Published in final edited form as:

Shock. 2017 January ; 47(1): 70–78. doi:10.1097/SHK.0000000000000730.

THE AMPK ACTIVATOR AICAR AMELIORATES AGE-DEPENDENT MYOCARDIAL INJURY IN MURINE HEMORRHAGIC SHOCK

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Abstract

The development of myocardial dysfunction in patients with hemorrhagic shock is significantly impacted by the patient age. AMP-activated protein kinase (AMPK) is a pivotal orchestrator of energy homeostasis, which coordinates metabolic recovery after cellular stress. We investigated whether AMPK-regulated pathways are age-dependent in hemorrhage-induced myocardial injury and whether AMPK activation by 5-amino-4-imidazole carboxamide riboside (AICAR) affords cardioprotective effects. Anesthetized C57/BL6 young (3–5 months old) and mature male mice (9–12 months old) were subjected to hemorrhagic shock by blood withdrawing followed by resuscitation with shed blood and Lactated Ringer's solution. Mice were sacrificed at 3 hours after resuscitation, and plasma and hearts were harvested for biochemical assays. Vehicle-treated mature mice exhibited higher myocardial injury and higher levels of plasma biomarkers of cardiovascular injury (endocan and follistatin) when compared with young mice. Cardiac cell mitochondrial structure was also markedly impaired in vehicle-treated mature mice when compared to young mice. At molecular analysis, an increase of the phosphorylated catalytic subunit pAMPK α was associated with nuclear translocation of the peroxisome proliferator-activated receptor γ co-activator- α in young, but not mature mice. No changes in autophagy were observed as evaluated by the conversion of the light-chain (LC)3B-I protein to LC3B-II form. Treatment with AICAR ameliorated myocardial damage in both age groups. However, AICAR therapeutic effects were less effective in mature mice compared to young mice and involved distinct mechanisms of action. Thus, our data demonstrate that during hemorrhagic shock AMPK-dependent metabolic mechanisms are important for mitigating myocardial injury. However, these mechanisms are less competent with age.

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Conflict of interest: None declared

Keywords

Hemorrhagic shock; AMP-activated protein kinase (AMPK); 5-amino-4-imidazole carboxamide riboside (AICAR); peroxisome proliferator-activated receptor γ co-activator- α (PGC-1 α); autophagy; mitochondria

INTRODUCTION

Aging is a risk factor for multiple organ failure (1, 2). Epidemiological studies have, in fact, reported that adult trauma victims, including patients with hemorrhagic shock, have considerably higher mortality rate (12–15%) than pediatric population (0–6%) (3, 4). In normal physiological conditions, aging is associated with changes in myocardial function, biochemistry and structure (5). Experimental studies in rodents have demonstrated that these changes are exacerbated in pathophysiologic conditions of ischemia and reperfusion as the aging myocardium is highly susceptible to injury (6, 7).

Mitochondrial dysfunction and impairment of its primary role of ATP synthesis is a fundamental mechanism underlying cardiac failure. Because the heart is a mitochondria-rich organ, mitochondrial damage contributes to cardiac dysfunction and myocyte injury via a loss of metabolic capacity and by the production and release of reactive oxygen species (8). However, the underlying mechanisms that link age to myocardial dysfunction are not well understood.

AMP-activated protein kinase (AMPK) is an important regulatory enzyme for mitochondrial homeostasis, which serves as a fuel sensor to adapt to environmental stress such as exercise, cold and fasting or pathological conditions of anoxia and ischemia in order to maintain adequate adenosine triphosphate (ATP) production (9). This kinase consists of a catalytic α -subunit and two regulatory β - and γ subunits. In the heart, activation of AMPK under normal conditions is low, but in response to different insults of ischemia or pressure overload the catalytic α -subunit is phosphorylated leading to activation of the enzyme (10).

AMPK is known to regulate two major metabolic pathways - mitochondrial biogenesis and autophagy. Autophagy is a cellular catabolic process that leads to the breakdown and recycling of unnecessary or dysfunctional cellular components into energetic metabolites (11). AMPK also regulates metabolic recovery through mitochondrial biogenesis by activation of peroxisome proliferator-activated receptor γ co-activator α (PGC-1 α) (12). Being one of the AMPK-pathways downstream signaling amplifier, PGC-1 α is predominantly expressed in tissues with high energy oxidative capacity like heart, liver, skeletal muscles, brain and controls fatty acid oxidation and oxidative phosphorylation gene expression (13).

Among pharmacological agents that may activate AMPK the 5-amino-4-imidazolecarboxamideriboside-1- β -D-ribofuranoside (AICAR) is an AMP analogue that increases the AMP/ATP ratio in the cell and mimics that high energy expenditure condition necessary for enzyme activation (14).

In view of the metabolic role of AMPK, the purpose of our study was to provide insights into the age-dependent molecular mechanisms of mitochondrial quality control in the heart in a clinically relevant model of hemorrhagic shock in young (3–5 months old) and middle-aged mature (9–12 months old) mice. Also, purpose of our study was to investigate whether pharmacological treatment with AICAR would ameliorate hemorrhage-induced myocardial injury. Our data demonstrate that myocardial AMPK metabolic signaling declines in aged animals and correlates with myocardial injury. However, despite these age-dependent changes of AMPK, pharmacological activation of the enzyme by AICAR may afford cardio-protective effects and may represent a novel treatment in the management of trauma and hemorrhagic shock.

MATERIALS AND METHODS

Murine model of hemorrhagic shock

The experiments conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (8th edition, 2011) and had the approval of the Institutional Animal Care and Use Committee. C57/BL6 young (3–5 months) and mature male mice (9–12 months) were obtained from Charles River Laboratories (Wilmington, MA). Sixty male C57/BL6 mice, including 30 young age (26.7 ± 1.8 g body weight), and 30 mature (38.3 ± 4.2 g body weight) were acclimatized for at least 48 h. Animals were housed with an inverse 12 hour day-night cycle in a temperature ($22 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) controlled room, and were allowed free access to water and a maintenance diet.

Before procedure, each animal was placed on a circulating warming water blanket to maintain its temperature at physiological conditions throughout experiment. Mice were anesthetized with pentobarbital (80 mg/kg) intraperitoneally (IP). Either the left or right femoral artery was cannulated (PE-10 tube) and connected to a blood pressure transducer (PowerLab, ADInstruments, Colorado Springs, CO) for measurement of mean arterial blood pressure (MABP) and heart rate (HR). Hemorrhagic shock was induced by blood removal until MABP reached 30 ± 5 mmHg (15, 16). The mice were kept in this MABP range for 90 minutes by additional blood removal or small volume transfusion. At the end of the shock period, young and mature mice were randomly assigned to two groups: a vehicle-treated group received the vehicle compound (distilled water IP); an AICAR-treated group received AICAR (100 mg/kg IP). The mice were then resuscitated by infusing their shed blood and twice that amount in Lactate Ringer's solution over a 10-minute period. The mice were further monitored for 3 hours for MABP and HR values. Sham mice were anesthetized and underwent cannulation, but were not hemorrhaged. At 3 hours after resuscitation, mice were sacrificed and blood and heart tissue were obtained for biochemical assays.

Cardiovascular disease biomarkers

Plasma levels of biomarkers of cardiovascular injury, including Troponin T, Troponin I, PIGF-2, Follistatin, Endocan-1 and CXCL16 were measured by a multiplex array system according to the manufacture's protocol (Milliplex, Millipore Corporation, Billerica, MA).

Histopathologic analysis

Heart tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin and evaluated by light microscopy. To have a quantitative estimation of the histological myocardial damage, myocardial injury scores were quantified by four blinded reviewers. Scores of myocardial injury included: perivascular edema, neutrophil infiltration, myofibril derangement and nuclear hydrops. A score of 0 indicated no injury and scores of 1, 2, 3 represented minimal (<33% of myocardial involvement), moderate (33%-66% of myocardial involvement) and severe (>66% of myocardial involvement) injury, respectively. The overall score was based on the sum of all scores. The sum of four variables for each histological characteristic represented the myocardium injury score (total score, 0–48).

Transmission electron microscopy

Heart tissue samples were fixed in 3% glutaraldehyde and postfixed in 1% osmium tetroxide in sodium phosphate buffer. Sections were stained with 2% uranyl acetate and lead citrate and were photographed on Hitachi H-7650 transmission electron microscope at 120 kV. The total number of mitochondria, and the presence of abnormal mitochondria or thin elongated with loose matrix, fragmented cristae and membranes were determined in four consecutive cells in four different sections for each animal by using NIH ImageJ analysis (17).

Western blot analysis

Cytosol and nuclear content of AMPK α 1/ α 2 and its phosphorylated active form pAMPK α 1/ α 2, and PGC1- α were determined by immunoblot analyses. Proteins were loaded on a 10% Bis-Tris gel and separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with Odyssey blocking buffer and incubated with specific primary antibodies; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was concomitantly probed as loading control. Membranes were washed and incubated with LI-COR secondary antibodies. The immunoreaction was visualized by fluorescence with an Odyssey LI-COR scanner (LI-COR Biotechnology, Lincoln, NE). Cytosol contents of light-chain (LC)3B-I and LC3B-II proteins, as markers of autophagy, were also determined by immunoblot analyses. Proteins were loaded on a 16% Tris-glycine gradient gel, separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 5% nonfat dried milk in Tris-buffered saline (TBS) and incubated with primary antibodies. Membranes were washed and incubated with secondary peroxidase-conjugated antibody; the immunoreaction was visualized by chemiluminescence. Membranes were also reprobed with primary antibody against GAPDH to ensure equal loading. Densitometric analysis of blots was performed using Quantity One (Bio-Rad Laboratories, Des Plaines, IL).

Measurement of NF- κ B activity

NF- κ B activity was analyzed by a TransAM Transcription Factor assay kit specific for the activated form of p65 using the NF- κ B consensus site (50–GGG ACTTTC-30) according to the manufacturer's protocol (Active Motif, Carlsbad CA).

Materials

The AMP analog, AICAR, was obtained from LC Laboratories (Woburn, MA). Primary antibodies for AMPK α 1/ α 2, pAMPK α 1/ α 2, and LC3B-I and LC3B-II were obtained from Cell Signaling Technology (Danvers, MA). Primary antibodies for GAPDH and for PGC1- α were obtained from Abcam (Cambridge, MA). The Odyssey blocking buffer, LI-COR goat anti-rabbit IR-800 and goat anti-mouse IR-680 antibodies, and the 4 $\times$ Protein Sample Loading Buffer were obtained from LI-COR Biotechnology (Lincoln, NE). The NuPAGE[®] LDS Sample Buffer (4 $\times$), and Western blot gels were purchased from Life Technologies (Grand Island, NY). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Statistical analysis

Statistical analysis was performed using SigmaPlot for Windows Version 12.5 (Systat Software, San Jose CA). Data are represented as means $\pm$ SD or SEM, or as medians with interquartile range of $n = 3$ –16 animals for each group. For multiple group analysis at a single time point, one-way analysis of variance (ANOVA) with Student-Newman-Keuls correction was used. For multiple group analysis at different time points, a two-way ANOVA with Student-Newman-Keuls correction was performed. If data failed to follow a normal distribution, a Mann-Whitney Rank Sum test or an ANOVA on ranks test was performed. P values less than 0.05 were considered significant.

RESULTS

AICAR ameliorates blood pressure in young, but not in mature mice

Since the degree of hypoperfusion influences the severity of the inflammatory response, we chose an experimental protocol of pressure-controlled hemorrhage to a target MABP of 30 mmHg in vehicle- and AICAR-treated mice of both age groups. After resuscitation with blood and Lactate Ringer's solution, MABP partially recovered in vehicle-treated mice of both age groups but did not reach baseline levels. Treatment with AICAR at the time of resuscitation ameliorated MABP in young mice when compared with vehicle treatment. Interestingly, AICAR treatment did not affect MABP in mature mice (Table 1). Heart rate was significantly higher after resuscitation when compared to baseline rate at time 0 in all groups. However, vehicle-treated mature mice appeared more tachycardic than young mice at all time points after resuscitation. Treatment with AICAR did not affect heart rate in either age group (Table 1).

Age-dependent myocardial pathological changes

At 3 hours after resuscitation, histological analysis revealed that both vehicle-treated young and mature mice had myocardial injury. However, following hemorrhagic shock tissue injury was more severe in vehicle-treated mature mice than young animals and was characterized by perivascular edema, myofibril derangement and neutrophil adhesion to vascular endothelium. Treatment with AICAR significantly ameliorated myocardial damage in both mature and young mice (Fig. 1).

Age-dependent effect of AICAR on cardiovascular disease biomarkers

To evaluate the hemorrhage-induced cardiovascular inflammatory response, a panel of cardiovascular injury markers was measured. At 3 hours after resuscitation, plasma levels of endocan-1 were significantly increased in vehicle-treated mature, but not in young mice when compared to age-matched controls. AICAR treatment significantly decreased levels of endocan-1 in mature group (Fig. 2A). Plasma levels of follistatin were significantly increased in both vehicle-treated young and mature adult mice compared to age-matched controls; however, the magnitude of elevation was higher in mature mice when compared to the young group (Fig. 2B). AICAR treatment significantly decreased levels of follistatin in both age groups. Plasma levels of the placental growth and angiogenic factor PIGF-2 and the chemokine CXCL16 were significantly increased in both vehicle-treated young and mature adult mice compared to age-matched controls. AICAR treatment significantly decreased levels of PIGF-2 in both age groups whereas it did not affect plasma levels of CXCL16 (Fig. 2C and D). Plasma levels of Troponin-I and Troponin-T were slightly, but not significantly, increased in both vehicle-treated young and mature adult mice compared to age-matched controls. After AICAR treatment, levels of Troponin-I and Troponin-T returned to baseline levels of age-matched controls (Fig. 2E and F).

Age-dependent changes of cardiac cell mitochondrial ultrastructure

Because cardiac cells are highly metabolically active, we evaluated their mitochondrial structure by electron microscopy (Fig. 3). At basal conditions, mitochondria of mature mice had reduced cristae density when compared with hearts of young mice. After hemorrhagic shock, electron microscopy analysis revealed a marked increase of thin and elongated mitochondria and reduction of the average volume-density of the organelles in both young and mature vehicle-treated mice when compared with mitochondria of age-matched control animals. Vehicle-treated mature mice also exhibited a higher percentage of damaged mitochondria with membrane loss or disorganized cristae when compared to age-matched control. AICAR treatment ameliorated mitochondrial damage and increased mitochondrial size, which returned to basal levels in both age groups.

Age-dependent cellular localization and activation of AMPK α

Because of the central role of AMPK in metabolic regulation, we measured the expression of the catalytic α -subunit and its active phosphorylated form pAMPK α in heart homogenates. After hemorrhagic shock, pAMPK α significantly increased in the cytosol and the nuclear compartments in vehicle-treated young, but not in mature adult mice, when compared to basal levels of age-matched control mice. Interestingly, the pAMPK α /AMPK α ratio of mature adult mice was significantly higher at basal conditions and after hemorrhagic shock in the nuclear compartment when compared with young mice, thus suggesting a distinct age-dependent cellular localization of the active pAMPK α . AICAR treatment further increased the cytosol and nuclear pAMPK α in young mice when compared to vehicle treatment. However, in mature mice AICAR treatment did not affect pAMPK in the nucleus, but restored levels in the cytosol, which were maintained similarly to basal levels of age-matched controls (Fig. 4A, B and C).

Age-dependent nuclear translocation of PGC-1 α

Given the role of PGC-1 α as a crucial regulator of mitochondrial function, we also evaluated the expression of this co-factor in heart homogenates. Vehicle-treated young mice exhibited a significant increase of the nuclear content of PGC-1 α at 3 hours after resuscitation when compared with basal levels of age-matched control mice. On the contrary, in vehicle-treated mature adult animals PGC-1 α was significantly downregulated in the nucleus when compared with age-matched control mice, thus suggesting an impairment of its nuclear translocation. AICAR treatment increased PGC-1 α nuclear translocation in both young and mature adult mice. However, levels of PGC-1 α of AICAR-treated mature adult mice were significantly lower than AICAR-treated young mice (Fig. 4A, D and E).

Age-independent increase of autophagy in the heart

Since AMPK may also regulate autophagy through the mTOR pathway (11), we also quantified the cytosolic conversion of the light chain 3B protein (LC3B) isoform I to the isoform II, which is considered a marker for autophagosome formation. The LC3BII/I ratio increased following hemorrhagic shock in both young and mature vehicle-treated groups when compared to age-matched controls, thus suggesting the capability of autophagy. AICAR treatment did not affect the LC3BII/I ratio in either age group (Fig. 5A and B).

Age-independent increase of NF- κ B activation in the heart

Previous studies have suggested that AMPK can inhibit NF- κ B activation (18). Therefore, we evaluated the activity of this transcription factor. NF- κ B activation was significantly increased in both age groups following hemorrhagic shock when compared to age-matched controls. AICAR treatment did not affect NF- κ B activation in either age group (Fig. 5C).

Discussion

Age is an independent risk factor for development of multiple organ failure and mortality in trauma patients (1–5). This is related to inability of elderly population to compensate for the insult from injury in response to trauma (5, 6). Taking this into consideration, major trauma in pediatric population is better tolerated (3). Thus, it is conceivable that physiological cardiovascular aging (6) contributes to myocardial dysfunction and morbidity after hemorrhagic shock. Our study investigated the age-dependent characteristics of myocardial injury after hemorrhagic shock and validated the importance of AMPK signaling in mitochondrial quality control. Furthermore, our study demonstrated that AMPK may serve as a pharmacological target for improvement of myocardial tissue injury, despite different age-dependent molecular mechanisms.

In our study, we demonstrated that myocardial damage has peculiar age-dependent characteristics which are associated with dysregulation of AMPK activation. We found that, while in a vehicle-treated young group of mice myocardial injury was very mild and characterized mostly by interstitial edema, in mature mice tissue injury was significantly worse with myofibril disarrangement being the most prominent feature. Furthermore, we observed that vehicle-treated mature mice had higher HR than young mice after

resuscitation, a phenomenon which is consistent with age-related attenuation of parasympathetic control after stress (19). In young mice, AICAR provided a significant improvement of myocardial damage, reduction of circulating biomarkers of cardiovascular injury as well as hemodynamic stability. Our studies are in agreement with previous reports demonstrating that AICAR treatment results in increased survival, improvement of cardiovascular parameters during experimental hemorrhagic shock in young adult rats or pigs (20–22). However, these beneficial effects of AMPK activation have never been tested in aging models of hemorrhagic shock. In our study, administration of AICAR to a more mature group of mice significantly reduced the plasma levels of cardiokines and myocardial injury but at lesser degree when compared to young animals. Consistent with this less cardioprotective effect, AICAR did not ameliorate hypotension in the mature group. Interestingly, AICAR did not modify HR in either age group. As MABP is a product of cardiac output and systemic vascular resistance, thus it possible that AICAR improves hypotension in young mice through a compensatory upregulation of stroke volume and/or systemic vascular resistance. However, this possibility warrants further studies of other cardiovascular parameters.

The age-related pathological characteristics of cell damage well paralleled with distinct changes of cardiovascular disease biomarkers. For example, endocan and follistatin were significantly increased after hemorrhagic shock in vehicle-treated mature mice when compared to young mice. Endocan is a proteoglycan which is excreted by endothelial cells in response to inflammatory cytokines (23). Follistatin is a cardiokine expressed in the adult heart and is induced in response to injurious conditions that promote myocardial hypertrophy and heart failure (24). AICAR treatment attenuated this inflammatory response, but the effect was less prominent in the mature mice than the young group. On the contrary, levels of the placental growth factor PIGF2, an angiogenic factor (25), CXCL16, a chemokine involved in neutrophil recruitment (26) and the classical cardiac biomarkers troponin-I and troponin-T (27) were not different among vehicle or AICAR-treated groups of either age. Taken together, these data, therefore, suggest an important contribution of the inflammatory response in mature animals.

AMPK is a serine/threonine protein kinase, that serves as a cellular energy sensor, regulating ATP levels through activation of catabolic and inhibition of anabolic processes. It consists of three subunits: one catalytic α -subunit and two regulatory β - and γ subunits. After gauging fuel demand and specifically rise in the AMP/ATP ratio, phosphorylation of the α -subunit activates AMPK (9–12). AICAR is an adenosine analog that allosterically activates AMPK, mirroring energetic failure by raising the AMP/ATP ratio (14). Our study demonstrated that activated AMPK α has a specific age-dependent cardiac cell localization after hemorrhagic shock. Vehicle-treated young mice showed significant increase in cytosol and nuclear phosphorylated AMPK α when compared to age-matched control mice suggesting a concerted nucleo-cytoplasmatic activation of the kinase in response to cellular stress. Treatment with AICAR further augmented this activation in young mice. Interestingly, baseline levels of phosphorylated AMPK were higher in mature hearts compared to young mice. Despite this baseline upregulation, AMPK was not responsive to hemorrhage-induced stress. This is consistent with previous findings in mouse livers or brains, which also exhibited baseline elevations in pAMPK levels with age, but an absent response to hypoxia

or stroke (28, 29). It is possible, therefore, that the baseline increase in AMPK activity in aging may reflect a defense mechanism to the age-related oxidative damage and decrease of metabolic processes, causing however an inability to respond to acute stressors. In line with this age-dependent dysregulation of AMPK activation, treatment with AICAR did not cause any further increase of pAMPK, but induced only a peculiar redistribution of this kinase into the cytosol from the nucleus.

Consistent with the age-dependent dysregulation of AMPK activation, we also observed a dysregulation in the nuclear translocation of PGC-1 α , which is an important AMPK substrate responsible for mitochondrial biogenesis (13). Changes in AMPK α phosphorylation well corresponded to nuclear translocation in vehicle-treated young mice after hemorrhagic shock when compared with age-matched control mice. Similarly to pAMPK levels, baseline levels of nuclear PGC-1 α were higher in hearts of vehicle-treated mature mice compared to young mice. Interestingly, after hemorrhagic shock nuclear levels of PGC-1 α were significantly downregulated in mature mice, thus suggesting an inability to promote further nuclear translocation. This downregulation also may explain why at electron microscopic analysis percentage of damaged mitochondria in mature mice were higher in comparison with age-matched controls, suggesting poor biogenesis. On the other hand, amount of damaged mitochondria in vehicle-treated young animals was unchanged after hemorrhagic shock when compared to baseline mitochondria number, thus demonstrating an efficient compensatory mechanism of mitochondrial homeostasis. In contrast to percentage of damaged organelles, mitochondrial size was, however, similarly decreased and was associated with small and thin shape in both age groups after hemorrhagic shock. These changes in morphology may be consistent with persistence of oxidative stress-induced fission, resulting in the formation of small, round mitochondria that can persist when fusion is inhibited (30–32). AICAR treatment further increased nuclear PGC-1 α expression in young mice. Consistent with the nuclear-cytosol distribution of pAMPK, AICAR treatment was able also to increase nuclear PGC-1 α expression in mature adult mice although at lesser degree than the young group. This molecular event of PGC-1 α nuclear translocation was associated with mitochondrial expansion in both age groups as evidenced by increased mitochondrial size.

Another downstream pathway of AMPK activation is autophagy via inhibition of mTOR (the mammalian target of rapamycin) signaling. Autophagy is a cellular process that recycles mitochondria and other organelles by delivering them to the autophagosome for degradation. The conversion of microtubule-associated protein-1 light chain 3B (LC3B) from LC3B-I (free form) to LC3B-II (phosphatidylethanolamineconjugated form) is a major step in autophagosome formation (11, 33). Results of our study demonstrated upregulation of LC3B-II/LC3B-I ratio after hemorrhagic shock in both young and mature animals, thus suggesting the capability to mount an autophagic event in response to cellular stress. AICAR treatment did not significantly affect LC3B-II conversion in either age group. Although autophagy has been shown to decline in the heart with age (34), our data suggest that in mature middle-aged animals autophagy is still maintained and may be also regulated by AMPK-independent mechanisms. This important aspect of metabolic recovery and autophagic function needs further investigation.

The NF- κ B is the main signaling pathway of the systemic inflammatory response (35). Several studies have demonstrated that activation of AMPK downregulates NF- κ B (18). Therefore, inhibition of NF- κ B may represent a molecular target of the cardioprotective effects of AICAR. In this regard, we investigated the activation of NF- κ B in the heart. We observed a significant rise of NF- κ B activity after hemorrhagic shock in both age groups. Interestingly, AICAR treatment did not inhibit this inflammatory pathway in either age group after hemorrhagic shock. Our data, therefore, suggest that myocardial injury is most probably repaired by AMPK activation independently of NF- κ B-mediated inflammatory influences and that the metabolic recovery is a key component of the resolution of tissue damage.

In conclusion, our data suggest that during hemorrhagic shock myocardial injury is age-dependent and is associated with an age-dependent cellular localization and activation of AMPK and dysregulation of downstream metabolic pathways. AICAR treatment ameliorated myocardial damage in both young and mature mice. However, the cardioprotective effects and the molecular mechanisms of action of the drug were also age-dependent. Whether AMPK may represent a therapeutic target in hemorrhagic shock deserves further investigation.

Acknowledgments

Funding Support: This work was supported by grants from the National Institutes of Health (NIH) (R01 AG-027990, R01 GM-067202 and R01 GM-115973 to B.Z.).

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Figure 1.

Representative histology photomicrographs of heart sections. Normal myocardium architecture in control young (A) and mature mice (D). Myocardial damage in vehicle-treated young (B) and mature mice (E) after hemorrhagic shock presented with perivascular edema (green arrows), neutrophil infiltration (yellow arrows) and myofibril disarrangement (red arrows). Significant amelioration of heart architecture in AICAR-treated young (C) and mature mice (F). Magnification $\times 400$. A similar pattern was seen in $n=4-6$ different tissue sections in each experimental group. (G) Histopathologic scores of heart sections ($n=4-10$

mice for each group). Myocardial injury was scored from 0 (no damage) to 48 (maximum damage). Box plots represent 25th percentile, median, and 75th percentile; error bars define 10th and 90th percentiles; whiskers define minimum and maximum values. *Represents $P < 0.05$ versus age-matched control mice; #represents $P < 0.05$ versus vehicle-treated group of the same age; ‡represents $P < 0.05$ versus young group.

Figure 2.

Effect of *in vivo* treatment with AICAR on plasma levels of Endocan-1 (A), Follistatin (B), PIGF-2 (C), CXCL16 (D), Troponin-I (E) and Troponin-T (F) in young and mature mice after hemorrhagic shock. Each data represents the mean $\pm$ SEM of 4–6 animals for each group. *Represents $P < 0.05$ versus age-matched control mice; #represents $P < 0.05$ versus vehicle-treated group of the same age; ‡represents $P < 0.05$ versus young group.

Figure 3. Transmission electronic microscopy of cardiac cells. Normal cellular structure in young (*A*) with normal electron dense mitochondria presenting organized cristae; and mature mice with reduced cristae density (*D*). Structural changes in vehicle-treated young (*B*) and mature mice (*E*) after hemorrhagic shock with mitochondria presenting disrupted membrane and cristae and thin elongated shape (arrow heads) and autophagosomes (arrows). Amelioration of mitochondrial damage in AICAR-treated young (*C*) and mature mice (*F*) after hemorrhagic shock with normal electron dense mitochondria. Quantification of average mitochondrial

area (G) and damaged mitochondria (H) in cardiac cells as determined by using the NIH Image J software. Damaged mitochondria were determined as percentage of total number of mitochondria. Data are expressed as means $\pm$ SEM. *Represents $P < 0.05$ *versus* age-matched control mice; ‡represents $P < 0.05$ *versus* young group.

Figure 4.

Representative Western blot analysis of pAMPK α , AMPK α , PGC-1 α and GAPDH (used as loading control protein) in heart cytosol and nuclear extracts (A). Image analyses of pAMPK α /AMPK α ratio as determined by densitometry in the cytosol (B) and in the nucleus (C). Image analyses of PGC-1 α expression as determined by densitometry in the cytosol (D) and in the nucleus (E). Data are mean $\pm$ SEM of 4–6 animals for each group and are expressed as relative intensity units. *Represents $P < 0.05$ versus age-matched control

mice; #represents $P < 0.05$ versus vehicle-treated group of the same age; ‡represents $P < 0.05$ versus young group.

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Figure 5.

Representative Western blot analysis of LC3B-I and LC3B-II and GAPDH (used as loading control protein) in heart cytosol extracts (A). Image analyses of LC3B-II/LC3B-I ratio as determined by densitometry (B). Data are mean $\pm$ SEM of 4–6 animals for each group and are expressed as ratio of relative intensity units. Activity of the p65 subunit of NF- κ B in heart nuclear extracts at 3 hours after resuscitation (C). Data are mean $\pm$ SEM of 5–13 animals for each group and are expressed as optical density units. *Represents $P < 0.05$ versus age-matched control mice.

Table 1

Mean arterial blood pressure and heart rate measurements in young (3–5 months) and mature (9–12 months) mice subjected to hemorrhagic shock.

Time (minutes)	Young mice				Mature mice			
	Vehicle		AICAR		Vehicle		AICAR	
	MABP	HR	MABP	HR	MABP	HR	MABP	HR
During hemorrhage	0	63.2±3.0	381.7±60	62.4±1.5	374.8±29	363.3±45	62.1±2.2	374.3±43
	60	29.3±0.5 [*]	403.2±138	28.4±0.5 [*]	377.5±38	366.5±62	29.6±0.9 [*]	363.0±31
	90	29.4±0.7 [*]	418.7±120	28.9±0.5 [*]	377.5±32	409±58 [*]	29.7±1.1 [*]	402.3±76
During resuscitation	150	60.3±3.0	458.9±57 [*]	65.6±3.0	456.5±43 [*]	61.5±2.2	63.8±2.7	513.4±84 ^{*,‡}
	210	56.3±2.8 [*]	477.3±56 [*]	65.5±3.5 [#]	467.8±46 [*]	55.6±2.5 [*]	60.2±4.5	533.0±55 ^{*,‡}
	270	51.5±4.2 [*]	453.6±63 [*]	65.9±6.6 [#]	451.9±111 [*]	56.5±3.1 [*]	49.9±4.2 [*]	497.4±64 [*]

Data represents the mean ± SD of 10 mice in each group. Vehicle or AICAR (100 mg/kg) was administered IP at the time of resuscitation.

^{*} Represents $P < 0.05$ versus baseline at time 0 before hemorrhage of mice of the same age group;

[#] represents $P < 0.05$ versus vehicle treatment of mice of the same age group;

[‡] represents $P < 0.05$ versus young group.