ARTICLES

AICAR prevents heat-induced sudden death in RyR1 mutant mice independent of AMPK activation

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Mice with a knock-in mutation (Y524S) in the type I ryanodine receptor (Ryr1), a mutation analogous to the Y522S mutation that is associated with malignant hyperthermia in humans, die when exposed to short periods of temperature elevation (≥37 °C). We show here that treatment with 5-aminimidazole-4-carboxamide ribonucleoside (AICAR) prevents this heat-induced sudden death in this mouse model. The protection by AICAR is independent of AMP-activated protein kinase (AMPK) activation and results from a newly identified action of the compound on mutant Ryr1 to reduce Ca2+ leak from the sarcoplasmic reticulum to the sarcoplasm. AICAR thus prevents Ca2+-dependent increases in the amount of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) that act to further increase resting Ca2+ concentrations. If unchecked, the temperature-driven increases in resting Ca2+ concentrations and the amounts of ROS and RNS create an amplifying cycle that ultimately triggers sustained muscle contractions, rhabdomyolysis and death. Although antioxidants are effective in reducing this cycle in vitro, only AICAR prevents heat-induced death in vivo. Our findings suggest that AICAR is probably effective in prophylactic treatment of humans with enhanced susceptibility to exercise- and/or heat-induced sudden death associated with RyR1 mutations.

An alarming increase in the number of exertional heat-related deaths among young, physically fit athletes, soldiers, policemen and even individuals conducting normal ‘everyday’ activities (for example, yard work or home maintenance) are reported each year in the mainstream media, raising questions as to whether some individuals are more susceptible to heat- and exercise-induced sudden death than others in the population. Recent findings suggest that at least 13 mutations affecting the skeletal muscle Ca2+ release channel, RyR1, are associated with life-threatening responses to exertion, heat challenge and febrile illness1–12. RyR1-associated disorders are not rare; the prevalence of genetic abnormalities in the RyR1 gene has been suggested to be as high as 1 in 3,000 individuals in the general world population7. Mutations in RyR1 are associated with a wide spectrum of human muscle disorders (for reviews, see refs. 8 and 13), including malignant hyperthermia, central core disease, myopathies, drugs that can be used prophylactically are needed.

We created a mouse model of malignant hyperthermia16,17 by knocking in a mutant version of the mouse Ryr1 gene (creating a Y524S mutation; Y522S is the analogous mutation in humans associated with this condition18). The heterozygous mice (Ryr1Y524S/WT, referred to here as YS mice) showed typical hallmarks of malignant hyperthermia (for example, whole-body contractures, elevated core temperature, rhabdomyolysis and death) upon exposure to inhalation anesthetics16. These mice also had an enhanced susceptibility to a heat-stroke–like response that led to sudden death after they were exposed to elevated environmental temperatures (>37 °C) or when exercising under warm (>25 °C) conditions16. In our search for agents that improve the myopathy19 in these mice, we found that AICAR protected the mice against EHR.

AICAR is an activator of AMPK, a kinase that functions as a cellular energy sensor that is activated by increases in the AMP-to-ATP ratio20. AICAR is converted to 5-aminimidazole-4-carboxamide ribonucleoside...
ZMP) in the cell, where it mimics AMP to activate AMPK and improves muscle endurance without exercise\textsuperscript{21–24}. We now report that acute AICAR treatment prevents the EHR in YS mice, but not through targeting of AMPK but rather at least partially by directly inhibiting RyR1-mediated Ca\textsuperscript{2+} leak and reducing oxidative or nitrosative stress.

RESULTS

AICAR prevents heat-induced sudden death in the YS mice

YS mice, if untreated, die after exposure to 37 °C for longer than 15 min\textsuperscript{17}. This heat-induced death is prevented by acute administration of AICAR (600 mg per kg of body weight) (Fig. 1). Administration of this same dose of AICAR after the onset of the heat-induced muscle contractures prevented death in four out of five YS mice exposed to a temperature of 37 °C. To more rigorously quantify the time course of the response of the YS mice to temperature and the protective effects of AICAR, we measured the oxygen consumption (VO\textsubscript{2}) of YS mice during exposure to 37 °C (Fig. 1a). The increase in VO\textsubscript{2} in response to exposure to elevated temperature was markedly greater in untreated YS mice than wild-type (WT) mice, and this increase was prevented by AICAR in YS mice (Fig. 1a). The AICAR dose we used for these experiments (600 mg per kg of body weight) is a commonly used dose that has not been reported to have serious side effects in acute or chronic studies in rodents\textsuperscript{19,21,24,25}. The half maximal dose of AICAR for survival in heat-challenged YS mice was approximately 163 mg per kg of body weight (Fig. 1b). YS mice also had an increase in VCO\textsubscript{2} elimination in response to exposure to 37 °C (Fig. 1c), and the respiratory exchange ratio,
calculated as \(\text{VCO}_2\text{eliminated}/\text{VO}_2\text{consumed}\) approached 1 in these mice (Fig. 1d), suggesting a shift toward anaerobic carbohydrate metabolism\(^{26,27}\). The 10-min exposure to 37 °C resulted in a significant increase in both serum [K\(^+\)] concentration (Fig. 1e) and rectal temperature (Fig. 1f) in YS mice. Administration of AICAR opposed all of these increases in both WT and YS mice (Fig. 1c–f). Although YS mice also died after exposure to volatile anesthetics, AICAR pretreatment (600 mg per kg of body weight) did not prevent this effect (five out of five pretreated mice died), suggesting that either volatile anesthetics are a stronger trigger than heat or that the heat-induced mechanism is different than the anesthetic-induced response.

**AICAR prevention of the EHR is not due to AMPK activation**

As AICAR is thought to be an activator of AMPK, we evaluated the role of this kinase in the rescue of the YS mice from the EHR. We did so by modifying the AMPK assay using the SAMS peptide (a modified peptide corresponding to the sequence around the AMPK target site in rat acetyl-CoA carboxylase (HMRSAMGSLHLVKRR)) for use in muscle homogenates (Online Methods). To verify the assay, we determined the \(V_{\text{max}}\) (the maximum velocity of the reaction) and the \(K_m\) (the substrate concentration that produces an initial velocity of the reaction that is half of the \(V_{\text{max}}\)) in homogenates of soleus and extensor digitorum longus (EDL) muscles of heat challenged WT and YS mice (Fig. 2a). The \(K_m\) values for each group were as follows: 145 ± 38 \(\mu\)M (n = 4) for WT soleus, 120 ± 44 \(\mu\)M (n = 3) for WT soleus, 137 ± 37 \(\mu\)M (n = 3) for WT EDL and 149 ± 36 \(\mu\)M (n = 3) for YS EDL (all values are mean ± s.e.m.). These \(K_m\) values were not significantly different among muscle samples, but the \(V_{\text{max}}\) (Fig. 2b) was significantly higher in the EDL of YS mice than of WT mice (\(P < 0.05\), presumably as a result of ongoing muscle contractions in YS mouse EDL muscles and activation of AMPK by phosphorylation (Supplementary Fig. 1a–f). Consistent with the higher amounts of muscle contractions in YS mice (even in the absence of heat challenge) compared to WT mice, glyco- gen concentrations were lower in both the soleus and EDL muscles of YS mice compared to WT mice (Supplementary Fig. 2). Glycogen is an inhibitor of AMPK\(^{28}\). The values of AMPK activity we obtained in this study are comparable or higher than those obtained in skeletal muscle in other laboratories\(^{29,30}\).

To assess the role of AMPK activation in AICAR rescue, we used WT mice, YS mice and both WT and YS mice crossed with mice expressing a muscle-specific dominant-negative AMPK-\(\alpha_2\) (DN)\(^{29,31}\). We injected each group of mice (WT, YS, WT-DN and YS-DN) with either saline or AICAR and exposed them to 37 °C for 10–15 min. Mice that showed signs of EHR were humanely killed at the onset of the involuntary sustained contractions (presenting as muscle rigidity, arched back and extended legs). The presence of dominant-negative AMPK-\(\alpha_2\) did not prevent AICAR protection of the EHR response in YS-DN mice (Fig. 2c). We also screened the mice for changes in inspired \(\text{VO}_2\) during heat challenge (Fig. 2d; values obtained after 10 min at 37 °C). Untreated YS and YS-DN mice showed the classic muscle signs of the EHR, and the \(\text{VO}_2\) in these two groups of mice was significantly higher (\(P < 0.001\)) compared to WT mice. The \(\text{VO}_2\) of YS-DN mice was, however, lower than that of YS mice, suggesting that AMPK activity contributes to the increased metabolism of YS muscle during heat challenge. The \(\text{VO}_2\) in both YS and YS-DN mice decreased in response to treatment with AICAR. None of the mice treated with AICAR showed an adverse reaction to elevated temperature. The decreased \(\text{VO}_2\) values of the YS and YS-DN mice treated with AICAR correlated well with the increased survival of these two groups of mice upon AICAR treatment during the heat challenge.

To further confirm that the AICAR rescue was not caused by AMPK activation, we isolated and homogenized the EDL and soleus muscles of mice treated with AICAR (Fig. 2c,d) and determined the initial rate (\(v_0\)) of phosphorylation of 150 \(\mu\)M SAMS peptide at both

**Figure 3** The effect of AICAR on Ryr1 in the presence of AMP-PCP. (a) Representative plots of [\(^3\)H]-ryanodine binding to the sarcoplasmic reticulum membranes from WT and YS mice with increasing AICAR concentrations in the absence of AMP-PCP. (b) Representative plots of [\(^3\)H]-ryanodine binding to the sarcoplasmic reticulum membranes from WT mice with increasing concentrations of AMP-PCP in the absence or presence (+A) of 1 mM AICAR. (c) Representative plots of [\(^3\)H]-ryanodine binding to the sarcoplasmic reticulum membranes of YS mice with increasing concentrations of AMP-PCP in the absence or presence of 1 mM AICAR. EC\(_{50}\) values stated in the text represent the mean of three independent preparations. (d) Representative single-channel recordings of WT Ryr1 in the presence of 1 mM AMP-PCP before and after the addition of 1 mM AICAR. (e) One subclass of single YS Ryr1 channels (other subclasses are listed in Supplementary Fig. 4) in the presence of 1 mM AMP-PCP before and after treatment with 1 mM AICAR. (f–h) Ryr1 probability of opening (\(P_o\)) (f), mean channel open time (\(t_{\text{open}}\)) (g) and mean channel closed time (\(t_{\text{close}}\)) (h) for the channels from WT and YS mice. AICAR treatment is indicated with the subscript ‘A’, and the n numbers of channels in each group are indicated in f–h as numbers in the bars. Data are means ± s.e.m. *** \(P < 0.001\) using Student’s t test.
23 °C (Supplementary Fig. 1g) and 37 °C (Fig. 2e,f). This brief, acute in vivo AICAR treatment did not significantly activate AMPK in either the EDL or soleus muscles. Mice expressing the dominant-negative AMPK-δ (WT-DN and YS-DN mice) treated with AICAR had lower AMPK activity in both their soleus and the EDL muscles than WT and YS mice not expressing the dominant-negative AMPK-δ. Despite the lack of an increase in AMPK activity in response to AICAR treatment in any of the groups of mice, all of the treated mice survived the heat challenge. We confirmed these findings (Fig. 2e,f) by western blot for phosphorylated AMPK (pAMPK) and AMPK (Supplementary Fig. 1).

We conclude that activation of AMPK is not responsible for the ability of AICAR to rescue YS mice from the heat challenge. We also tested the effects of chronic AMPK activation arising from expression of the mutant AMPK γ1 subunit (a muscle-specific noncatalytic γ1 subunit mutant (R70Q) of AMPK) and found that although AMPK activity was higher in muscle from these mice compared to non-mutant AMPK mice, expression of this mutant AMPK subunit in YS mice did not rescue the mice when exposed to 37 °C (we designated these mice ‘YS-CA’ mice; Supplementary Fig. 1).

AICAR inhibits RyR1 in the presence of nonhydrolyzable ATP

Because AMPK activation is not involved in the mechanism by which AICAR rescues YS mice during heat challenge, we next determined whether AICAR had a direct effect on Ry1. ATP is a known activator of the RyR proteins, and other adenine nucleotides (ADP, AMP, cAMP, adenosine and adenosine) function as weak partial agonists. As AICAR is a precursor of ZMP, it may also interact with Ry1 at the ATP binding site. We examined the effects of AICAR on [3H]-ryanodine binding to sarcoplasmic reticulum membranes from muscles from WT and YS mice. Ryanodine binds preferentially to the open state of RyR1, allowing the [3H]-ryanodine binding affinity to be used as an indirect measure of channel activation. In the absence of ATP, treatment with AICAR produced a small concentration-dependent increase in [3H]-ryanodine binding in the membranes from both WT and YS mice (Fig. 3a), and 1 mM AICAR produced a significant (P < 0.001 for WT and P < 0.01 for YS) rightward shift in the concentration dependence of AMP-PCP (adenylyl methylenediphosphonate, a nonhydrolyzable form of ATP) on the enhancement of [3H]-ryanodine binding (Fig. 3b,c). The half-maximal effective concentration (EC50) values were as follows: 153 ± 5 µM and 330 ± 27 µM for YS mice and 100 ± 5 µM and 318 ± 51 µM for WT mice without or with the presence of 1 mM AICAR, respectively (n = 3 for each group, and all values are mean ± s.e.m.). We also examined the effects of AICAR on the Ca2+ dependence of [3H]-ryanodine binding to membranes from WT and YS mice (Supplementary Fig. 3a). Ca2+ regulates RyR1 activity in a bimodal fashion, with low Ca2+ concentrations (~1–10 µM) activating

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**Figure 4** The effect of AICAR on the concentrations of Ca2+, ROS and RNS in single isolated FDB fibers from WT and YS mice. (a) The peak of Ca2+ transient triggered by in vitro application of 4-cmc. F360, fluorescence emission at 360 nm; F380, fluorescence emission at 380 nm. (b) Resting cytosolic Ca2+ in the indicated fibers. (c) Representative images of single fibers loaded with Fura-2-acetoxymethyl ester. Scale bars, 20 µm. Vertical linear scales represent free Ca2+ at concentrations of 0–1.7 µM. (d) Estimation of the changes in the concentration of resting Ca2+ (nM) with changes in temperature. In vitro AICAR treatment is indicated (+A) (n = 3–6 fibers per group). (e,f) The DAF fluorescence ratio as a measure of RNS production (e) and the DCF fluorescence ratio as a measure of ROS production (f) in FDB fibers. (g) Representative OxyBlot (top, green) and the pseudocolored red coomassie-stained gel (bottom, only the nonspecific band) in WT (W) and YS (Y) mouse muscle homogenates. Mice were injected either with saline (−) or AICAR (+) and were heat challenged at 37 °C (+) or not heat challenged (−). Oxidative stress in soleus (h) and EDL (i) muscle homogenates as quantified by OxyBlot densitometry. In vitro AICAR application is indicated with a subscript ‘A’ in a, b, e, f, h and i, and the n numbers of fibers from at least three mice in each group are indicated in these panels as numbers in the bars. Data are means ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 using Student’s t test.
channel activity and high Ca\(^{2+}\) concentrations (~0.1–1 mM) inhibiting channel activity\(^{17,34,35}\). As previously reported\(^{17}\), the Ca\(^{2+}\) enhancement of the \(^{[3]}\text{H}\)-ryanodine binding curve is shifted to the left in membranes from YS mice compared to those from WT mice. AICAR, however, does not alter the Ca\(^{2+}\) sensitivity of \(^{[3]}\text{H}\)-ryanodine binding, suggesting that AICAR does not prevent the EHR by decreasing the Ca\(^{2+}\) affinity of Ryr1. AICAR had no effect on \(^{[35]}\text{S}\)-FKBP12 (the FK506 binding protein 1a) binding or the caffeine sensitivity of \(^{[3]}\text{H}\)-ryanodine binding to the sarcoplasmic reticulum membranes from either WT or YS mice or the caffeine sensitivity of the sarcoplasmic reticulum Ca\(^{2+}\) release in flexor digitorum brevis (FDB) fibers from either group of mice (data not shown).

The inhibitory effect of AICAR on \(^{[3]}\text{H}\)-ryanodine binding suggests that the protective effect of AICAR could be a decrease in Ca\(^{2+}\) leakage from mutant Ryr1 channels at cellular concentrations of ATP. To test this possibility, we determined the effect of AICAR in the presence of 1 mM AMP-PCP on the single-channel activity of Ryr1 channels from WT and YS mice after reconstitution into planar lipid bilayers (Fig. 3d,e). We treated the Ryr1 preparations with the reducing agent dithiothreitol, and as a result, Ryr1 was not reversibly oxidized or S-nitrosylated in these experiments. The channels from the heterozygous YS mice showed a variety of single-channel behaviors (Supplementary Fig. 4), as may be expected for heterologer WT:YS tetramers. However, all channels in WT and YS mice were inhibited by AICAR in the presence of AMP-PCP. Treatment with AICAR significantly reduced the open probability (\(P_o\)) of channels from YS mice (Fig. 3f), primarily by reducing the mean channel-open time (Fig. 3g), and had a small effect on the closed times of WT channels (Fig. 3h). These findings show that treatment with AICAR reduces Ryr1 channel activity in the presence of AMP-PCP.

**AICAR prevents Ca\(^{2+}\) leak, ROS and RNS generation in YS myofibers**

We explored the possibility that AICAR prevents temperature-driven Ca\(^{2+}\) leakage through Ryr1. We measured representative Ca\(^{2+}\) transients elicited by 4-chloro-m-creosol (4-cmc, an activator of Ryr1 Ca\(^{2+}\) release) with Fura-2 in FDB fibers from WT and YS mice (Supplementary Fig. 5). Treatment of FDB fibers from YS mice with 1 mM AICAR for 10–20 min decreased the temperature-dependent increase in the resting Fura-2 ratio (\(F_{360}/F_{380}\)) and prevented a decrease in the 4-cmc-induced ‘readily releasable Ryr1 Ca\(^{2+}\) pool’ in FDB fibers from YS mice (Fig. 4a,b). These findings are consistent with a role for AICAR in dampening Ryr1 Ca\(^{2+}\) leak from the sarcoplasmic reticulum in the presence of cellular ATP. To assess the effects of AICAR on Ca\(^{1.1}.\) Ryr1 signaling during excitation-contraction coupling, we determined the effect of 1 mM AICAR on L-type Ca\(^{2+}\) currents and on voltage-gated sarcoplasmic reticulum Ca\(^{2+}\) release in WT myotubes using whole-cell voltage clamp experiments (Supplementary Fig. 3b,c and Supplementary Table 1). Although pretreatment with 1 mM AICAR produced a modest increase in the maximal L-type Ca\(^{2+}\) channel conductance, it had no effect on either the voltage dependence of this conductance or on the magnitude and voltage dependence of Ryr1-mediated sarcoplasmic reticulum Ca\(^{2+}\) release (Supplementary Fig. 3b,c and Supplementary Table 1). Thus, we conclude that AICAR normalizes the enhanced Ca\(^{2+}\) leak properties of Ryr1 channels in YS mice.

We used an in situ calibration to determine the magnitude of the temperature-dependent increase in the resting myoplasmic Ca\(^{2+}\) concentration at 32 °C and 37 °C compared to 23 °C (Fig. 4c,d). The results from this analysis indicated that the temperature-dependent increase in resting Ca\(^{2+}\) in FDB fibers from YS mice was as high as 100–250 nM and that 1 mM AICAR markedly reduced this increase in resting Ca\(^{2+}\).

To determine whether AICAR treatment affects the temperature-dependent increase in ROS and RNS production in fibers from YS mice, we assessed the effects of AICAR on 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF) and 5-carboxy-2′,7′-dichlorodihydrofluorescein (DCF) fluorescence (Fig. 4e,f). The temperature-dependent increases in both RNS and ROS production in fibers from YS mice were prevented by treatment with 1 mM AICAR (Fig. 4e,f).

Proteins in the soleus and EDL muscles of YS mice showed higher amounts of oxidative modifications than muscles from WT mice.

![Figure 5](#) The effects of NOX or NOS inhibition in single isolated FDB fibers at 35 °C. Fibers from WT and YS mice were preincubated either with the NOX inhibitor gp91ds-tat peptide (g) or with the NOS blocker L-NAME (L); the blockers are shown as subscripts. (a,b) The DCF fluorescence ratio as a measure of ROS production in fibers incubated with gp91ds-tat peptide or the control peptide (a) or L-NAME (b). (c,d) The DAF fluorescence ratio as a measure of RNS production in fibers incubated with gp91ds-tat peptide or the control peptide (c) or L-NAME (d). (e,f) The Fura-2 ratio as a measure of the concentration of resting Ca\(^{2+}\) in fibers incubated with gp91ds-tat peptide or the control peptide (e) or L-NAME (f). (g,h) The Fura-2 ratio as a measure of Ca\(^{2+}\) transient peak triggered by in vitro application of 4-cmc in fibers incubated with gp91ds-tat peptide or the control peptide (g) or L-NAME (h). The \(n\) numbers of fibers from at least three mice in each group are indicated in all panels as numbers in the bars. Data are means ± s.e.m. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) using Student’s \(t\) test.
Sources of reactive species that contribute to RyR1 Ca\(^{2+}\) leak

The nitric oxide synthase (NOS) isoforms endothelial NOS and neuronal NOS are activated by Ca\(^{2+}\) through calmodulin in skeletal muscle, suggesting that increases in the concentration of myoplasmic Ca\(^{2+}\) are responsible for the temperature-dependent increases in ROS and NOS production observed in YS mice. The ability of ryanodine to block the temperature-dependent increases in both DAF and DCF fluorescence supports this mechanism. There are a number of potential sources of ROS production in muscle, including mitochondria, NADPH oxidases (NOX) and xanthine oxidase. A temperature-dependent increase in mitochondrial superoxide production in FDB fibers from YS mice was recently reported. We used the gp91ds-TAT peptide to inhibit the effects of NOX and a scrambled gp peptide to control for nonspecific effects and L-NAME to inhibit the effects of NOS, on the temperature-dependent increases in DCF fluorescence. The gp91ds-TAT peptide, Ryr1 Y524S, and increased the peak Ca\(^{2+}\) release triggered by 4-cmc in FDB fibers from YS mice. Inhibiting the effects of either NOX or NOS prevented the temperature-dependent increases in both DAF and DCF fluorescence in the fibers from YS mice, suggesting that the major source of ROS in muscle from YS mice exposed to heat is NOX. Blocking either Ryr1 or ROS production decreased the concentration of resting Ca\(^{2+}\) and increased the magnitude of 4-cmc–induced Ca\(^{2+}\) release in FDB fibers from YS mice at 35°C, supporting a feed-forward cycle whereby Ca\(^{2+}\) increases ROS and ROS production and, in turn, RNS and ROS increase Ryr1 Ca\(^{2+}\) leakage.

DISCUSSION

Mutations in RYR1 underlie a life-threatening sensitivity to heat and exercise in humans. Similarly, enhanced heat and exercise sensitivity is found in mice with the Y524S Ryr1 knock-in mutation. We show that this Y524S mutation enhances Ryr1 Ca\(^{2+}\) leakage, especially at higher temperatures, which, in turn, drives an increase in oxidative and nitrosative stress. Oxidative and nitrosative modifications of Ryr1 and other muscle proteins result in a feed-forward cycle that drives both myopathy and the EHR.

Several groups have suggested that Ca\(^{2+}\) influx may contribute to the sustained increases in Ca\(^{2+}\) concentration that are associated with the malignant hyperthermia response, but the possibility that Ca\(^{2+}\) influx through stretch-, store- or voltage-operated Ca\(^{2+}\) channels contributes to the EHR in YS mice has not been investigated.

There are no known drug interventions that prevent heat-induced death in humans. We show that AICAR, a compound that improves muscle performance, prevents heat-induced sudden death in YS mice. We also show that the ability of AICAR to protect these mice is not a result of activation of the energy-sensing kinase AMPK but, rather, is a result of a direct inhibition of Ca\(^{2+}\) leakage through Ryr1, which prevents heat-induced increases in the concentration of resting Ca\(^{2+}\), Ca\(^{2+}\) store depletion and increases in RNS and ROS production.

Despite the finding that ROS and RNS are involved in the feed-forward cycle, antioxidants such as N-acetylcysteine, which blocks increases in the production of both ROS and RNS in myotubes and FDB fibers from YS mice, delays but does not prevent heat-induced sudden death in YS mice. This lack of in vivo efficacy probably reflects a limited bioavailability of antioxidants, which has been a drawback to antioxidant therapy for a number of diseases (for a review, see ref. 48). In contrast, AICAR, which improves muscle function with only very mild side effects, is 100% effective in preventing heat-induced sudden death in YS mice.

In summary, we show that AICAR interacts with Ryr1 to decrease Ca\(^{2+}\) leakage in the presence of cellular concentrations of ATP (a more efficacious agonist). AICAR rescues the EHR of YS mice at least partially by reducing Ryr1 Ca\(^{2+}\) leakage and oxidative and nitrosative stress to amounts sufficient to disrupt the destructive feed-forward cycle that, when unchecked, leads to sustained contractures, rhabdomyolysis and death. We propose the potential use of AICAR for prophylactic treatment in humans with an enhanced susceptibility to exercise- and/or heat-induced sudden death due to mutations in RYR1.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.
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AUTHOR CONTRIBUTIONS

J.T.L. designed and analyzed the experiments shown in Figures 1a–d and 3a–c, analyzed data, produced Supplementary Figure 1a–f, wrote the initial draft of the paper and edited the final draft. D.K.G. developed the new AMPK assay, designed, performed and analyzed the experiments in Figure 2, wrote an intermediate draft of the paper, prepared the supplementary information, and helped write and edit the final draft of the manuscript. A.D.-A. designed, performed and analyzed the experiments in Figures 4a–f and 5a,b and participated in the writing of the manuscript. A.A. designed, performed and analyzed data from the experiments in Figure 4d. Q.C. made the initial AICAR discovery and performed the experiments in Figure 1e and Supplementary Figure 2. A.D.J. generated and analyzed the data in Figure 4g–i and Supplementary Figure 2. T.C. performed the bilayer experiments. V.Y. designed, performed and analyzed data from the experiments in Supplementary Table 1 and Supplementary Figure 3b,c. J.M.O. performed the experiments shown in Figure 5c,d. C.S.L. designed, performed and analyzed data (using western blots and quantitative RT-PCR) to show that calcium-handling proteins are not changed by the Y524S mutation or by the presence of AICAR. T.O.M. designed the experiments and performed and analyzed many of the pAMPK and AMPK western blots shown in Supplementary Figure 1. A.S. performed all of the mouse dissections, tested the endurance of mice on running wheels, performed indirect calorimetry and contributed to the preparation of the manuscript. K.D. handled all mouse matings and genotyping. S.L.H. supervised all experiments, reanalyzed all data for accuracy, plotted all figures and wrote the final draft of the manuscript. All authors reviewed and approved the final version of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

**Mice.** For the experiments involving mice, we used 6–10-week-old male heterozygous Ryr1<sup>Y524S/WT</sup> knock-in (YS) mice and their WT littermates (on a C57BL/6 background)<sup>16</sup>. We also crossed YS mice with transgenic mice that expressed either a muscle-specific constitutively active AMPK-γ1<sup>-E171N</sup> (CA)<sup>25</sup> or dominant-negative AMPK-α2<sup>-D177A</sup> (DN)<sup>25,31</sup>. We housed all mice at 22–24 °C with a 12 h light, 12 h dark cycle and provided food and water <i>ab libitum</i>. We injected AICAR (Toronto Research Chemicals) and saline subcutaneously. The dose of AICAR used was 600 µg per kg of body weight, unless otherwise stated, and it was administered 10 min before heat challenge. All procedures were approved by the Animal Care Committee at Baylor College of Medicine.

**AMPK activity assay.** We analyzed AMPK activity by assessing the incorporation of <sup>32</sup>P radiolabeled phosphate from ATP (PerkinElmer) into 150 µM SAMS (AnaSpec) peptide for 15 min at 37 °C as previously described<sup>49</sup>, with modifications. The assay was on 5 µg homogenates, 1 µM thapsigargin (Sigma-Aldrich) and 10 mM ethylene glycol tetra-acetic acid (Sigma).

**Indirect calorimetry.** We assessed O₂ consumption and CO₂ production of individual mice undergoing heat challenge at 37 °C at 1 min intervals for up to a 15 min period as previously described<sup>17</sup>. We performed all metabolic experiments at approximately the same time each day (between 10 am and 1 pm), and the mice were not fasted.

**Serum K⁺.** We measured serum K⁺ in the Center for Comparative Medicine at Baylor College of Medicine with a Roche COBAS INTEGRA 400 plus instrument according to the instructions of the manufacturer.

**Radioligand binding.** We performed equilibrium <sup>3</sup>H-ryanodine<sup>17,47</sup> (5 nM) and <sup>35</sup>S-FKBP12 (ref. 50) (20 nM) binding studies with skeletal muscle membranes as previously described.<sup>17</sup>

**Single-channel recordings and analyses.** We performed single-channel measurements by fusing proteoliposomes containing purified Ryr1 from skeletal muscles with lipid bilayers bathed in 250 mM Cs-HEPES (Sigma), pH 7.4 (<i>cis</i>) and 50 mM Cs-methanesulphonate (<i>trans</i>) (Sigma) as previously described<sup>31</sup>. Free Ca<sup>2+</sup> was at a concentration of 6–8 µM, as neither Ca<sup>2+</sup>- nor ethylene glycol tetra-acetic acid were added. After single-channel activity became evident in the presence of 1 mM AMP-PCP (Sigma) (in both chambers), we adjusted the concentration of Cs-methanesulphonate in the <i>trans</i> chamber to 250 mM, and we added 1 mM AICAR to both sides of the membrane. We recorded Ryr1 current at a holding potential of +30 mV, digitally filtered at 2 KHz, and acquired the sampling rate at 10 KHz using CLAMPTEX 10.0 (Molecular Devices). For illustration purposes, we digitally filtered the records shown at 300 Hz. We analyzed the recordings using Clampfit 10.0 (Molecular Devices). We calculated the open probability for each channel from the events analyses as a ratio of the total open time (sum of all open times, including partial openings of the channels to a sub-conductance state) to the total time of the record. We calculated the mean open and closed times by fitting the dwell time histograms to a single exponential log probability function.

**OxyBlot.** We assessed oxidative stress using the OxyBlot Protein Oxidation Detection Kit (Millipore) in 10 µg soleus and 10 µg EDL muscle homogenates according to the protocol provided by the manufacturer.

**Fiber isolation.** We isolated and plated single fibers from the FDB muscle bundles as previously described<sup>25</sup>.

**Measurements of ROS and RNS.** To assess the amounts of ROS and RNS, we loaded the FDB fibers with DCF (CM-H<sub>2</sub>DCFDA) (Invitrogen) or DAF (DAF-FM diacetate) (Invitrogen) dyes as previously described<sup>17</sup>. Imaging details are described in the Supplementary Methods. We also tested the effects of 1 mM AICAR, 50 µM L-NAME (Sigma) and 5 µM gp91ds-tat peptide or the corresponding scrambled peptide<sup>40</sup> (Biopolymer Core, University of Maryland) on DCF and DAF fluorescence after 1 h of preincubation at room temperature.

**Ca<sup>2+</sup> measurements.** We measured resting Ca<sup>2+</sup> and the readily releasable Ryr1 Ca<sup>2+</sup> pool in single FDB fibers loaded with Fura-2-acetoxyethyl ester (Invitrogen) using a conventional epifluorescence technique (Supplementary Methods).

**Measurements of the temperature dependence of resting Ca<sup>2+</sup>.** Resting Fura-2 fluorescence ratios (<i>R = F<sub>340</sub>/F<sub>380</sub></i>) were converted to free Ca<sup>2+</sup> concentrations using an <i>in situ</i> calibration approach<sup>14</sup> and the following equation: \[ [\text{Ca}^{2+}] = K_f \times \beta \times ((R - R_{\text{min}})/R_{\text{max}} - R) \], where <i>K_f</i> is the Ca<sup>2+</sup> affinity of Fura-2, <i>β</i> is the ratio of the 380-nm emission recorded under Ca<sup>2+</</sup>-free and Ca<sup>2+</sup> saturating conditions, <i>R_{\text{min}}</i> is the emission ratio under Ca<sup>2+</sup>-free conditions, and <i>R_{\text{max}}</i> is the emission ratio under Ca<sup>2+</sup> saturating conditions. The values of <i>β</i>, <i>R_{\text{min}}</i> and <i>R_{\text{max}}</i> were determined experimentally. The <i>K_f</i> was used taken from the <i>in vitro</i> calibration of Fura-2 in the presence of 27 mg ml<sup>−1</sup> of aldolase (428 nM)<sup>15</sup> and was assumed to be independent of the temperature in intact cells as shown previously<sup>53</sup> (Supplementary Methods).

**Statistical analyses.** We performed the statistical analyses of two groups by Student’s <i>t</i> test. <i>P < 0.05</i> was considered statistically significant.

**Additional methods.** Detailed methodology is described in the Supplementary Methods.

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