZMP).

The measurements of AMPK activity from all hearts that contained cytosolic [AMP] < 3 µM were merged into two new groups, with and without AICAraboside. The AICAraboside hearts, which had [ZMP] = 3.3 ± 0.9 mM, displayed an AMPK activity 50% higher (\( p = 0.004; 3.3 \pm 1.3 \text{ pmol min}^{-1} \text{ mg protein}^{-1} \)) than hearts (2.0 ± 1.2 pmol min\(^{-1}\) mg protein\(^{-1}\)) without ZMP. Thus, ZMP of ~3.5 mM increased AMPK activity without increasing [AMP].

The increase of [ZMP] correlated with increased cytosolic [AMP] (Figure 4C). The relationship among AMPK activity, [ZMP] and [AMP] was complex. Inspection of the group
data from hearts perfused with low [AICAraboside] (0.15 mM and 0.25 mM for up to 32 min), which had ZMP < 3 mM and [AMP] < 3 μM, shows that the AMPK activity was unchanged (Table 3). Whereas, hearts perfused with [AICAraboside] (0.25 mM for 48 min and 0.5 mM for 20 min), which had [ZMP] < 5 mM and [AMP] < 4 μM, had increased AMPK activity. In hearts with [ZMP] greater than ~5 mM, [AMP] increased (approaching the A0.5 for AMP) and the increase in AMPK activity can not be ascribed to increased [ZMP] alone. For example, the AMPK activity of the GB hearts was equal to that of the 2.0 mM AICAraboside hearts, which had equal [AMP] (Table 3).

There was a 6 fold increase in α2 isoform specific AMPK activity for the hearts perfused with 1 mM AICAraboside (Table 3). This was consistent with the five fold increase in total activity measured for the 1 mM AICAraboside hearts.

**Relationship between total AMPK activity and cytosolic [ZMP] (Figure 4D)-** The dependence of the *in vitro* total AMPK activity on the *in vivo* cytosolic [ZMP] was determined by fitting the individual measurements from all hearts to the Hill equation,

\[
v = V_{basal} + V_{max} \frac{[ZMP]^h}{(A_{0.5})^h + [ZMP]^h}
\]

where v = AMPK activity, A0.5 = [ZMP] at 50% AMPK activation, Vbasal = basal activity, Vmax = maximal activity, and h = Hill coefficient.

Fitting the data from Glc group hearts ([ZMP] = 0) and all AICAraboside perfused hearts to equation 2 (Figure 4D) yields the following (best fit ± standard error): A0.5 = 4.1 ± 0.6 mM,
ZMP activation of AMPK in the heart

\[ V_{\text{max}} = 5.2 \pm 1.0 \text{ pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}; \ V_{\text{basal}} = 1.65 \pm 0.44 \text{ pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1} \] and \( h = 4 \pm 1.9 \). The [AMP] varied in these hearts especially when [ZMP] > 5 mM (see Fig. 4C).

Note that the ZMP and AMPK activity can best be fit with a sigmoid equation. Modeling of the AMPK cascade predicted that the AMP to AMPK activity relationship should also be described by a sigmoid equation (12). To date our measurements of the \textit{in vivo} [AMP] and AMPK activity relationship (Fig, 4A) have been best fit with a hyperbola (8).

Relative magnitude of AMPK activity resulting from [ZMP] (Figure 5)- In the oxygenated mouse heart the AMPK activity resulting from either increased [ZMP] and/or [AMP] is less than 35% of the AMPK activity resulting from 8 min of no flow ischemia (Figure 5). This difference in the AMPK activity in the oxygenated and hypoxic or ischemic heart is consistent with our recent observation in the hypoxic rat heart of a two-fold increase in AMPK activity that is independent of the effect of increased [AMP] (8). The AMPK activity from the 1 mM [AICAraboside] group was the highest of the groups with [ZMP]. These hearts had elevated [AMP], which undoubtedly contributes to the AMPK activity.

The increase in AMPK activity and phosphorylation reflects covalent modification of AMPK resulting from ZMP binding \textit{in vivo}. ZMP may also allosterically increase \textit{in vivo} AMPK activity, which will not be revealed in the \textit{in vitro} activity or in the \textit{in vivo} αThr\textsuperscript{172} phosphorylation. To emulate the \textit{in vivo} allosteric effects of ZMP in the \textit{in vitro} activity assay, 5 mM ZMP was added to the \textit{in vitro} AMPK activity assay for the 8 min ischemic hearts. The added [ZMP] increased the
in vitro activity two fold (Figure 5). It is quite possible that the in vivo AMPK activity in hearts with ZMP is up to two times greater than the in vitro activity.

Phosphorylation of AMPK α Thr$^{172}$ and ACC Ser$^{79}$ in mouse hearts with and without [ZMP] (Figure 6) - The phosphorylation state of Thr$^{172}$ on the α subunit of AMPK was determined by western blot analysis of Glc hearts and of hearts perfused with [AICArriboside] of 0.5 mM and below (Figure 6A). The AMPK α Thr$^{172}$ phosphorylation for hearts perfused with 0.5 mM and 0.25 mM [AICArriboside] for 48 min was greater than that of Glc hearts and 0.15 and 0.25 mM (32 min) hearts (Figure 6B). The phosphorylation is consistent with the activity (Table 3). AMPK phosphorylates Ser$^{79}$ on ACC to regulate its activity. The phosphorylation state of ACC Ser$^{79}$ was also determined (Figure 6C). ACC phosphorylation increased in the hearts with ZMP. The relative phosphorylation of Ser$^{79}$ was nearly complete in the hearts exposed to 0.5 mM AICArriboside for 48 min (Figure 6D). The magnitude of the increase in ACC phosphorylation of the 0.15 mM and the 32 min 0.25 mM [AICArriboside] hearts is relatively greater than the AMPK phosphorylation of these hearts (Figure 6B). This disproportionate increase in the phosphorylation of ACC indicates that ZMP allosterically increased AMPK activity in vivo. These hearts had cytosolic [ZMP] of ~3 mM (Table 3).
ZMP activation of AMPK in the heart

Discussion

The AMPK protein kinase cascade responds to [AMP] increases (10, 11). Increases in [AMP] activate the cascade by four mechanisms (4, 6, 14). First, AMP allosterically activates AMPKK (12). This mechanism is somewhat controversial. LKB1 has been identified as an AMPKK, however, it does not respond to AMP (13). Second, AMP binds to AMPK, which promotes phosphorylation of Thr$^{172}$ of the AMPK $\alpha$ subunit by AMPKK. This phosphorylation correlates with AMPK activity. Third, AMP binds to AMPK, which makes it a poorer substrate for protein phosphatases. Fourth, AMP allosterically activates phosphorylated AMPK. The activating effects of AMP are antagonized by high concentrations of ATP (10). This study examines the effect of cytosolic [ZMP] on mechanisms two and four; and cytosolic [AMP] on mechanism two. This study can not rule out an effect of ZMP on the upstream AMPKK (mechanism 1). If mechanism 1 is operative, however, its [ZMP] dependence will not occur at lower [ZMP] than the effect that we have assigned to mechanism 2.

ZMP or AICArribotide is a structural analog of AMP. ZMP has been used in numerous studies to pharmacologically activate AMPK. The present study tested two hypotheses using the isolated perfused mouse heart: first, that AICArriboside metabolism forms sufficient [ZMP] to increase AMPK activity and phosphorylation; and second, that ZMP accumulation alters cellular energetics and increases cytosolic [AMP]. This study defined the relationship among AMPK activity measured in vitro, cytosolic [ZMP] and cytosolic [AMP] measured in vivo of isolated mouse hearts.
In the mouse heart we found a substantial increase in [ZMP] that was time and [AICArabinoside] dependent. The $A_{0.5}$ of ZMP for the phosphorylation of AMPK was $4.1 \pm 0.6$ mM, whereas, the $A_{0.5}$ for AMP was $5.5 \pm 1.6$ $\mu$M. Cytosolic ZMP binding to AMPK was, therefore, 800 fold less effective than AMP in promoting AMPK phosphorylation by AMPK\(\alpha\). These results explain why AICArabinoside does not increase AMPK activity in the isolated rat heart. The rat heart ZMP contents reported were equivalent to a [ZMP] of 0.2 to 0.4 mM (20). This [ZMP] is too low relative to the $A_{0.5}$ for ZMP to promote AMPK phosphorylation. The $A_{0.5}$ of ZMP for phosphorylation of AMPK has been reported in two other cell types. In hepatocytes the $A_{0.5}$ of ZMP for phosphorylation of AMPK was $\sim$2 mM (4). In addition a ZMP/ATP of about 0.2 was required for half-maximal activation of AMPK in INS-1 cells (12). In the mouse heart the ZMP/ATP ratio for half maximal AMPK activity was $0.43 \pm 0.08$; the ZMP/ATP+ZTP ratio for half maximal AMPK activity was $0.36 \pm 0.05$ (not shown). Thus, it appears that the $A_{0.5}$ of ZMP for AMPK phosphorylation (covalent modification) in the heart is about twice that reported in hepatocytes and INS-1 cells.

Since the state of in vivo AMPK allosteric activation of AMPK is not preserved during protein isolation it can only be inferred from AMPK actions on its target proteins. Hearts with [ZMP] of $\sim$3 mM exhibited increased ACC phosphorylation relative to their AMPK phosphorylation. This is consistent with a ZMP $A_{0.5}$ for allosteric activation of 3 mM or lower. In the rat heart [ZMP] of 0.2 to 0.4 mM increased phosphorylation of ACC (20). This implies ZMP $A_{0.5}$ of $\sim$0.3 mM or lower. In vitro determinations of the allosteric activity using purified AMPK have yielded a range of values. Henin et al reported an $A_{0.5}$ for ZMP of $\sim$ 5 mM in the presence of 3 mM ATP (16); Corton et al reported an $A_{0.5}$ for ZMP of $1.5 \pm 0.6$ mM at 4 mM.
ZMP activation of AMPK in the heart

ATP with an $A_{0.5}$ for AMP of $29 \pm 14 \, \mu$M (4). Thus, the in vivo results from the mouse heart with [ATP] of ~10 mM are generally consistent with reported in vitro measurements of the ZMP $A_{0.5}$ for allosteric activation.

In the mouse heart as in some other tissues AICAraboside metabolism resulted in ZMP accumulation to millimolar concentrations (4, 16, 24, 29). The reduction in [ZMP] with removal of AICAraboside or by addition of iodotubercidin, an inhibitor of adenosine kinase, indicates an active futile cycle of AICAraboside phosphorylation and ZMP dephosphorylation (28). The dephosphorylation of ZMP to AICAraboside in the heart is most likely catalyzed by cytosolic 5’nucleotidase activity. The lack of appreciable formation of ZMP in the rat heart suggests the possibility that adenosine kinase activity may be too low relative to cytosolic 5’nucleotidase activity. Alternatively, the transport of AICAraboside, which occurs via the adenosine transporter (9), may be much lower in the rat heart. Low [ZMP] accumulation may not be limited to the rat heart. ZMP was not detectable in rabbit cardiomyoctes exposed to 500 $\mu$M AICAraboside (18). In the heart the extent of ZMP formation is species dependent.

ZMP can be metabolized in several ways (28). One possible fate of ZMP is conversion into ZDP and ZTP. Substantial [ZTP] formed in the mouse heart. We estimate that [ZTP] was 3 to 5 mM in hearts perfused with 0.5 to 2 mM [AICAraboside] for 48 min. [ZMP], an intermediate in the de novo synthesis of purine nucleotides, can also be converted into inosine monophosphate (IMP). IMP can be converted into the nucleotides guanine monophosphate (GMP) and AMP (anabolic pathway) or uric acid (catabolic pathway). AICAraboside increased IMP and ATP in post- ischemic dog myocardium even though [ZMP] was low (27). In normal
dog myocardium treated with AICAraboside when [ZMP] was < 70 µM, IMP was predominantly converted to adenine nucleotides (27); when [ZMP] was > 190 µM, IMP was predominantly converted to uric acid (24). The lack of ZMP metabolism to adenine nucleotides in our study of the mouse heart may result from the high [ZMP] achieved, which were well above 190 µM.

In the mouse heart [ZMP] accumulation, which was time and [AICAriboside] dependent, resulted in variable decreases of [PCr] and [ATP]. Some studies of AICAriboside metabolism in hepatocytes (4) and skeletal muscle (22) have found no change in ATP with ZMP formation. Others have reported that hepatocytes exposed to 0.5 mM AICAriboside had unchanged ATP yet exposure to 2mM AICAriboside decreased ATP 40% (29). It is evident that the AICAriboside metabolism induced decrease in myocardial [PCr] and [ATP] translated into increased cytosolic [AMP].

In conclusion, AICAriboside formed sufficient [ZMP] to increase AMPK activity in the mouse heart. The [ZMP] was time and AICAriboside concentration dependant. The in vivo $A_{0.5}$ of ZMP for AMPK activity (covalent modification of AMPK) was 4.1 mM. AICAriboside metabolism decreased [ATP] and increased the [AMP].
Acknowledgements

The authors wish to thank Professor Joanne Ingwall for a critical reading the manuscript. The present address of Markus Frederich is the University of New England, Biological Sciences, Biddeford, ME 04005.

Grants

NIH Grant HL46033 to J.A.B supported this work.
ZMP activation of AMPK in the heart

References

ZMP activation of AMPK in the heart


Figure legends

Figure 1 Heart Perfusion Protocols to vary [ZMP] and [AMP]

Panel A- Hearts perfused without AICArriboside in the KH. All hearts were paced at a heart rate (HR) of 420 beats per min (bpm) unless noted. Five groups of hearts were studied: 1) hearts perfused with KH during the entire protocol (Glc group, n=15); 2) hearts perfused with KH at a HR of 420 bpm for 8 min, followed by a HR of 600 bpm for 40 min (Glc 600 group, n = 8); 3) hearts perfused with KH for 8 min, followed by KH plus AOA without pyruvate (Pyr) for 40 min (GA group; n = 5); 4) hearts perfused with KH for 16 min followed by KH containing BrO without Pyr for 24 min (GB group, n = 6); 5) hearts perfused with KH for 16 min followed by KH containing BrO without Pyr for 24 min, followed by KH without Pyr plus AOA and dobutamine (Db) for 16 min at a HR of 600 bpm (GBA group, n = 5). These conditions created variable cytosolic [AMP]. At the end of the perfusion hearts were freeze-clamped with Wollenberger tongs cooled in liquid nitrogen and stored at –80 ºC until further measurements.

Panel B- Hearts perfused with KH containing AICArriboside. All hearts were paced at a heart rate (HR) of 420 beats per min (bpm). Nine groups of hearts were perfused with KH for 8 to 24 min. The perfusate was then switched to KH containing one of the following [AICArriboside] for 48 min unless noted otherwise: 1) 0.15 mM, n = 7; 2) 0.25 mM for 24 min, n = 3; 3) 0.25 mM for 32 min, n = 4; 4) 0.25 mM for 48 min, n = 4; 5) 0.5 mM for 20 min, n = 7; 6) 0.5 mM for 48 min, n = 4; 7) 1 mM, n = 11; 8) 1.5 mM, n = 4; and, 9) 2 mM, n = 5. Perfusion of hearts with KH solutions containing different [AICArriboside] for varying times created variable [ZMP]. At the end of the perfusion hearts were freeze-clamped with Wollenberger tongs cooled in liquid nitrogen and stored at –80 ºC until further measurements.
**Figure 2**

**Panel A-** $^{31}$P NMR Spectra obtained from isolated mouse heart. The bottom spectrum is that of a mouse heart perfused with Krebs Henseleit (KH). The top spectrum (KH + 1.5 mM AICAraboside) is that of the same mouse heart 20 min after the perfusion was switched to KH containing 1.5 mM AICAraboside (5-aminoimidazole-4-carboxamide-ribose). $^{31}$P NMR resonances are identified as follows, going from left to right: 5-aminoimidazole-4-carboxamide-ribotide (ZMP); phosphocreatine (PCr); the $\gamma$-phosphate of ATP; the $\alpha$-phosphate of ATP; NAD$^+$ and NADH (NAD); and the $\beta$-phosphate of ATP.

**Panel B-** $^{31}$P NMR Spectra obtained the extracts of isolated mouse hearts perfused with AICAraboside. Bottom spectrum (I), extract of 3 mouse hearts perfused with KH containing AICAraboside. Middle spectrum (II), same extract after addition of 5 mM ZMP. Top spectrum (II – I), the difference spectrum results from the subtraction of I from II. $^{31}$P NMR resonances are identified as follows, going from left to right: ZMP, inorganic phosphate (Pi), and PCr.
Figure 3

Panel A- $^{31}$P NMR measured [ZMP] of isolated mouse hearts. Perfusion began with Krebs Henseleit (KH) medium, at time = 0 min the perfusion medium was switched to KH containing one of the following concentrations of AICArabinose: 0.15 mM (▲) (n=7), 0.25 mM (■) (n=15), 0.5 mM (●) (n=10), 1.0 mM (◆) (n=17), or 2.0 mM (▲) (n=5). Mean ± SEM.

Panel B- $^{31}$P NMR measured concentrations of ZMP (■) and PCr (○) for isolated perfused mouse hearts (n = 4). Perfusion began with Krebs Henseleit (KH) medium. At time zero the perfusion was switched to KH medium containing 1 mM AICArabinose. At 48 min the perfusion was switched back to KH. Mean ± SD.

Panel C- $^{31}$P NMR measured [ZMP] of isolated perfused mouse hearts (n=6). Perfusion began with KH medium. At time zero the perfusion was switched to KH medium containing 1 mM AICArabinose. At 48 min the perfusion for five hearts (■, Mean ± SD) was switched to KH containing 1 mM AICArabinose plus 50 µM iodotubercidin, an inhibitor of adenosine kinase. One heart (□) was perfused with KH + 1 mM AICArabinose continuously from time zero.
Figure 4

Panel A- **Total AMPK activity as a function of [AMP]:** A hyperbolic curve of the equation (1): \[ v = \frac{V_{\text{max}} \times [\text{AMP}]}{A_{0.5} + [\text{AMP}]} \] where \( v \) = AMPK activity, \( A_{0.5} = [\text{AMP}] \) at 50% AMPK activation, and \( V_{\text{max}} = \) the maximal activation was fit to the measurements of *in vivo* [AMP] and *in vitro* total AMPK activity from the individual hearts. Fitting the total AMPK activity from hearts without ZMP (□, \( n = 40 \)) to equation 1 (dashed line) produced the following parameters (best fit ± standard error): \( A_{0.5} = 5.5 \pm 1.6 \mu M, V_{\text{max}} = 9.6 \pm 1.6 \) pmol × min\(^{-1}\) × mg protein\(^{-1}\), 95% confidence intervals (\( A_{0.5} = 2.3 \) to 8.6 \( \mu M, V_{\text{max}} = 7.6 \) to 11.6 pmol × min\(^{-1}\) × mg protein\(^{-1}\)), \( R^2 = 0.70 \). Fitting the total AMPK activity from the hearts with ZMP (■, \( n = 48 \)) to equation 1 (solid line) produced the following parameters: \( A_{0.5} = 1.9 \pm 0.7 \mu M, V_{\text{max}} = 7.5 \pm 0.8 \) pmol × min\(^{-1}\) × mg protein\(^{-1}\), 95% confidence intervals (\( A_{0.5} = 0.5 \) to 3.2 \( \mu M, V_{\text{max}} = 6.0 \) to 9.1 pmol × min\(^{-1}\) × mg protein\(^{-1}\)), \( R^2 = 0.38 \).

Panel B- **An expansion of Total AMPK activity as a function of [AMP] shown in panel A.** The shift of the curve (solid line) to the left results from an apparent reduction in the \( A_{0.5} \) for AMP for the hearts with ZMP (■). This indicates that ZMP is increasing activity at lower [AMP]. As [AMP] increase the effect of ZMP on activity was reduced.

Panel C- **[AMP] and [ZMP] in hearts:** The cytosolic [AMP] and [ZMP] measured from hearts perfused with AICAraboside. The [AMP] at 0 mM ZMP are from the Glc group hearts, which were not exposed to AICAraboside. The dashed line is meant only to guide the eye. It does not imply a defined relationship between [AMP] and [ZMP]. The increase in [AMP] becomes significant near [ZMP] of 5 mM.
**Panel D-**  **Total AMPK activity as a function of [ZMP]:** A curve of the Hill equation:

\[ v = V_{basal} + V_{max} \times \frac{[ZMP]^h}{(A_{0.5})^h + [ZMP]^h} \]

where \( v \) = AMPK activity, \( A_{0.5} = [ZMP] \) at 50% AMPK activation, \( V_{basal} \) = basal activity and \( V_{max} \) = the maximal activity was fit to the combined AMPK activity measurements of the Glc group hearts (for basal activity at 0 mM ZMP) and AICAraboside perfused hearts (\textit{in vivo} [ZMP]). Fitting the data to the equation yields the following (best fit ± standard error): \( A_{0.5} = 4.1 \pm 0.6 \) mM, \( V_{max} = 5.2 \pm 1.0 \) pmol × min\(^{-1}\)× mg protein\(^{-1}\); Basal Activity = 1.65 ± 0.44 pmol × min\(^{-1}\)× mg protein\(^{-1}\) and \( h = 4 \pm 1.9 \). The 95% confidence intervals were (\( V_{basal} = 1.0 \) to 2.5 pmol × min\(^{-1}\)× mg protein\(^{-1}\), \( V_{max} = 3.1 \) to 7.3 pmol × min\(^{-1}\)× mg protein\(^{-1}\), \( A_{0.5} = 3.0 \) to 5.2 mM, \( h = 0.1 \) to 4.0), \( R^2 = 0.57 \).
Figure 5

AMPK activity (pmol min\(^{-1}\) mg protein\(^{-1}\)) measured for mouse hearts from the Glc, 1 mM AICAraboside (6.9 mM cytosolic [ZMP]), 8 min of no flow ischemia (8 min Isch,) and from 8 min Isch groups with 5 mM ZMP added to the in vitro assay (8 min Isch + 5 mM ZMP). Mean ± SD, * p< 0.05 measurement vs. Glc group, # p< 0.05 measurement vs. 1 mM AICAraboside group, ‡ p< 0.05 measurement vs. 1 mM AICAraboside group; † p< 0.05 measurement vs. 8 min Isch group
Figure 6

Western blot analysis of the phosphorylation of AMPK α Thr^{172} and ACC Ser^{79} of mouse heart after perfusion with and without AICAriboside:

Panel A- Representative western immunoblot used to measure the phosphorylation of Thr^{172} on the α subunit of AMPK from hearts perfused with the conditions, Glc (KH only) and KH containing the following [AICAriboside] and (exposure time): 0.5 mM (48 min), 0.25 mM (48 min), 0.15 mM (48 min) and 0.25 mM (32 min) hearts. 50 µg of protein was loaded in each lane.

Panel B- A plot of the relative phosphorylation of the AMPK α subunit Thr^{172} from western immunoblot shown in panel A. The AMPK-P signal values were normalized as percentages of the optical density of one 0.5 mM [AICAriboside] heart sample: Mean ± SD, * p< 0.05 measurement vs. Glc group.

Panel C- A representative western immunoblot used to measure the phosphorylation of Ser^{79} on ACC from hearts perfused with the conditions, Glc (KH only) and KH containing the following [AICAriboside] and (exposure time): 0.5 mM (48 min), 0.25 mM (48 min), 0.15 mM (48 min) and 0.25 mM (32 min) hearts. 50 µg of protein was loaded in each lane.

Panel D- The relative phosphorylation of Acc Ser^{79} from western immunoblot shown in panel C. The ACC-P signal values were normalized as percentages of the density of one 0.5 mM [AICAriboside] sample on the blot: Mean ± SD, * p< 0.05 measurement vs. Glc group, † p< 0.05 measurement vs. 0.15 mM and 0.25 mM 32 min groups.
ZMP activation of AMPK in the heart

Table 1: HPLC measured content of Z nucleotides and adenine nucleotides of mouse hearts perfused with and without AICAraboside

<table>
<thead>
<tr>
<th></th>
<th>ZMP</th>
<th>ZDP</th>
<th>ZTP</th>
<th>ZTP +ATP</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>AMP/ATP (×10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>27.56±3.71</td>
<td>27.56±3.71</td>
<td>5.21±0.30</td>
<td>0.83±0.14</td>
</tr>
<tr>
<td>0.25 mM [AICAraboside]</td>
<td>11.63±1.20</td>
<td>1.08±0.45</td>
<td>7.40±0.53</td>
<td>32.63±2.00</td>
<td>25.23±2.04</td>
<td>5.92±0.33</td>
<td>0.67±0.11</td>
<td>26.52±3.74</td>
</tr>
<tr>
<td>0.5 mM [AICAraboside]</td>
<td>15.67±3.52 †</td>
<td>1.43±0.18 †</td>
<td>11.20±1.72 †</td>
<td>35.26±7.56 *</td>
<td>24.06±7.56</td>
<td>5.71±1.45</td>
<td>0.85±0.15</td>
<td>37.05±8.92</td>
</tr>
<tr>
<td>1.5 mM [AICAraboside]</td>
<td>16.77±1.31 †</td>
<td>2.12±1.06 †</td>
<td>13.15±2.35 †</td>
<td>32.69±6.36</td>
<td>19.54±4.29 *</td>
<td>4.89±1.28</td>
<td>0.84±0.31</td>
<td>45.78±22.97</td>
</tr>
<tr>
<td>2.0 mM [AICAraboside]</td>
<td>16.43±1.14 †</td>
<td>1.89±0.60 †</td>
<td>13.25±1.62 †</td>
<td>32.27±3.29</td>
<td>19.02±1.71 *</td>
<td>4.90±0.73</td>
<td>0.85±0.23</td>
<td>44.60±9.03</td>
</tr>
</tbody>
</table>

Mean ± SD * p< 0.05 measurement vs. Glc group; † p< 0.05 measurement vs. 0.25 mM [AICAraboside];
5-aminoimidazole-4-carboxamide-ribotide (ZMP; Z monophosphate); Z diphosphate (ZDP); Z triphosphate (ZTP); Adenosine Monophosphate (AMP);
ZMP activation of AMPK in the heart

Table 2A: Concentration of heart $^{31}$P NMR measured metabolites and intracellular pH hearts perfused with KH containing AICAriboside.

<table>
<thead>
<tr>
<th>[AICAriboside]</th>
<th>[ZMP] (mM)</th>
<th>[PCr] (mM)</th>
<th>[ATP] (mM)</th>
<th>pH$_i$</th>
<th>[AMP] (μM)</th>
<th>AMP/ATP ($\times 10^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 mM</td>
<td>3.0 ± 0.7</td>
<td>12.1 ± 1.7</td>
<td>10.0 ± 0.2</td>
<td>7.04 ± 0.03</td>
<td>1.4 ± 1.0</td>
<td>13.2 ± 7.9</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>2.6 ± 0.4</td>
<td>12.1 ± 2.9</td>
<td>10.1 ± 0.2</td>
<td>7.06 ± 0.01</td>
<td>2.3 ± 1.3</td>
<td>22.8 ± 12.9</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>3.2 ± 0.5</td>
<td>13.0 ± 1.5</td>
<td>10.0 ± 0.1</td>
<td>7.07 ± 0.04</td>
<td>1.0 ± 0.3</td>
<td>10.4 ± 2.8</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>4.8 ± 0.9†</td>
<td>7.9 ± 1.1 *</td>
<td>9.7 ± 0.1 *</td>
<td>7.02 ± 0.04 *</td>
<td>3.6 ± 1.1</td>
<td>37.4 ± 11.3</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>4.5 ± 0.4†</td>
<td>10.4 ± 1.1*</td>
<td>10.0 ± 0.4*</td>
<td>7.09 ± 0.03</td>
<td>2.4 ± 0.9</td>
<td>24.3 ± 9.1</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>5.9 ± 0.7†</td>
<td>5.9 ± 1.0 *</td>
<td>9.2 ± 0.2 *</td>
<td>7.06 ± 0.07</td>
<td>8.8 ± 2.5*†</td>
<td>95.6 ± 25.1</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>6.9± 1.2†</td>
<td>5.4 ± 0.7 *</td>
<td>8.5 ± 0.3 *</td>
<td>7.04 ± 0.04 *</td>
<td>9.3 ± 3.2*†</td>
<td>111.8 ± 37.5 *†</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>8.1± 1.3†</td>
<td>5.3 ± 0.2 *</td>
<td>7.2 ± 0.1 *</td>
<td>7.09 ± 0.02</td>
<td>9.8 ± 0.9*†</td>
<td>135.8 ± 11.3 *†</td>
</tr>
<tr>
<td>2.0 mM</td>
<td>8.4± 1.1†</td>
<td>4.4 ± 1.1*</td>
<td>6.9 ± 0.1 *</td>
<td>7.09 ± 0.03</td>
<td>18.8 ± 12.6*†</td>
<td>271.0 ± 182.0*†</td>
</tr>
</tbody>
</table>

Mean ± SD * p< 0.05 measurement vs. Glc group (Table 2B); † p< 0.05 measurement vs.0.15 mM 48 min; 5-aminoimidazole-4-carboxamide-ribotide (ZMP); Phosphocreatine (PCr), Intracellular pH (pH$_i$), Adenosine Monophosphate (AMP);
Table 2B: Concentration of heart $^{31}$P NMR measured metabolites and intracellular pH of KH perfused hearts.

<table>
<thead>
<tr>
<th>Group</th>
<th>[PCr] (mM)</th>
<th>[ATP] (mM)</th>
<th>pH$_i$</th>
<th>[AMP] (μM)</th>
<th>AMP/ATP ($\times 10^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc</td>
<td>13.1 ± 1.8</td>
<td>9.6 ± 0.6</td>
<td>7.08 ± 0.04</td>
<td>1.1 ± 0.4</td>
<td>11.7 ± 5.1</td>
</tr>
<tr>
<td>Glc 600</td>
<td>10.7 ± 2.0 *</td>
<td>10.1 ± 1.4</td>
<td>7.10 ± 0.04</td>
<td>2.4 ± 1.0</td>
<td>25.0 ± 11.3</td>
</tr>
<tr>
<td>GA</td>
<td>6.6 ± 1.1 *</td>
<td>8.7 ± 1.4 *</td>
<td>7.07 ± 0.02</td>
<td>6.8 ± 2.6 *</td>
<td>79.0 ± 29.7</td>
</tr>
<tr>
<td>GB</td>
<td>5.6 ± 1.4 *</td>
<td>10.1 ± 0.6</td>
<td>7.11 ± 0.02</td>
<td>18.4 ± 10.9 *</td>
<td>183.0 ± 100.9*</td>
</tr>
<tr>
<td>GBA</td>
<td>2.0 ± 0.1 *</td>
<td>4.1 ± 0.2 *</td>
<td>6.98 ± 0.03 *</td>
<td>33.6 ± 9.9 *</td>
<td>819.6 ± 230.9*</td>
</tr>
</tbody>
</table>

Mean ± SD, * p< 0.05 measurement vs. Glc group; Phosphocreatine (PCr), Intracellular pH (pHi), Adenosine Monophosphate (AMP); Krebs Henseleit (KH) buffer
Heart Groups: Glc = Krebs Henseleit (KH), Glc 600 = KH paced at 600 bpm, GA = KH without pyruvate plus amino-oxyacetate, GB = KH without pyruvate plus bromo-octanoate, GBA = KH without pyruvate plus bromo-octanoate and amino-oxyacetate and dobutamine.
Table 3: AMP-activated protein kinase activity of mouse hearts perfused with and without AICArriboside

<table>
<thead>
<tr>
<th>Group</th>
<th>Total AMPK activity (pmol×min⁻¹×mg protein⁻¹)</th>
<th>[AMP] µM</th>
<th>[ZMP] mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc</td>
<td>1.6 ± 0.6</td>
<td>1.1 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>Glc 600</td>
<td>3.0 ± 1.2</td>
<td>2.4 ± 1.0</td>
<td>0</td>
</tr>
<tr>
<td>GA</td>
<td>6.6 ± 1.6 *</td>
<td>6.8 ± 2.6</td>
<td>0</td>
</tr>
<tr>
<td>GB</td>
<td>5.4 ± 1.7 *</td>
<td>18.4 ± 10.9</td>
<td>0</td>
</tr>
<tr>
<td>GBA</td>
<td>9.2 ± 3.1 *</td>
<td>33.6 ± 9.9</td>
<td>0</td>
</tr>
<tr>
<td>[AICArriboside]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 mM 48 min</td>
<td>2.7 ± 1.0</td>
<td>1.4 ± 1.0</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>0.25 mM 24 min</td>
<td>3.1 ± 0.3</td>
<td>2.3 ± 1.3</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>0.25 mM 32 min</td>
<td>2.3 ± 1.0</td>
<td>1.0 ± 0.3</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>0.25 mM 48 min</td>
<td>5.3 ± 0.9* †</td>
<td>3.6 ± 1.1</td>
<td>4.8 ± 0.9 †</td>
</tr>
<tr>
<td>0.5 mM 20 min</td>
<td>4.3 ± 0.9*</td>
<td>2.4 ± 0.9</td>
<td>4.5 ± 0.4 †</td>
</tr>
<tr>
<td>0.5 mM 48 min</td>
<td>6.0 ± 1.5* †</td>
<td>8.8 ± 2.5* †</td>
<td>5.9 ± 0.7 †</td>
</tr>
<tr>
<td>1 mM 48 min</td>
<td>7.9 ± 2.8 * †</td>
<td>9.3 ± 3.2* †</td>
<td>6.9± 1.2 †</td>
</tr>
<tr>
<td>1.5 mM 48 min</td>
<td>3.9 ± 0.8*</td>
<td>9.8 ± 0.9* †</td>
<td>8.1± 1.3 †</td>
</tr>
<tr>
<td>2.0 mM 48 min</td>
<td>5.9 ± 1.4* †</td>
<td>18.8 ± 12.6* †</td>
<td>8.4 ± 1.1 †</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>α₂ isoform AMPK activity (pmol×min⁻¹×mg protein⁻¹)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc</td>
<td>2.2 ± 0.8</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>1 mM 48 min</td>
<td>13.3 ± 2.2*</td>
<td>9.7 ± 3.9*</td>
</tr>
</tbody>
</table>

Mean ± SD, * p< 0.05 measurement vs. Glc, † p< 0.05 measurement vs. 0.15 mM [AICArriboside] 48 min; AMP activated protein kinase (AMPK); Adenosine Monophosphate (AMP); 5-aminoimidazole-4-carboxamide-ribotide (ZMP); Krebs Henseleit (KH) buffer

Heart Groups: Glc = Krebs Henseleit (KH), Glc 600 = KH paced at 600 bpm, GA = KH without pyruvate plus amino-oxyacetate, GB = KH without pyruvate plus bromo-octanoate, GBA = KH without pyruvate plus bromo-octanoate and amino-oxyacetate and dobutamine.
**Figure 1.**

**Heart perfusion protocol**

**A. without AlCAriboside**

<table>
<thead>
<tr>
<th>KH changes to Increase [AMP]</th>
<th>Baseline</th>
<th>Glc 600 600 bpm</th>
<th>GA - Pyr + AOA</th>
<th>GBA - Pyr + BrO + AOA + Dba 600 bpm</th>
<th>GB - Pyr + BrO</th>
</tr>
</thead>
<tbody>
<tr>
<td>(min)</td>
<td>0</td>
<td>24</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR = 420 bpm unless noted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B. with AlCAriboside**

<table>
<thead>
<tr>
<th>[AlCAriboside] 0.15, 0.25, 0.5, 1, 1.5, or 2 mM</th>
<th>KH (min)</th>
<th>0 24 32 48</th>
</tr>
</thead>
<tbody>
<tr>
<td>[AlCAriboside] 0.5 0.25 0.25 0.25 0.25 All</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

↑ Hearts freeze-clamped for AMPK activity and phosphorylation
Figure 2.

A. KH + 1.5 mM AlCAriboside

B. ZMP Pi PCr

III- I

II

I
Figure 3.
ZMP activation of AMPK in the heart

Figure 4.
Figure 5.
Figure 6.

A. 

B. 

C. 

D. 

ZMP activation of AMPK in the heart